

# **Redoxregulation of Prostanoid Biosynthesis**

Dissertation

zur Erlangung des akademischen Grades des  
Doktors der Naturwissenschaften (Dr. rer. nat.)  
im Fachbereich für Biologie  
an der Universität Konstanz

vorgelegt von

**Stefan Schildknecht**

Tag der mündlichen Prüfung: 15.7.2005

1. Referent: Prof. Dr. Volker Ullrich
2. Referent: Priv. Doz. Dr. Christian Schudt
3. Referent: Prof. Dr. Urs Ruegg

# **Acknowledgments**

The present study was performed during the years 2002-2005 at the chair of biological chemistry under supervision of Prof. Dr. Volker Ullrich.

My special thanks go to Prof. Ullrich for his stimulatory ideas, his patience in discussing and writing our manuscripts and the academic freedom he allowed to follow own ideas. During the time in the laboratory, I was deeply impressed by his broad knowledge and his enthusiasm for science.

I am furthermore deeply indebted to Dr. Markus Bachschmid for his ideas, technical advice and motivation during the years.

I am grateful to my colleagues for their excellent support, especially to R. Hölz, E. Müsig, D. Frein and G. Klumpp and indeed G. Naschwitz for unforgettable discussions.

I was pleased to become a member of the “Graduiertenkolleg Biochemische Wirkstoffforschung” and profited much from its seminars and courses which allowed insights into different interesting scientific fields. I would like to thank the organizers Prof. Dr. A. Wendel and Prof. Dr. K. Schäfer for their encouragement during the Graduiertenkolleg.

The last and final thanks go to my parents who made all this possible.

# Redoxregulation of Prostanoid Biosynthesis

The results of this cumulative thesis are published in the following papers:

1. Schildknecht S., Bachschmid M., Baumann A., Ullrich V. COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. *FASEB J.* 2004 Apr;18(6):757-9. Epub 2004 Feb 20.
2. Schildknecht S., Bachschmid M., Weber K., Maass D., Ullrich V. Endotoxin elicits nitric oxide release in rat but prostacyclin synthesis in human and bovine vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 2005 Feb 4;327(1):43-8.
3. Schildknecht S., Bachschmid M., Ullrich V. Peroxynitrite provides the peroxide tone for PGHS-2 dependent prostacyclin synthesis in vascular smooth muscle cells. *FASEB J.*, accepted, in press.
4. Bachschmid M., Schildknecht S., Heinz K., Hamacher J., Kavakli C., Daiber A., Ullrich V. Autocatalytic Tyrosine Nitration of Prostaglandin Endoperoxide Synthase-2 in LPS-stimulated RAW 264.7 Macrophages. Submitted to *J. Biol. Chem.*
5. Schildknecht S., Bachschmid M., Hamacher J., Ullrich V. Nitration of PGHS-2 inhibits prostanoid release in rat alveolar macrophages. Submitted to *J. Immunol.*
6. Frein D., Schildknecht S., Bachschmid M., Ullrich V. Redox regulation: A new challenge for pharmacology. *Biochemical Pharmacology.* Review, in press.

7. van der Loo B., Bachschmid M., Skepper J.N., Labrugger R., Schildknecht S., Hahn R., Muessig E., Gygi D., Luescher T. „Mitochondrial trapping“ of Sod 1 in oxidative stress associated vascular ageing. Submitted to *J. Clin. Invest.*

If not otherwise indicated, all results presented in this thesis have been performed by the author.

# Contents

|   |    |
|---|----|
| <b>1. Introduction</b> .....  | 1  |
| 1.1. The Circulatory System.....  | 1  |
| 1.2. Regulation of Vascular Tone by the Endothelium .....                                       | 3  |
| 1.3. Contraction and Relaxation of Vascular Smooth Muscle Cells.....                            | 7  |
| 1.4. The Pathophysiology of Sepsis .....  | 10 |
| 1.5. Eicosanoid Biosynthesis.....   | 13 |
| 1.6. Catalytic Mechanism of Prostaglandin H <sub>2</sub> Synthase.....                          | 16 |
| 1.7. Catalytic Mechanism of Nitric Oxide Synthase.....  | 19 |
| 1.8. Formation of Superoxide in the Vasculature.....  | 21 |
| 1.8.1. NADPH Oxidase .....  | 22 |
| 1.8.2. Xanthine Oxidase .....   | 24 |
| 1.8.3. Mitochondria as Source of $\bullet\text{O}_2^-$ and $\bullet\text{NO}$ .....             | 25 |
| 1.9. Redoxregulation of Prostanoid Biosynthesis by $\bullet\text{NO}/\bullet\text{O}_2^-$ ..... | 27 |
| 1.10. The Biochemical Basis for LPS-Induced Endothelial Dysfunction.....                        | 29 |
| 1.11. The Vasculature in Sepsis .....   | 32 |
| 1.11.1 The Physiological Situation .....  | 32 |
| 1.11.2 Endothelial Cell Activation .....  | 33 |
| <br>  |    |
| <b>2. Materials and Methods</b> .....   |    |
| 2.1. Materials.....   | 35 |
| 2.2. Smooth Muscle Cell Culture .....   | 36 |
| 2.3. Preparation of Rat Alveolar Macrophages .....  | 38 |
| 2.4. Western-Blot Analysis .....  | 38 |

|   |              |
|---|--------------|
| 2.5. Quantitative PCR Analysis.....   | 39           |
| 2.6. PGHS Activity.....   | 41           |
| 2.7. NOS Activity Assay.....  | 42           |
| 2.8. Measurement of Prostanoids and •NO-Products in<br>Cell Culture Supernatants.....   | 43           |
| 2.9. Immunoprecipitation.....   | 44           |
| 2.10. HPLC Analysis of Protein-bound 3-NT.....  | 44           |
| 2.11. GC-MS.....  | 45           |
| 2.12. Statistical Analysis.....   | 46           |
| <br>  |              |
| <b>3. Aims of the Study.....</b>  | <b>47</b>    |
| <br>  |              |
| <b>4. Results and Discussions.....</b>  | <b>.....</b> |
| <br>  |              |
| 4.1. Prostacyclin Release by LPS Exposed Vascular Smooth Muscle Cells.....  | .....        |
| Introduction.....   | 49           |
| Results.....  | 51           |
| Discussion.....   | 59           |
| <br>  |              |
| 4.2. Peroxynitrite Provides the Peroxide Tone for PGHS-2 Dependent<br>Prostacyclin Synthesis in Vascular Smooth Muscle Cells..... | .....        |
| Introduction.....   | 64           |
| Results.....  | 67           |
| Discussion.....   | 74           |

|  |     |
|--|-----|
| 4.3. Endotoxin Elicits Nitric Oxide Release in Rat but Prostacyclin<br>Synthesis in Human and Bovine Vascular Smooth Muscle Cells..... |     |
| Introduction .....   | 79  |
| Results .....  | 81  |
| Discussion .....   | 86  |
| 4.4. Autocatalytic Tyrosine Nitration of Prostaglandin Endoperoxide<br>Synthase-2 in LPS-Stimulated RAW 264.7 Macrophages.....         |     |
| Introduction .....   | 88  |
| Results .....  | 91  |
| Discussion .....   | 98  |
| 4.5. Nitration of PGHS-2 Inhibits Prostanoid Release in Rat<br>Alveolar Macrophages .....  |     |
| Introduction .....   | 102 |
| Results .....  | 105 |
| Discussion .....   | 111 |
| <b>5. General Discussion</b>   |     |
| 5.1. Prostacyclin Synthesis by Vascular Smooth Muscle Cells .....  | 115 |
| 5.2. Peroxynitrite Provides the Cellular Peroxide Tone for PGHS-2.....   | 121 |
| 5.3. Mutual Formation of either PGI <sub>2</sub> or •NO by Vascular Smooth<br>Muscle Cells from Different Species .....                | 125 |
| 5.4. Nitration of PGHS-2 by Nitrite .....  | 126 |

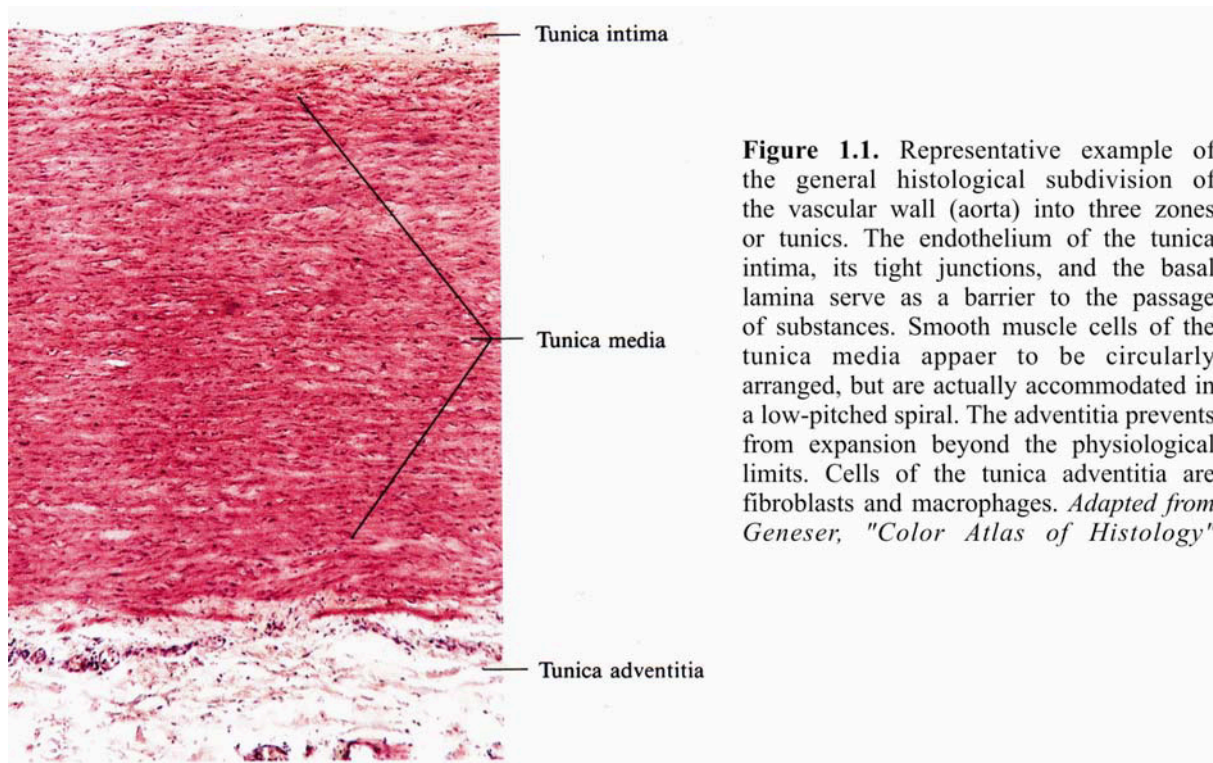
|   |            |
|---|------------|
| 5.5. Nitration of PGHS-2 in Alveolar Macrophages..... | 129        |
| 5.6. Conclusions.....                                 | 130        |
| <br>  |            |
| <b>6. Summary.....</b>                                | <b>132</b> |
| <br>  |            |
| <b>7. Zusammenfassung.....</b>                        | <b>134</b> |
| <br>  |            |
| <b>8. References.....</b>                             | <b>137</b> |

# 1. Introduction

## 1.1. The Circulatory System

The blood circulatory system is made up of the heart, the arterial network that distributes blood to virtually every part of the body and the venous system which returns it back to the heart to close the circuit. The microvascular bed of the various tissues and organs begins with arterioles which convey the blood into the dense network of capillaries from which it is collected in venules. The circulatory system serves very different physiological tasks reaching from the exchange of gases and nutrients to an involvement in the host immune response. The latter function requires a tremendous complexity of interactions between parts of the vascular wall and cells of the immune system and forms the center of the present investigation.

The vessel walls as shown in Figure 1.1. are made up of three coats called tunicae: 1) The **tunica intima** consists on its inner surface of the endothelium which is separated by a thin basal lamina from the subendothelial layer of loose tissue, 2) The **tunica media** which in case of a muscular artery is composed of circularly arranged smooth muscle cells. The intercellular substance holding the smooth muscle cells together is made by the smooth muscle itself and includes mainly elastin. 3) The **tunica adventitia** which particularly contains extracellular elastic fibers and collagen secreted by adventitial fibroblasts. The adventitia exerts an innervation by nerve fibers of the autonomous nerve system, blood vessels (vaso vasorum) and also harbors appreciable amounts of macrophages. The relative thickness of each of these coats depends on the type of vessel, arterioles for example can consist only of the endothelium and its basement membrane and a media which is made up of only one or two circular layers of smooth muscle cells.



Arterial vessels are classified as 1) conducting **elastic arteries** for which the aorta, heart carotid arteries and pulmonary arteries are prominent examples. This vessel type is mainly involved in the maintenance of the diastolic blood pressure, 2) **muscular arteries** that distribute blood to the entire body and 3) **arterioles** which are defined as arteries with an internal diameter of less than 30 $\mu$ m. Veins of the same diameter as their accompanying arteries usually have thinner walls which however consist of the same three basic tunicae as described for the arteries. The veins of lower regions of the body have a thicker media with more smooth muscle cells, which is of importance for the active propulsion of the blood back to the heart. In contrast, the veins of the upper regions of the body have a thin media and essentially drain the blood back to the heart.

Under physiological conditions, local vascular tone is mainly regulated by the single layer of endothelial cells lining the inner surface of a vessel. The main regulators released by an intact endothelium are prostacyclin (PGI<sub>2</sub>) and nitric oxide ( $\bullet$ NO) which both evoke relaxation of the adjacent smooth muscle. Under severe pathophysiological conditions, the endothelium can

become activated which evokes an impairment of its regulatory function as conductor of vascular homeostasis. This “endothelial cell activation” has been the subject of previous investigations of our group. In the present thesis it was established for the first time that vascular smooth muscle cells can act as an alternative regulator of vascular tone by the sustained release of PGI<sub>2</sub>. The biochemistry involved turned out to exert significant differences compared with the endothelium and allowed to describe new aspects of redox signaling.

## **1.2. Regulation of Vascular Tone by the Endothelium**

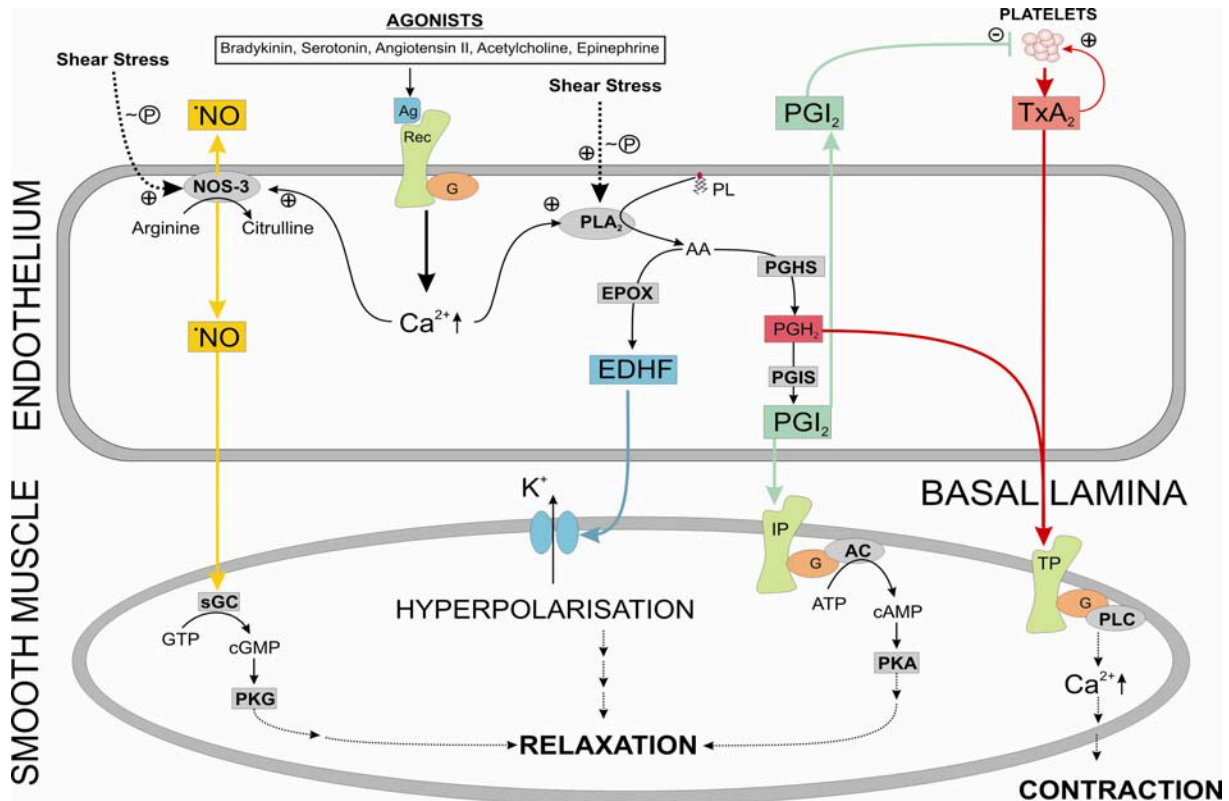
Endothelial cells which cover the vast surface of the whole vascular system can be regarded as the conductor of local vascular tone and are also involved in the recognition and defence of invaded pathogens by orchestrating a complex series of events enabling leukocytes to transmigrate from the blood to the sites of infection. Under normal conditions, the endothelium senses blood pressure through mechanoreceptors which activate the formation of the most relevant endothelial mediators prostacyclin (PGI<sub>2</sub>) and nitric oxide (•NO) (Vane 1990; Cines 1998; Vane 1993). Besides their anti-aggregatory and anti-adhesive properties, PGI<sub>2</sub> and •NO both promote relaxation of vascular smooth muscle cells (SMC). PGI<sub>2</sub> binds to its corresponding (IP) receptor on the surface of SMC thus activating intracellular formation of cAMP by adenylyl cyclase which finally results in the lowering of intracellular free Ca<sup>2+</sup>. In contrast, •NO can freely diffuse across membranes and activates soluble guanylyl cyclase in SMC. The subsequent rise of cGMP via several intermediary steps also lowers the levels of intracellular free Ca<sup>2+</sup> in SMC and therefore causes vasodilation. A third mechanism of vasorelaxation can be observed mainly after inhibition of the •NO and PGI<sub>2</sub> pathways. The so-

called endothelial derived hyperpolarizing factor(s) (EDHF) are not yet characterized in detail however they act by promoting an efflux of potassium from smooth muscle cells (SMC) (Fisslthaler 1999; Pfister 1998; Matoba 2003).

Contraction of the smooth muscle can be evoked by a plethora of mediators among the most prominent ones are thromboxane A<sub>2</sub> (TxA<sub>2</sub>), angiotensin II (Ang II), endothelins, thrombin or platelet activating factor (PAF). Since a steady influx of Ca<sup>2+</sup> into SMC is observed also under resting conditions without additional stimulation, relaxation can be regarded as an active process. Of particular interest with respect to the questions addressed in this work is the observation that the prostanoid precursor PGH<sub>2</sub> also acts as a potent vasoconstrictor by interacting with the TxA<sub>2</sub> (TP)-receptor on the surface of SMC or platelets. This scenario arises when PGI<sub>2</sub>-synthase as the major recipient of PGH<sub>2</sub> in endothelial cells is inhibited while cellular prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) activity is still high.

The major enzymatic source of •NO in the normal endothelium is NOS-3 (eNOS), but no definite answer to the question whether the constitutively expressed PGHS-1 or the inducible PGHS-2 mainly contribute to the basal formation of PGI<sub>2</sub> could be found so far. Difficulties arise from the limited validity of *in vitro* data with respect to the situation *in vivo*. In static endothelial cell cultures for example, PGHS-1 is solely expressed (Caughey 2001) whereas administration of a laminar shear force clearly induces PGHS-2 (Topper 1996; Okahara 1998). This induction not only depends on a transcriptional activation but also on a posttranscriptional stabilization of the corresponding mRNA (Inoue 2002). Clinical studies performed with healthy subjects indicated a significant decline in plasma and urinary 6-keto-PGF<sub>1α</sub> levels after oral administration of PGHS-2-specific inhibitors (McAdam 1999; Muscara 2000). Although the endothelium is a likely candidate for the observed PGI<sub>2</sub> formation, the exact sites of formation could not be elucidated in these studies. The initial rationale for developing PGHS-2-specific inhibitors was the assumption that physiological prostanoids are formed by the constitutive PGHS-1 whereas inflammatory prostanoids are

produced via the induction of PGHS-2. In two independent clinical trials (VIGOR, CLASS) (Bombardier 2000; Wright 2002; Silverstein 2000) comparing the effects of the new class of PGHS-2-specific inhibitors versus classical NSAIDs (Non Steroidal Anti Inflammatory Drugs), a significantly higher risk of myocardial infarction in the group receiving the newly developed coxibs was detected. Based on these novel findings, a reinterpretation of the role of PGHS-2 in the endothelium began to emerge. Although PGHS-2 was demonstrated to be induced by shear stress *in vitro* and systemic 6-keto-PGF<sub>1 $\alpha$</sub>  levels were lowered by PGHS-2-specific inhibitors in healthy subjects, an equally distributed induction of PGHS-2 in the vascular endothelium can be ruled out today by the fact



**Figure 1.2.** Biochemical pathways in the regulation of vascular tone. (AA: arachidonic acid; Ag: agonist; AC: adenylyl cyclase; PGHS: prostaglandin endoperoxide H<sub>2</sub> synthase; EDHF: endothelium-derived hyperpolarization factor; EPOX: epoxygenase; G: G protein; NOS: nitric oxide synthase; Rec: receptor; P: protein phosphorylation; PGH<sub>2</sub>: prostaglandin endoperoxide H<sub>2</sub>; PGI<sub>2</sub>: prostacyclin; PGIS: prostacyclin synthase; PKA: protein kinase A; PKG: protein kinase G; PL: phospholipids; PLA<sub>2</sub>: phospholipase A<sub>2</sub>; PLC: phospholipase C; sGC: soluble guanylyl cyclase; TxA<sub>2</sub>: thromboxane A<sub>2</sub>). Adapted from Bachschmid et al., Futura 2003.

that administration of PGHS-2-inhibitors would otherwise have rapid and detrimental effects on vascular homeostasis even in healthy subjects, which is definitely not the case. As a more convincing approach, it is now assumed that PGHS-2 is induced only in restricted areas like branching sites of the vasculature (Verma 2001). For example in patients with atherosclerosis, PGHS-2 expression in the lesions and plasma 6-keto-PGF<sub>1α</sub> levels were markedly elevated (Schonbeck 1999; Chenevard 2003; Husain 1998). Since the participants in the VIGOR and CLASS studies were at higher risk due to already existing coronary and vascular syndromes, the specific inhibition of PGHS-2-derived PGI<sub>2</sub> could have further triggered the preexisting disorders in these patients.

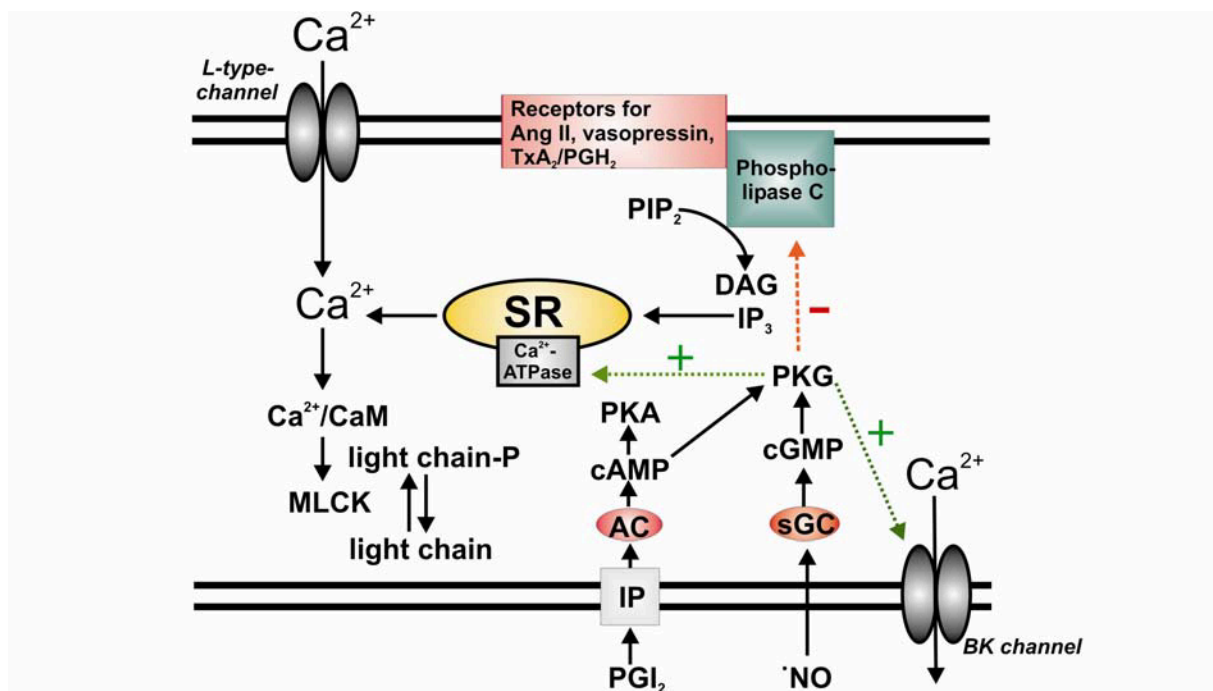
Classical NSAIDs like aspirin, which inhibit both isoforms of PGHS, did not evoke such an imbalance in vascular homeostasis. It has to be remembered that in the vascular system, a well balanced interplay between the vasodilatory, anti-adhesive and anti-aggregatory PGI<sub>2</sub> and the mainly platelet-derived TxA<sub>2</sub>, which evokes the opposing effects, exists. In contrast to the close coupling of endothelial PGI<sub>2</sub> synthesis to the induction of PGHS-2 (Ueno 2001), TxA<sub>2</sub> nearly exclusively originates from PGHS-1 in platelets. Both mediators interact by several pathways in a complex and not yet fully understood network which can be regarded as a perfect example of the Yin-Yang principle. In human syndromes with platelet activation for example, biosynthesis of PGI<sub>2</sub> is elevated due to an upregulation of endothelial PGHS-2 expression by TxA<sub>2</sub> (Fitzgerald 1991, 2001). Arachidonate or PGH<sub>2</sub> released by platelets were demonstrated to be processed by endothelial cells to PGI<sub>2</sub> (Marcus 1980). Furthermore, a cross-talk between the TP and IP-receptor-dependent pathways was observed. TP desensitization was shown to evoke sensitization to IP agonists whereas IP activation evokes desensitization of the TP-mediated signaling cascade (Murray 1990; Walsh 2000; Cheng 2002). Specific inhibition of PGHS-2 which at least under conditions of cellular activation provides PGI<sub>2</sub> synthase with substrate would therefore result in an imbalance of this finely

tuned and self-regulating Yin-Yang network which exists between vascular endothelial cells and platelets. Such an imbalance can also be observed after exposure of a vessel strip to endotoxin (LPS, lipopolysaccharide). Under these conditions, endothelial PGI<sub>2</sub> synthase undergoes a peculiar process of <sup>•</sup>NO/<sup>•</sup>O<sub>2</sub><sup>-</sup> dependent Tyr-nitration and inhibition (Zou 1996, 1997). PGH<sub>2</sub> is still formed under such circumstances and can cause contraction of the adjacent smooth muscle and aggregation of platelets after about 1h of LPS exposure. Since this inhibition of endothelial PGI<sub>2</sub> synthase was at variance with clinical observations of a PGI<sub>2</sub>-mediated hypotension in septic patients, vascular smooth muscle cells are suggested in this work as an alternative regulator of vascular tone under severe pathophysiological conditions.

### **1.3. Contraction and Relaxation of Vascular Smooth Muscle Cells**

Smooth muscle cells can be found in almost every organ of the mammalian body. In the vasculature they are responsible for the generation of the vascular tone under conductorship of the endothelium. Single smooth muscle fibers are long, spindle-shaped cells with pointed ends (Small 1977) whose mechanical properties arise from the cytoskeleton and the contractile apparatus. The cytoskeleton is composed of longitudinal fibrils, consisting of intermediate filaments, cytoplasmic actin, and dense bodies. Branching of some intermediate filaments between fibrils and attachment to the membrane skeleton serves to maintain the three-dimensional integrity of the cytoskeleton (Bagby 1983). The contractile apparatus is composed of polar myosin filaments and actin filaments which are anchored at the cytoplasmic dense bodies and at the membrane skeleton (Trybus 1991). Smooth muscle activation by angiotensin II, thromboxane A<sub>2</sub> (TxA<sub>2</sub>), endothelin etc. results in increased

intracellular levels of free  $\text{Ca}^{2+}$ . The resulting  $\text{Ca}^{2+}$ /calmodulin complex activates myosin light chain kinase (MLCK) which subsequently phosphorylates the myosin light chain and therefore enables a myosin/actin-interaction necessary for contraction. The increase of intracellular  $\text{Ca}^{2+}$  can either be provoked by voltage-gated  $\text{Ca}^{2+}$ -channels (Hamaguchi 1992, Bolotina 1994) or through an inositol trisphosphate ( $\text{IP}_3$ )-mediated release from the sarcoplasmic reticulum (Krippeit-Drews 1992, Raeymaekers 1988, Sarcevic 1989).  $\text{IP}_3$  is formed by the catalytic action of phospholipase C (PLC) which is linked via G-proteins with the receptors of the corresponding agonists located on the surface of the smooth muscle cell.  $\text{IP}_3$  acts as a receptor agonist on intracellular  $\text{Ca}^{2+}$  storage sites causing a transient increase of  $\text{Ca}^{2+}$ .



**Figure 1.3. Contraction and relaxation of smooth muscle cells.**  $\text{Ca}^{2+}$  regulates contraction of smooth muscle cells by mediating a  $\text{Ca}^{2+}/\text{CaM}$ -dependent activation of the myosin light chain kinase (MLCK). Phosphorylation of the myosin light chain (MLC) by MLCK is a prerequisite for the interaction with actin and therefore smooth muscle contraction. Intracellular free  $\text{Ca}^{2+}$  is either elevated by an influx from the extracellular space via L-type channels or it is liberated from the sarcoplasmic reticulum (SR). This release from endogenous storage compartments is mediated by an agonist-triggered activation of phospholipase C (PLC). It catalyzes the conversion of phosphatidylinositol-bisphosphate ( $\text{PIP}_2$ ) to diacylglycerol (DAG) and inositol trisphosphate ( $\text{IP}_3$ ). Nitric oxide (NO) activates soluble guanylyl cyclase (sGC), the resulting elevated levels of cGMP activate protein kinase G (PKG). Receptor-mediated activation of protein kinase A (PKA) occurs in response to elevated cAMP levels after prostacyclin ( $\text{PGI}_2$ ) binding to its IP-receptor. PKA and PKG mediate phosphorylation and inactivation of MLCK. Additionally, PKG may inhibit PLC activation, stimulate removal of intracellular free  $\text{Ca}^{2+}$  by activating BK channels which export cytosolic  $\text{Ca}^{2+}$  to the extracellular space or stimulate  $\text{Ca}^{2+}$ -ATPase activity thus increasing the uptake of cytosolic  $\text{Ca}^{2+}$  by the SR.

Relaxation of smooth muscle cells is mainly mediated by endothelium-derived nitric oxide ( $\bullet\text{NO}$ ) or prostacyclin ( $\text{PGI}_2$ ). While  $\text{PGI}_2$  activates its corresponding receptor on the surface of the cell resulting in a G-protein-mediated activation of adenylyl cyclase and the subsequent increase of intracellular cAMP,  $\bullet\text{NO}$  can freely diffuse into the cytosol of the target cell. Murad and Ignarro first identified  $\bullet\text{NO}$  as the activator of soluble guanylyl cyclase resulting in an increase of intracellular cGMP (Katsuki 1977, Gruetter 1979). Lincoln and Johnson reported later that cGMP leads to a lowering of intracellular free  $\text{Ca}^{2+}$  by activating the energy-dependent uptake of cytosolic  $\text{Ca}^{2+}$  into the sarcoplasmic reticulum (Lincoln 1983, Johnson 1985).

The major intracellular targets for cGMP and cAMP are protein kinase G (PKG) and protein kinase A (PKA), respectively. The original hypothesis that cAMP-mediated relaxation occurs in response to phosphorylation of MLCK by PKA (Conti 1981), which inhibits binding of the  $\text{Ca}^{2+}$ /calmodulin complex, has been modified by newer findings demonstrating that cAMP mediates SMC relaxation mainly by the activation of PKG (Francis 1988, Lincoln 1990). It was found that PKG expression is lost upon passaging of rat smooth muscle cells and this loss was accompanied by a declined capacity of the cells to lower intracellular free  $\text{Ca}^{2+}$ . Agents that elevate cAMP failed to reduce  $\text{Ca}^{2+}$  levels even though PKA was present. When PKG was restored in these passaged cells, the elevation in cAMP resulted again in the reduction of intracellular free  $\text{Ca}^{2+}$ .

It can be summarized that among all intracellular effectors of the  $\bullet\text{NO}$  signaling pathway the most important is PKG.  $\text{Ca}^{2+}$ -mobilization, activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and reorganization of the cytoskeleton have already been attributed to PKG activity whereas a sensitivity adjustment of the contractile apparatus to  $\text{Ca}^{2+}$  or a regulation of gene expression is still under debate.

## 1.4. The Pathophysiology of Sepsis

Sepsis and its associated syndromes is the leading cause of death in critically ill patients in western countries (Angus 2001). It was defined as “the systemic inflammatory response syndrome that occurs during infection” (Bone 1992). According to the current view, the systemic inflammatory response syndrome (SIRS), sepsis and septic shock represent a continuum of severity of the host response, however no clear-cut parameters can be defined to distinguish between the different stages (Wort 1999). Mortality rises from 7% for patients with SIRS to 50-90% for those with septic shock (Rangel-Frausto 1995; Bone 1991). Endotoxin is regarded as the initiator of mortality which arises mainly from an overproduction of secondary induced host mediators (Warren 1997).

In higher vertebrates the Gram-negative bacterial cell envelope constituent endotoxin or lipopolysaccharide (LPS) evokes a plethora of host-defence mechanisms which often end in life-threatening events like vascular collapse when present systemically (Raetz 1990; Raetz 2002). As the name implies, LPS is a glycolipid composed of lipid A and a polysaccharide region which can be divided into a core region and a O-specific chain. The O-specific chain elicits the specific immune response of the host and composes of a polymer of repeating oligosaccharides which can vary largely from strain to strain and even within a serotype (Rietschel 1992, 1993, 1994). Lipid A is a phosphoglycolipid composed of two glucosamine sugars, each containing a phosphate group and fatty acid chains and is the portion of the LPS molecule detected by the TLR4 receptor (Toll-Like Receptor) of the innate immune system and responsible for most of its detrimental biological properties. In an *in vivo* model of sepsis, a biological inactive analog of lipid A protected from LPS-induced reactions of the host (Soejima 1996). In cells of the innate immune response, membrane-bound CD14 (mCD14) is the receptor which supports binding of LPS and activation of the cell (Viriyakosol 1995). Soluble CD14 (sCD14) is present in the blood and responsible for activation of cells that do

not express mCD14 (Frey 1992; Arditi 1993). For efficient binding of LPS to the CD14 receptor, the so-called LPS binding protein (LBP), a hepatocyte-derived acute phase protein present in human serum, is believed to shuttle LPS to the cell surface where it supports binding of LPS to mCD14 (Schumann 1994; Schumann 1990). As a second task, LBP conveys LPS into lipoprotein particles thus supporting neutralization of LPS (Wurfel 1994).

LPS has been demonstrated to accelerate the movement of radiolabeled tracers across the vascular endothelial barrier. Such changes were reported to depend on F-actin depolymerization associated with intercellular gap formation in a protein synthesis independent way (Goldblum 1993). The sensitivity of endothelial cells towards LPS not only depends on the species or the anatomical site within a given species but was also reported highly sensitive towards the presence of serum (Meyrick 1986; Ishii 1995; Arditi 1993). While in the presence of 10% fetal calf serum LPS concentrations of 0.5ng/ml were sufficient to evoke a transendothelial flux of marker molecules, up to 100µg/ml of LPS were required to achieve comparable effects in the complete absence of serum. The orchestrated sequence of events LPS triggers in confluent vascular endothelial cell barriers finally ends up in intercellular gap formation (Brigham 1986), increased transendothelial transport (Goldblum 1993) and in some cases even in the partial detachment of endothelial cells (Reidy 1983; Gaynor 1970). This altogether allows LPS access to the underlying tunica media and was one of the rationales for studying the involvement of vascular smooth muscle cells in the pathogenesis of septic shock.

A first approach to block the effects of LPS was the production of monoclonal IgM antibodies directed against the lipid A portion of LPS (Ziegler 1991; Greenman 1991). Following first promising studies, the attempt failed due to the relatively low affinity of the antibodies used and the fact that such treatment can only be performed in response to bacterial infections. A

single initial activation of vascular endothelial cells by LPS was demonstrated sufficient for the detrimental outcome of sepsis without significant benefit from the removal of endotoxin in progressive stages of exposure. Another promising pharmacological target in focus of many research groups could be the LPS receptor CD14. CD14 mediates signaling to the nucleus of the responding cell by yet poorly understood mechanisms which however could become subject of pharmacological interventions in the future (Warren 1997; Viriyakosol 1995; Ulevitch 1994). The disappointing results obtained with antiinflammatory agents in the treatment of the sepsis syndrome, for example corticosteroids (Bone 1987), anti-endotoxin antibodies (Ziegler 1991), TNF-antagonists (Fisher 1996; Abraham 1995) or IL-1 receptor antagonists (Fisher 1994) raised the question whether death of patients with sepsis really results from an uncontrolled systemic inflammation. The multiple organ dysfunction syndrome (MODS) is usually regarded as the major cause of death in response to septic shock. However, although severe pathological disorders like myocardial depression, respiratory distress syndrome, renal failure or liver dysfunction can be observed in septic patients, the exact cause of death remains elusive (Hotchkiss 1999).

It is therefore more and more believed that the failure of these strategies may be due to a change in the syndrome over time (Lederer 1999; Oberholzer 2001). Initially, sepsis may be indeed characterized by the increase of inflammatory mediators as assumed in the past, but as sepsis persists, one could predict a shift towards an antiinflammatory, immunosuppressive state. This current view has to be considered when the formation of prostanoids is discussed in association with endotoxemia or septic shock conditions. Prostanoids have been implicated as important mediators in some of the pathophysiological events occurring during endotoxic shock. In several animal models of acute endotoxemia, plasma levels of PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub>, PGF<sub>2α</sub> and TxB<sub>2</sub> were elevated (Coker 1983, Bult 1980). Decreased systemic vasomotor tone and normal cardiac output are characteristic hemodynamic features of human septic shock. PGHS inhibitors were shown to negatively affect this hypotension in systemic endotoxemia

(Fletcher 1977, Fletcher 1978, Wise 1980). From these observations cautious optimism may arise regarding the clinical efficacy of PGHS-inhibitors as a means of pharmacological intervention for the treatment of the sepsis syndrome. However, time-dependent changes in the formation of prostanoids, which could be caused by variations in the induction pattern or by posttranslational modifications of the enzymes involved, would question a generalized strategy for the treatment of such patients with inhibitors of the arachidonic acid cascade.

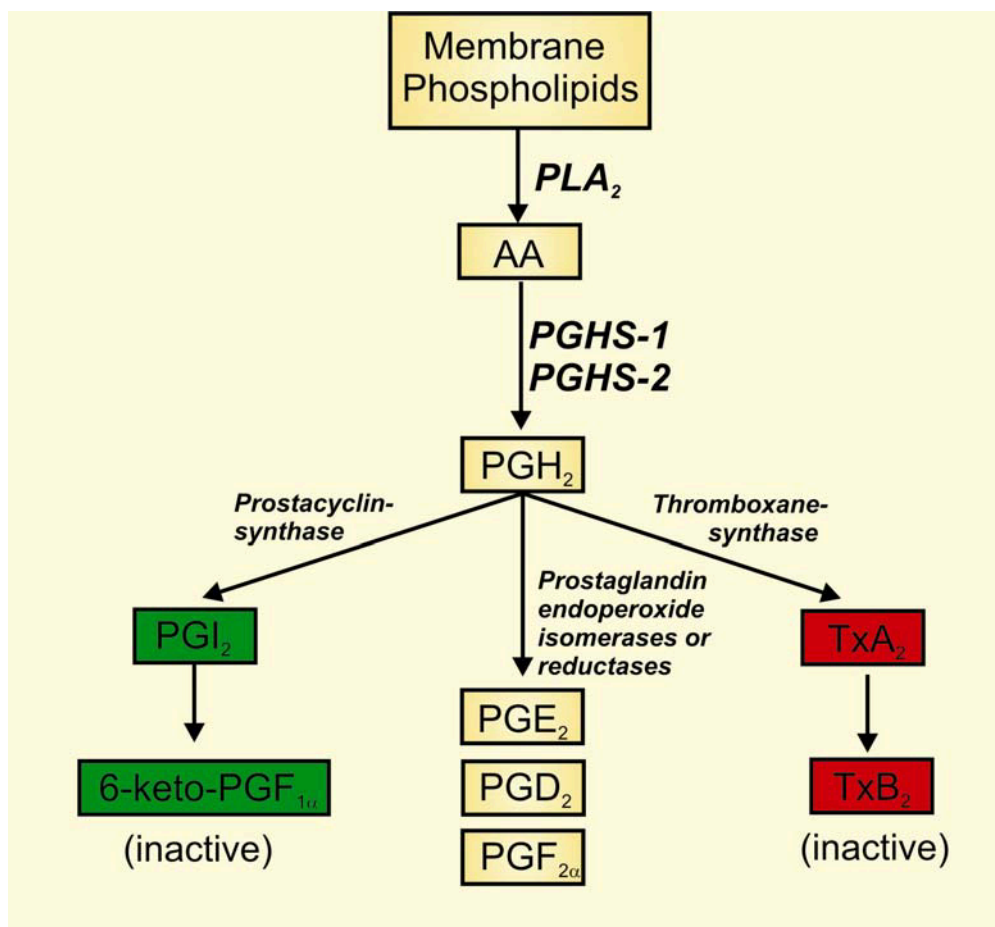
### **1.5. Eicosanoid Biosynthesis**

The biosynthesis of prostanoids includes a sequence of three enzymatic steps which is initiated by the release of arachidonic acid from membrane-glycerophospholipids by phospholipase A<sub>2</sub> followed by the oxygenation of arachidonate by prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) yielding the prostaglandin endoperoxide H<sub>2</sub> (PGH<sub>2</sub>) and finally its conversion to the primary prostanoids prostacyclin (PGI<sub>2</sub>), PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) via their corresponding synthases.

Arachidonic acid (20:4 n-6) which is usually found in the sn-2 position of membrane phospholipids serves as the major precursor of prostanoid biosynthesis and is liberated by PLA<sub>2</sub>-mediated deacylation (Dennis 1994; Dennis 1997). One interesting feature of arachidonic acid is that it cannot be synthesized *de novo* in human cells but must be either obtained through the diet or through conversion of linoleic acid by elongation and desaturation (Jeffcoat 1979). Two main types of PLA<sub>2</sub> are classified with respect to their biological properties: secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) (Cupillard 1997; Tischfield 1997) which exerts no significant fatty acid selectivity and cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) (Leslie 1997; Clark 1995) which possesses a preference for phospholipids containing arachidonate. cPLA<sub>2</sub> translocates to membranes in response to increased intracellular Ca<sup>2+</sup> levels and is activated after

phosphorylation by MAP-kinases. A general assumption on a specific coupling of one PLA<sub>2</sub> isoform to the synthesis of certain prostanoids is hard to deduce however in those cells that contain sPLA<sub>2</sub> most of the arachidonate release appears to be catalyzed by sPLA<sub>2</sub>, while activation of cPLA<sub>2</sub> seems to be a prerequisite for full sPLA<sub>2</sub> activity (Balsinde 1996; Balsinde 1994; Naraba 1998).

Arachidonic acid is present in relatively high concentrations in plasma at levels ranging from 1-5µg/ml (Manku 1983), though, as it is tightly protein-bound, it cannot exert pharmacological effects in this state. In contrast, the intracellular level of free arachidonate in resting cells is very low due to the activity of acyl transferases that facilitate the reincorporation of unesterified arachidonate into phospholipids (Chilton 1987).



**Figure 1.4.** The biosynthesis of prostanooids involves three enzymatic steps. Arachidonate (AA) is released by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and converted by prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) to PGH<sub>2</sub> which is the substrate for specific synthases to generate prostacyclin (PGI<sub>2</sub>), PGE<sub>2</sub>, PGD<sub>2</sub>, and thromboxane A<sub>2</sub> (TxA<sub>2</sub>).

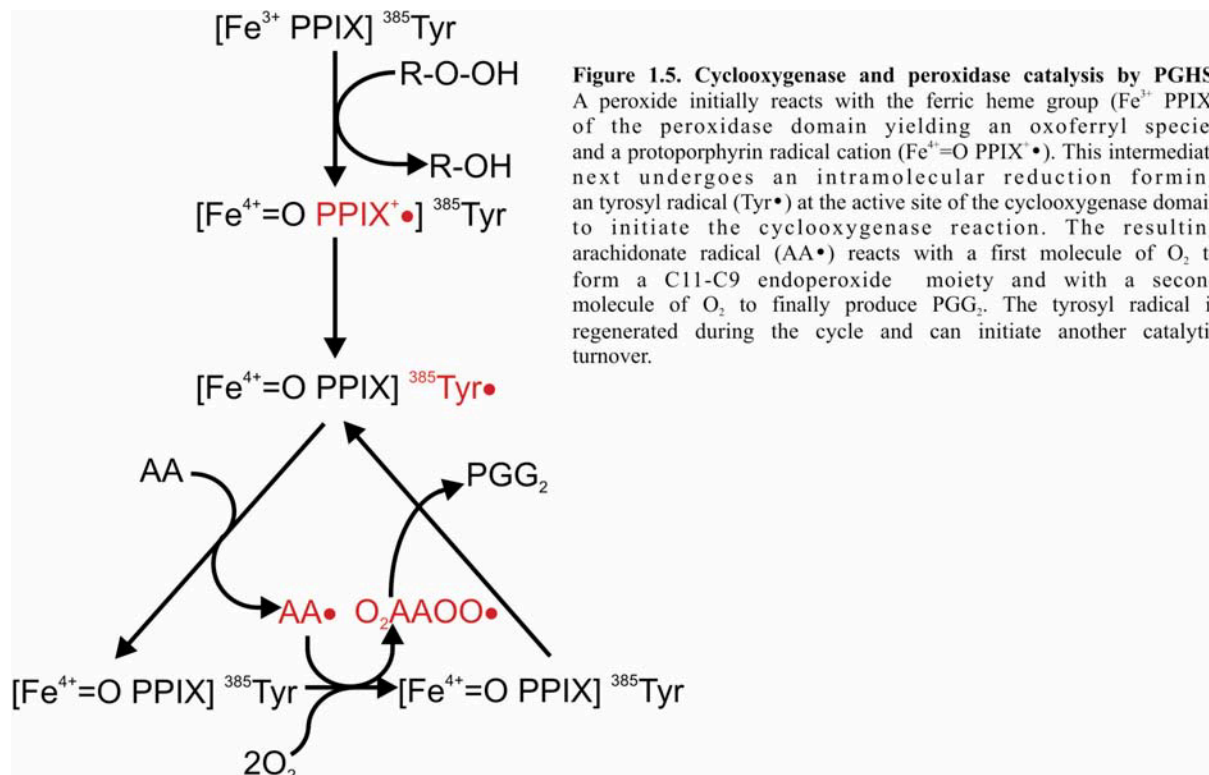
The committed step in prostanoid biosynthesis is mediated by prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) which catalyzes the irreversible cyclization and reduction to form the 15-hydroxy-9,11-endoperoxide (PGH<sub>2</sub>). The enzyme, which is the main target of NSAIDs (Non-Steroidal Anti Inflammatory Drugs) like aspirin or diclofenac (Lecomte 1994, Vane 1998) is expressed either constitutively (PGHS-1) in most mammalian tissues or it is induced in response to endotoxin, cytokines, growth factors, hormones or tumor promoters (PGHS-2). The crystal structures of PGHS-1 and PGHS-2 are remarkably similar (Picot 1994), with two small variations in the amino acid sequence that lead to a larger side-pocket for substrate access in the cyclooxygenase-domain of PGHS-2. Ile in PGHS-1 is exchanged for Val in PGHS-2 at the positions 434 and 523 (Luong 1996, Kurumbail 1996). Due to the reduced sterical inhibition of the cyclooxygenase channel by Val, PGHS-2 can accept a wider range of fatty acids as substrates than PGHS-1 (Otto 1995). PGH<sub>2</sub> is then transferred by a variety of different terminal synthases and nonenzymatic mechanisms into the primary prostanoids which are guided outside the cell in a carrier-mediated process and activate their corresponding heterotrimeric G-protein linked receptors on target cells (Wright 2001).

## **1.6. Catalytic Mechanism of Prostaglandin H<sub>2</sub> Synthase**

Prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) which is also known as cyclooxygenase (COX) is a homodimeric, membrane-bound enzyme which catalyzes the committed step in prostanoid biosynthesis (Smith 2000; Garavito 2003; Marnett 1999; Smith 1996). The discovery of this enzyme and its mechanistic and crystallographic investigation was stimulated by its identification as the major target of acetylsalicylic acid (aspirin) and other NSAIDs by Smith and Lands in the 1970s (Smith 1972; Garavito 1999; Hamberg 1973; Vane 1971). A second wave of attention was focused on this bifunctional enzyme in the early 1990s when a second inducible isoform (PGHS-2) was identified (Fu 1990; Xie 1991; Kujubu 1991; O'Bannon 1991). Sequence analysis revealed 60-65% identity between the two isoforms within the same species while sequence identity between different species varies from 85-90% for the compared isoforms respectively (Smith 1996; Otto 1996; Spencer 1999; Picot 1994; Luong 1996; Kurumbail 1996). This similarity is also reflected in the catalytic and structural similarities that exist between PGHS-1 and PGHS-2.

The PGHS enzyme consists of two distinct but interconnected catalytic sites: the cyclooxygenase domain is localized in a hydrophobic channel near the core of the enzyme whereas the peroxidase active site is located on the surface of the enzyme (Marnett 2000). The mechanism which connects the cyclooxygenase and the peroxidase domain has been discussed controversial (Bakovic 1994; Wei 1995). A branched-chain reaction mechanism for PGHS, which is generally accepted today, was first proposed by Ruf and colleagues and further confirmed by Kulmacz (Karthein 1988; Dietz 1988; Wei 1995). According to this model, the catalytic cycle is initiated by a peroxide which oxidizes the heme prosthetic group

of the peroxidase domain to form Intermediate I ( $\text{Fe}^{4+}=\text{O PPIX}^{+\bullet}$ ) which carries one oxidizing equivalent on the ferryl iron and the other as a protoporphyrin radical cation (Landino 1997; Lambeir 1985; Lu 1999).



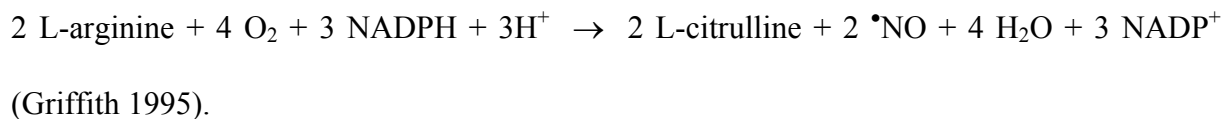
The connection between the cyclooxygenase and the peroxidase site is provided by an intramolecular electron transfer from  $\text{Fe}^{4+}=\text{O PPIX}^{+\bullet}$  to a tyrosine residue (Tyr<sup>385</sup> in PGHS-1, Tyr<sup>371</sup> in PGHS-2) in the cyclooxygenase site yielding Intermediate II ( $\text{Fe}^{4+}=\text{O PPIX}$ ) and a tyrosyl radical (Tyr $\bullet$ ) (Tsai 2000; Tsai 1994; Shimokawa 1990; Xiao 1997; Tsai 1998). When arachidonate as the preferred fatty acid substrate is bound at the cyclooxygenase active site, the tyrosyl radical initiates the cyclooxygenase reaction by abstracting a hydrogen atom at C-13 of arachidonate yielding an arachidonate radical (Tsai 1998). This radical reacts with a first molecule of dioxygen to form a C9- C11 endoperoxide bond and then with a second molecule of dioxygen to generate a hydroperoxy-endoperoxide at C15 ( $\text{PGG}_2$ ) which is subsequently reduced by the peroxidase domain to yield the 15-hydroxy-9,11-endoperoxide ( $\text{PGH}_2$ )

(Hamberg 1973; Hamberg 1974). Under *in vitro* conditions, the interplay between the cyclooxygenase and the peroxidase sites represents a cycle which requires a first initiation by peroxides and would afterwards maintain catalysis in an autocatalytic manner since PGG<sub>2</sub> is itself a hydroperoxide with the capacity to regenerate Intermediate II (Fe<sup>4+</sup>=O PPIX Tyr<sup>•</sup>) and therefore allow a new catalytic cycle (Camper 1985; Montesano 1985). In a cell however, a large excess of antioxidants and peroxide scavengers like urate, ascorbate or glutathione peroxidase/ glutathione exists which efficiently keep the intracellular hydroperoxide level low (Capdevila 1995; Buckley 1991). Therefore, for sustained formation of PGH<sub>2</sub> the presence of constant intracellular levels of peroxides for reactivation of the enzyme is required. This necessity of peroxides for full PGHS catalytic turnover was summarized in literature with the term “peroxide tone”. Interestingly, the peroxide tone for the constitutively expressed PGHS-1 (21nM) was described 10-fold higher than that of the inducible PGHS-2 (2nM) (Kulmacz 1995). As a result of this work, this difference has profound effects on the outcome of prostanoid biosynthesis both under physiological and pathophysiological conditions.

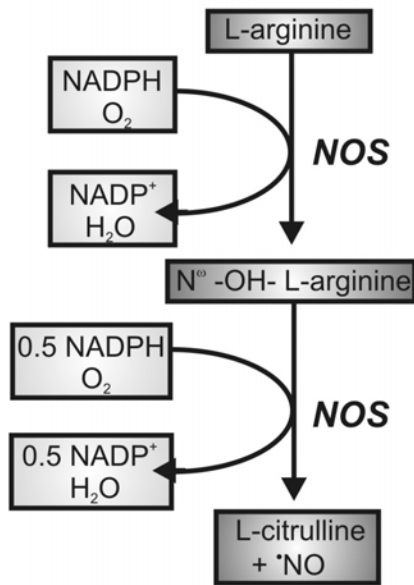
## 1.7. Catalytic Mechanism of Nitric Oxide Synthases

In the early 1980s, Tannenbaum and colleagues realized that animals excreted more nitrate than they were supplied with the food (Tannenbaum 1978; Green 1981). A couple of years later the group of Furchgott described an endothelium-dependent mediator evoking relaxation of vascular smooth muscle cells (Furchgott 1984). Since the chemical structure was unknown at that time, the metabolite was termed “Endothelium Derived Relaxing Factor” (EDRF) and later identified as nitric oxide ( $\bullet\text{NO}$ ) (Ignarro 1987).

The ubiquitous intracellular messenger  $\bullet\text{NO}$  is produced by the oxygenation of L-arginine to L-citrulline by one of three described isoforms of the heme-containing NO-synthase according to the equation:

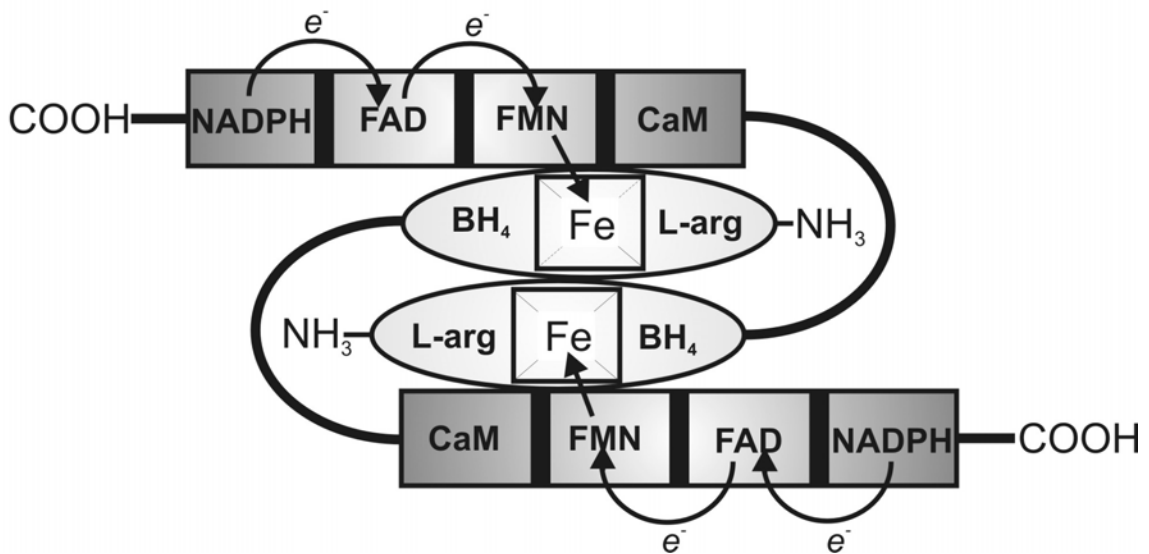


While neuronal NOS-1 (nNOS) and endothelial NOS-3 (eNOS) (Bredt 1990; Schmidt 1991) are constitutively expressed and activated by increased levels of intracellular free  $\text{Ca}^{2+}$ , inducible NOS-2 (iNOS) (Hevel 1991; Stuehr 1991) is usually induced in response to various pathological stimuli and tightly binds  $\text{Ca}^{2+}$ /calmodulin which allows independence from varying intracellular  $\text{Ca}^{2+}$  levels. Active NO-synthases possess a homodimeric structure in a head to head subunit alignment connected by a central  $\text{Ca}^{2+}$ /calmodulin binding region (Marletta 1993). The distal C-terminal half of a NOS monomer functions as a reductase domain transferring electrons from NADPH to the heme prosthetic group of the oxygenase domain. Electrons from the reductase domain of one subunit are shuttled via four redox active prosthetic groups (FAD, FMN, PPIX and  $\text{BH}_4$ ) to the oxidase domain of the other subunit to finally activate  $\text{O}_2$  at the heme (Siddhanta 1998).

**A****Figure 1.6. Mechanisms of <sup>•</sup>NO-synthesis**

**A)** All three isoforms of NOS are thought to catalyze the production of <sup>•</sup>NO by the same biochemical pathway. One molecule of L-arginine is oxidized to an N<sup>ω</sup>-OH-L-arginine intermediate which is further oxidized to yield one molecule of <sup>•</sup>NO and L-citrulline. 1.5NADPH and 2O<sub>2</sub> are converted to 1.5 NADP<sup>+</sup> and 2 H<sub>2</sub>O.

**B)** The functional NOS enzyme consists of two identical subunits arranged in a head-to-head alignment. The NOS subunits are comprised of two domains connected by a central Ca<sup>2+</sup>/CaM binding region. The reductase domains are attached as independent extensions to the dimeric oxygenase core. The oxygenase domain contains binding sites for L-arginine, tetrahydrobiopterin (BH<sub>4</sub>) and heme. The C-terminal half represents a reductase domain that binds FMN, FAD and NADPH. Electrons from NADPH pass along the reductase domain via FAD and FMN. The reduced heme of the oxygenase domain can then bind and activate O<sub>2</sub> for <sup>•</sup>NO synthesis (*Adapted from Stuehr 2001*).

**B**

The NOS heme is bound at the bottom of a substrate binding channel with free access to the surrounding milieu which allows binding of O<sub>2</sub> (Crane 1997; Abu-Soud 2000). To generate <sup>•</sup>NO, NOS must bind and activate O<sub>2</sub> twice (Stuehr 1991). The NOS oxy-heme intermediates are unstable and tend to release <sup>•</sup>O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in the absence of substrate L-arginine. In case of reduced availability of L-arginine or under oxidative conditions accompanied by decreased levels of the cofactor BH<sub>4</sub> which guarantees rapid reduction of the oxycomplex, the electron

transfer can become “uncoupled” from  $\bullet\text{NO}$  synthesis and instead of  $\bullet\text{NO}$ , NOS can become a source of  $\bullet\text{O}_2^-$  (Stuehr 2001). Several reports suggested  $\text{BH}_4$  as a target for oxidation by peroxynitrite due to a 6-10 times faster reaction than with ascorbate or thiols (Kuzkaya 2003). Alternatively, peroxynitrite was shown to release zinc from the zinc-thiolate cluster of eNOS resulting in the formation of disulfide bonds between the monomers (Zou 2002). In both cases an initial exposure to peroxynitrite results in an uncoupling of NO synthase which then releases both  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$ .

### **1.8. Formation of Superoxide in the Vasculature**

Besides formation of  $\bullet\text{NO}$ , the release of  $\bullet\text{O}_2^-$  is essentially involved in redoxregulation of prostanoid biosynthesis by the vasculature.  $\bullet\text{O}_2^-$  is far less reactive against biological macromolecules than one would expect from its radical nature. Due to the diffusion-limited reaction with  $\bullet\text{NO}$ ,  $\bullet\text{O}_2^-$  represents a central regulatory molecule which can directly adjust the levels of free  $\bullet\text{NO}$  and therefore indirectly the formation of prostanoids as described in this work.

### 1.8.1. NADPH Oxidase

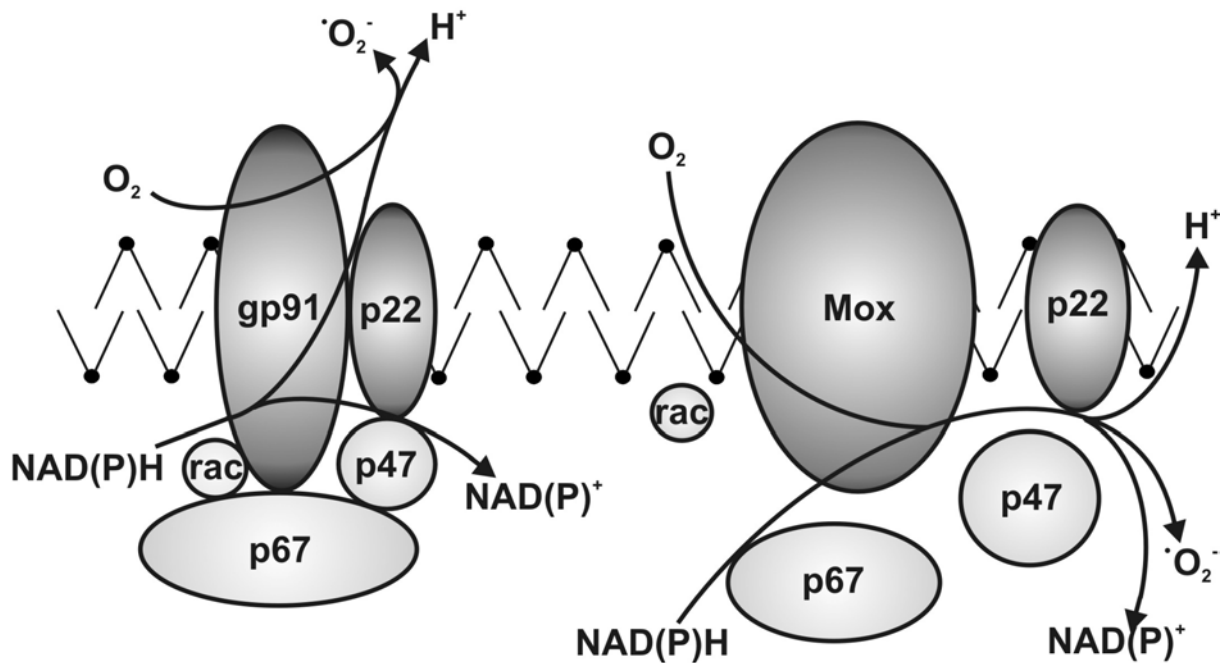
During the past years more and more evidence indicated NADPH oxidase as a major source of  $\bullet\text{O}_2^-$  generation in various cell types and tissues according to the equation :



Originally discovered in neutrophil granulocytes involved in host defence mechanisms (Woodman 1991), it has also been identified abundantly in vascular endothelial cells (Pagano 1993), smooth muscle cells (Griendling 1994) and fibroblasts (Pagano 1997) with a capacity of about one third compared with the neutrophil oxidase (Griendling 1998). In the resting phagocyte the subunits  $\text{p40}^{\text{phox}}$ ,  $\text{p47}^{\text{phox}}$  and  $\text{p67}^{\text{phox}}$  exist as a complex whereas  $\text{p22}^{\text{phox}}$  and  $\text{gp91}^{\text{phox}}$  are located in the membranes of secretory vesicles where they occur as cytochrome  $\text{b}_{558}$  (Griendling 2000). Such separation of the two components prevents from uncontrolled activation of the entire complex in resting cells. Following stimulation,  $\text{p47}^{\text{phox}}$  becomes phosphorylated and the entire cytosolic complex migrates to the membrane to associate with cytochrome  $\text{b}_{558}$ . As the central subunit,  $\text{gp91}^{\text{phox}}$  couples the oxidation of NADPH via an electron shuttle over flavin- and heme- containing proteins with the final one-electron reduction of  $\text{O}_2$  (Babior 1999). An increase in the assembly of the subunits to form the active vascular NADPH oxidase complex was observed by Ang II,  $\text{TNF-}\alpha$ , PAF, LPS and proceeds within minutes to hours (Pagano 1998; Ohara 1993) whereas neutrophil NADPH oxidase can release  $\bullet\text{O}_2^-$  after 20-30 seconds (Jones 1994).

## Neutrophil NAD(P)H Oxidase

## Vascular NAD(P)H Oxidase



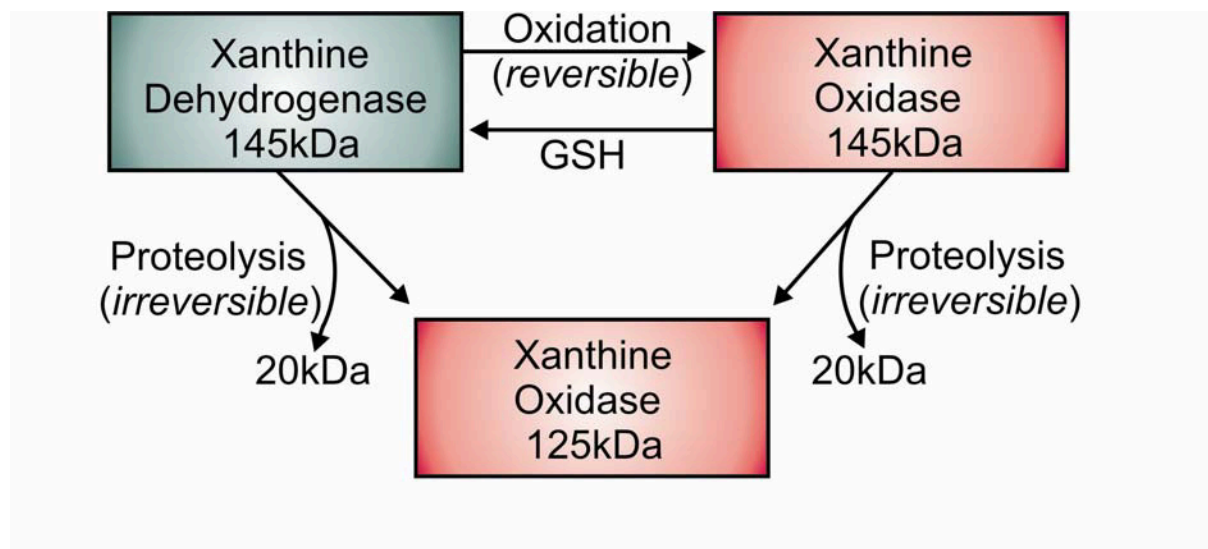
**Figure 1.7. Structure of NADPH oxidases.** The neutrophil NADPH oxidase is comprised of gp91<sup>phox</sup> and p22<sup>phox</sup> which together form the electron transfer component of the oxidase and p47<sup>phox</sup>, p67<sup>phox</sup> and the G-protein rac which are cytosolic components that modulate the activity of the complex. In contrast to the neutrophil oxidase, the vascular complex as identified in vascular smooth muscle cells releases  $\cdot\text{O}_2^-$  mainly into the cytosol. This indicates a profound role of the vascular NADPH-oxidase complex in redoxregulation of prostanoid biosynthesis. (*Adapted from Griendling 2000*)

Activation of protein kinase C (PKC) by  $\cdot\text{O}_2^-$  which additionally phosphorylates the cytosolic p47<sup>phox</sup> and p67<sup>phox</sup> subunits indicates on a positive feed-back loop allowing a sustained activation of the neutrophil NADPH oxidase complex (Griendling 2000). Although vascular oxidases are similar to that in neutrophils, they represent a separate family of enzymes. The major functional difference however is that neutrophil oxidases utilize intracellular NADH or NADPH and transfer the electrons across the membrane to extracellular oxygen whereas cardiovascular NADPH oxidases mainly produce intracellular  $\cdot\text{O}_2^-$  (Zafari 1998). Since  $\cdot\text{O}_2^-$  cannot easily pass biological membranes, the large quantities of intracellular  $\cdot\text{O}_2^-$  generated by the vascular NADPH oxidase complex represent a key regulator of the intracellular redox potential.

### 1.8.2. Xanthine Oxidase

The widely distributed enzyme xanthine oxidoreductase (XOR) was first recognized in association with its involvement in nucleic acid degradation but has in the meantime emerged as a potent source of  $\bullet\text{O}_2^-$  in the vascular system (Parks 1986). Ever since McCord and Fridovich reported that superoxide dismutase functions primarily as an endogenous free radical scavenger (McCord 1968; McCord 1969), XOR became of interest as a cellular generator of superoxide and therefore a potential mediator of physiological and pathophysiological processes *in vivo*. The name xanthine oxidoreductase implies the two distinct forms of the enzyme which can be found as a homodimer in two forms: the constitutively expressed xanthine dehydrogenase (XDH) and the posttranslational modified form xanthine oxidase (XO) usually found under pathological conditions (Hille 1995; Euroth 2001). Transition from the dehydrogenase to the oxidase form requires either reversible thiol oxidation of sulfhydryl residues or irreversible proteolytic cleavage of a segment of XDH (Ichida 1993). A pathophysiological involvement of XO has been found associated with ischemia-reperfusion injury (McCord 1985). During the phase of insufficient supply with  $\text{O}_2$ , both the conversion from XDH to XO and the accumulation of hypoxanthine as a AMP degradation product can be observed (Meneshian 2002). Following reperfusion,  $\text{O}_2$  becomes again available and is effectively reduced by XO yielding high amounts of  $\bullet\text{O}_2^-$ . Apart from  $\bullet\text{O}_2^-$ , active XO also releases uric acid as an end product of adenosine nucleotide depletion which on the other hand is a potent scavenger of peroxynitrite. In contrast to the situation with active NADPH oxidase whose  $\bullet\text{O}_2^-$  also interacts with  $\bullet\text{NO}$  and therefore regulates the intracellular levels of peroxynitrite, activation of XO could rather be regarded as a  $\bullet\text{NO}$ -sink by supporting the formation of peroxynitrite which would subsequently be scavenged by its interaction with uric acid. Since peroxynitrite was elaborated a potent intracellular activator of PGHS in this thesis, the conversion of XDH to XO in progressive stages of cell stimulation

may limit prostanoid biosynthesis and should rather be associated with conditions of severe oxidative stress than with redoxregulation.



**Figure 1.8. Conversion of xanthine dehydrogenase (XDH) into xanthine oxidase (XO).** XDH is composed of two identical independent subunits. Each subunit contains one molybdopterin, two non-identical 2Fe/2S centres and FAD. The two iron-sulphur centres are located in a 20kDa N-terminal domain, the FAD is in a 40kDa intermediate domain and the molybdopterin is in a C-terminal 85kDa domain. XO exists in vivo predominantly as a NAD<sup>+</sup>-dependent dehydrogenase which can be transformed to a oxidase by oxidation, anaerobic conditions or sulfhydryl reagents.

### 1.8.3. Mitochondria as Sources of $\bullet\text{O}_2^-$ and $\bullet\text{NO}$

It is more and more realized that mitochondria are not only autonomous cell organelles but can actively participate in cellular signal transduction pathways. Apart from the described interaction of  $\bullet\text{NO}$  with mitochondrial cytochrome c oxidase and the release of cytochrome c into the cytosol, emphasis is focused on the mitochondrial formation of  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$ . Normal electron transport of the mitochondrial respiratory chain involves a coordinated four electron reduction of  $\text{O}_2$  to  $\text{H}_2\text{O}$  by electrons originating from NADH or succinate. Superoxide is continuously formed as a by-product of accidental one-electron dioxygen reduction and was estimated to account for 1-2% of the total electron flux (Loschen 1973; Loschen 1974). However, due to an exaggerated  $\text{O}_2$  pressure and elevated substrate availability in these *in*

*vitro* experiments, the reported values appear quite overestimated. Similar predictions by other groups were performed in the presence of electron transport chain inhibitors which favor an accumulation of upstream carriers in their reduced form and therefore trigger an elevated rate of autoxidation. Together with findings performed in subcellular fractions of *E. coli* under conditions comparable to those in mitochondria, an effective electron leakage of approximately 0.1% appears as a more plausible value (Fridovich 2004; Imlay 1991).

Mitochondrial NO-synthase (mtNOS) activity was first identified in the mid 1990s and although several doubts had been raised, numerous reports revealed the presence of a NOS in mitochondria (Ghafourifar 1997; Giulivi 1998). Deduced from immunocytochemical and knock-out studies, mtNOS was classified as a constitutively active eNOS-like isoform however different opinions exist (Bates 1995; Kanai 2001). Since all necessary cofactors like L-Arg, L-Arg-transporters,  $\text{Ca}^{2+}$ , calmodulin, NADPH and  $\text{O}_2$  are present (Freedland 1984; Hatare 1982; McCormack 1989), mitochondria can be regarded as a potential source of cellular  $\bullet\text{NO}$  production.  $\bullet\text{NO}$  reversibly inhibits oxygen consumption and ATP synthesis by competitive binding to cytochrome oxidase which stimulated the hypothesis that cellular energy supply might depend on the mitochondrial  $\bullet\text{NO}/\bullet\text{O}_2^-$  ratio (Sarkela 2001; Brown 1994). The impact of mitochondrial  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  production on the cellular redox potential appears rather difficult to estimate. Since  $\bullet\text{O}_2^-$  cannot easily pass biological membranes and due to a high mitochondrial Mn-SOD activity which prevents an accumulation of  $\bullet\text{O}_2^-$ , a direct involvement of mitochondrial  $\bullet\text{O}_2^-$  in cellular redoxregulation seems of minor biological importance. In contrast, intramitochondrially formed  $\bullet\text{NO}$  and peroxynitrite can diffuse into the cytosol and could affect redox-sensitive cytosolic processes. Due to the high sensitivity of the mitochondrial electron transport chain towards reactive nitrogen species, a flux vice versa affecting the respiratory chain by cellular  $\bullet\text{NO}$  derivatives may however play a more pronounced role.

## 1.9. Redoxregulation of Prostanoid Biosynthesis by $\bullet\text{NO}/\bullet\text{O}_2^-$

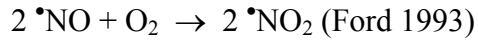
Redoxregulation of prostanoid biosynthesis seems to be basically mediated by  $\bullet\text{NO}$  and its derivatives originating from the interaction with molecular oxygen,  $\bullet\text{O}_2^-$  or other reactive oxygen species. The chemical properties of these players, their time- and compartment-specific formation and degradation as well as feedback inhibitory effects of the  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  forming enzymes exert profound consequences on the outcome of prostanoid biosynthesis (Salvemini 1995; Wen 2000; Stadler 1993). The simple molecule  $\bullet\text{NO}$  whose biological properties were known for a long time and described with the term “Endothelium Derived Relaxing Factor” (EDRF) turned out as a key intra- and intercellular messenger regulating vessel tension, thrombosis and neuronal activity (Gruetter 1979; Radomski 1990; Garthwaite 1988). The binding of  $\bullet\text{NO}$  evokes a large increase in the  $V_{\text{max}}$  of guanylyl cyclase enabling the conversion of GTP to cGMP. At low concentrations ( $<1\mu\text{M}$ ), such direct effects of  $\bullet\text{NO}$  predominate, whereas at higher concentrations ( $>1\mu\text{M}$ ) the indirect effects prevail. Indirect effects of  $\bullet\text{NO}$  usually accompany the induction of NOS-2 and include

**Nitrosations:** when formally  $\text{NO}^+$  is added to an amine, thiol or hydroxy aromatic group and  $\text{H}^+$  is released.

**Nitrations:** when  $\text{NO}_2^+$  is formally added to a molecule and  $\text{H}^+$  is released.

Due to its phenolic nature, tyrosine becomes a preferred target for nitrations. Nitrotyrosine formation has been demonstrated by immunohistochemical staining in numerous human diseases however only a relatively small number of proteins appear to be nitrated *in vivo*. Although peroxynitrite was thought to be the major nitrating agent *in vivo*, peroxynitrite-independent tyrosine-nitrations exist and include the peroxidatic activation of nitrite.

$\bullet\text{NO}$  is indeed also susceptible for autoxidation according to the equation:



forming two molecules of nitrogen dioxide, a potent oxidant involved in the regulation of prostanoid biosynthesis. In aqueous aerobic solutions  $\cdot\text{NO}_2$  is further converted to yield nitrite, however at the concentrations of  $\cdot\text{NO}$  released *in vivo* this third order kinetics becomes an extremely slow reaction under physiological conditions. While for a saturated 2mM  $\cdot\text{NO}$  solution under aerobic conditions a half-life time of approximately 1s was calculated,  $\cdot\text{NO}$  levels around 5nM, which were found sufficient for smooth muscle relaxation in the vasculature, exerted a  $t_{1/2}$  of nearly 70h (Beckman 1996; Malinski 1993). The information  $\cdot\text{NO}$  encodes therefore depends on its local concentration which implies that the molecule must be rapidly removed to prevent saturation. In higher vertebrates this is mainly accomplished by its interaction with oxyhemoglobin in red blood cells (Doyle 1981). Intracellular levels of  $\cdot\text{NO}$  can also be regulated by superoxide ( $\cdot\text{O}_2^-$ ) since both radicals combine in an almost diffusion-controlled reaction to form peroxynitrite ( $k= 6.7 \cdot 10^9/\text{Ms}$ ;  $-22\text{kcal/mol}$ ) (Huie 1993). Dismutation of  $\cdot\text{O}_2^-$  by superoxide dismutases (SODs) was observed with  $k= 10^7/\text{Ms}$  which is 2-3 orders of magnitude slower than the reaction with  $\cdot\text{NO}$  and allows to conclude the formation of peroxynitrite as soon as  $\cdot\text{NO}$  and  $\cdot\text{O}_2^-$  are available (Cudd 1982; Rigo 1975). The large Gibbs energy of peroxynitrite formation additionally makes the reaction nearly irreversible.

In contrast to the highly diffusible  $\cdot\text{NO}$ ,  $\cdot\text{O}_2^-$  cannot easily pass biological membranes. Since basal amounts of  $\cdot\text{O}_2^-$  are formed even under resting conditions by accidental electron transfer in the mitochondrial respiratory chain, the radical must be detoxified in the compartment in which it is generated. This task is performed by cellular SODs (MnSOD, Cu/ZnSOD) (Beyer 1991) which not only protect from destructive oxygen-derived toxicants but also allow the cell to utilize the remaining low levels of intracellular  $\cdot\text{O}_2^-$  as an adjustable redoxregulator (Ullrich 2000).

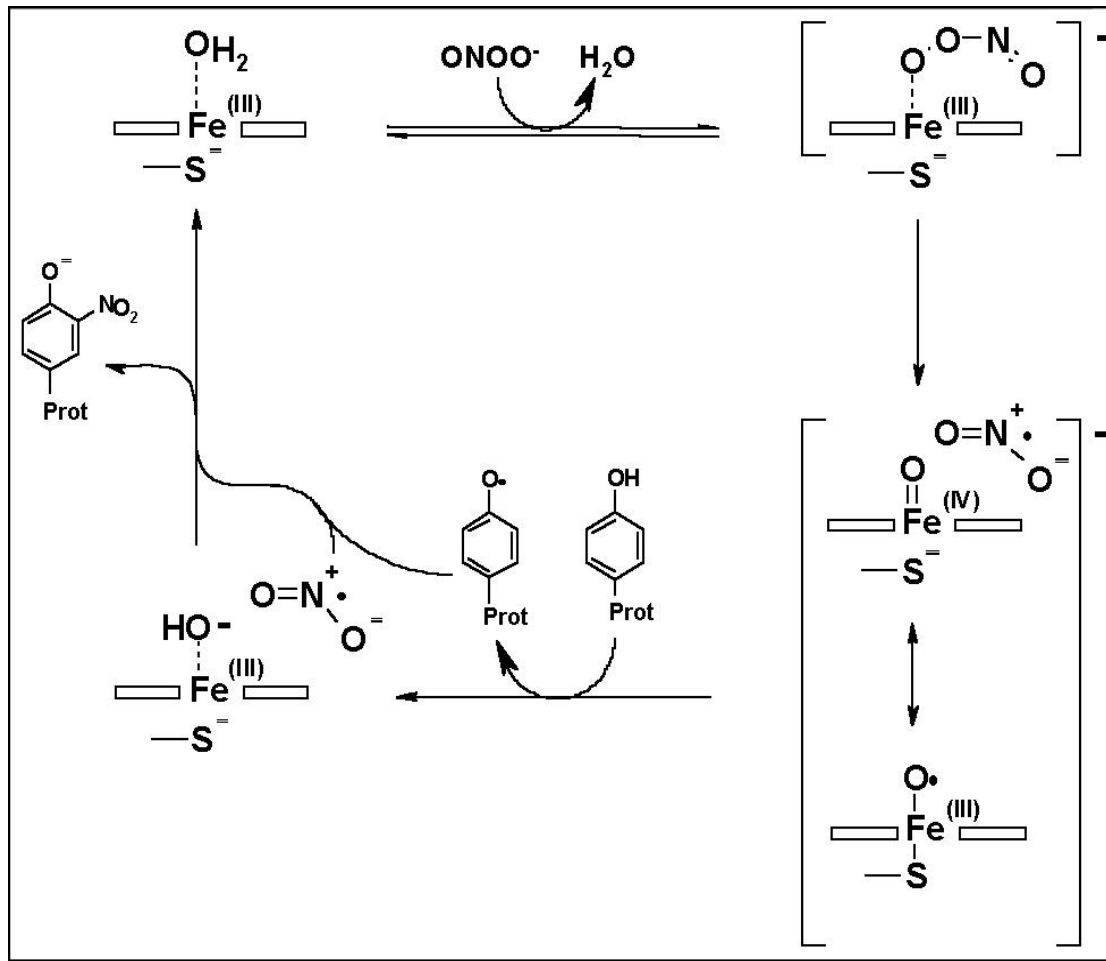
## 1.10. The Biochemical Basis for LPS-Induced Endothelial Dysfunction

Prostacyclin together with  $\bullet\text{NO}$  are generally accepted today as key regulators of vascular homeostasis and integrity. In several previous publications of our group, substantial evidence has emerged that vascular endothelial cells of atherosclerotic lesions, after ischemia-reperfusion or exposure to endotoxin demonstrated nitration of  $\text{PGI}_2$  synthase which was found to be accompanied by an inhibition of the enzyme and impaired relaxation of the vessel in response to angiotensin II stimulation (Zou 1999). Nitration of  $\text{PGI}_2$  synthase was found mediated by peroxynitrite, originating from the interaction of nitric oxide ( $\bullet\text{NO}$ ) and superoxide ( $\bullet\text{O}_2^-$ ) (Zou 1996). In endothelial cells, NOS-3 (eNOS) is the major source of  $\bullet\text{NO}$  production both under physiological and pathophysiological conditions. Superoxide however can originate from different sources following activation of the cell. Besides the assembly of the NADPH-oxidase complex, conversion of xanthine-dehydrogenase to xanthine-oxidase was reported to contribute essentially to the increased  $\bullet\text{O}_2^-$  release observed (Bachschnid 2003). In parallel, electron efflux from the mitochondrial electron transport chain and so-called “uncoupling” of NO synthases must also be taken into account.

Nitrations by peroxynitrite were observed in many proteins, however concentrations between  $100\mu\text{M}$  and  $1\text{mM}$  were usually required for such findings. In contrast, nitration of  $\text{PGI}_2$  synthase was detected at peroxynitrite levels of around  $50\text{-}100\text{nM}$  which can be formed in activated cells (Zou 1997; Schmidt 2003). Interestingly,  $\text{PGI}_2$  synthase was elaborated as the only nitrated protein in endothelial cells incubated with bolus additions of peroxynitrite in the nanomolar range. The requirement of such low concentrations can only be explained by the heme nature of the enzyme whose metal ions allow a kinetically preferred pathway leading to a ferryl species and nitrogen dioxide ( $\bullet\text{NO}_2$ ) which is assumed to subsequently nitrate a Tyr-

radical. It was further elucidated that the corresponding tyrosine residue (Y 430, bovine sequence) is localized near the active site in PGI<sub>2</sub> synthase. Since nitration maximally diminished PGI<sub>2</sub> release by roughly 80% but was never found capable to completely downregulate catalysis, a sterical inhibition by the nitro group seems the most plausible explanation. Despite its structural and mechanistic similarities, TxA<sub>2</sub> synthase was not found inhibited under comparable pathophysiological situations. Taken together, in the antagonistic interplay between PGI<sub>2</sub> and TxA<sub>2</sub>, TxA<sub>2</sub> represents the activated state in response to different stimuli whereas PGI<sub>2</sub> as the major counterplayer represents the mediator of the resting state. Formation of PGI<sub>2</sub> requires pro-reductive cellular conditions to prevent nitration and inhibition of PGI<sub>2</sub> synthase by peroxynitrite while TxA<sub>2</sub> synthesis is not markedly affected by such conditions (Salmon 1978; Tate 1984). Not only that PGI<sub>2</sub> release is inhibited by elevated levels of peroxynitrite, PGH<sub>2</sub> which is still formed under such circumstances was elaborated to bind and activate the TxA<sub>2</sub> (TP) -receptor on vascular smooth muscle cells (Mais 1985; Ogletree 1985).

The shift from a high cellular antioxidant potential to pro-oxidative conditions therefore not only prevents PGI<sub>2</sub> formation but additionally promotes a TxA<sub>2</sub>-like response by releasing elevated amounts of PGH<sub>2</sub>. Furthermore, the signal transduction capacity of the TP receptor was described to be increased by the oxidation of vicinal dithiols to disulfides under oxidative conditions (D'Angelo 1996; Dorn 1990) favoring a shift from PGI<sub>2</sub> towards the actions of its antagonists.



**Figure 1.9.** Tyrosine-nitration of prostacyclin (PGI<sub>2</sub>) synthase is catalyzed by the heme prosthetic group. The presence of a transition metal allows an energetically preferred pathway which leads to a ferryl species (Fe<sup>4+</sup>=O) and nitrogen dioxide (<sup>•</sup>NO<sub>2</sub>). Generation of the tyrosyl radical is catalyzed by the heme intermediary complex and subsequently reacts with the <sup>•</sup>NO<sub>2</sub> radical to form nitrotyrosine. Adapted from Ullrich 2001.

The vascular system was chosen as a well described system to exemplify the basic principles in the interaction of the <sup>•</sup>NO/<sup>•</sup>O<sub>2</sub><sup>-</sup> couple with prostanoid biosynthesis. These regulatory mechanisms indeed apply also for other cell systems and can be regarded as a general mechanism how redoxregulation can affect prostanoid-mediated physiological and pathophysiological responses of cells, tissues and entire organs.

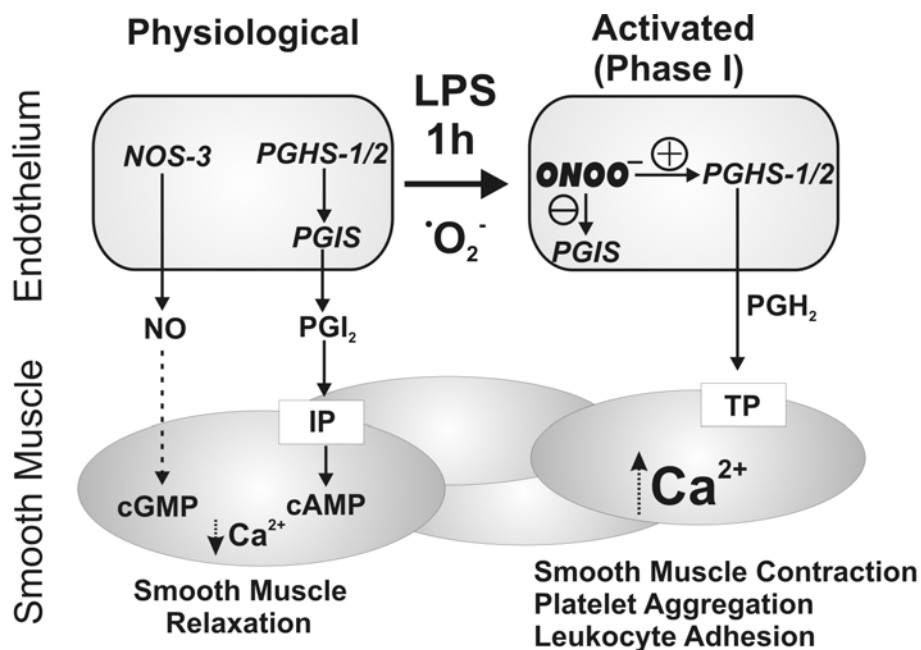
## 1.11. The Vasculature in Sepsis

### 1.11.1. The Physiological Situation

Under physiological conditions, the endothelium functions as the regulator of local vascular tone. It exerts antiadhesive, antiaggregatory and vasodilatory properties which are mainly maintained by a basal release of prostacyclin ( $\text{PGI}_2$ ) and nitric oxide ( $\bullet\text{NO}$ ) (Vallance 1995; Vane 1990). Both endothelial mediators evoke vasorelaxation of adjacent smooth muscle cells of the tunica media (Furchgott 1980; Palmer 1987). The highly diffusible  $\bullet\text{NO}$  activates intracellular soluble guanylyl cyclase in neighboring cells yielding elevated intracellular levels of cGMP (Ignarro 1991) while  $\text{PGI}_2$  binds to its corresponding receptor (IP) on the surface of smooth muscle cells resulting in the activation of adenylyl cyclase (Nakagawa 1994; Gorman 1979; Tateson 1977). Both cGMP and cAMP result in decreased intracellular  $\text{Ca}^{2+}$  levels and therefore cause smooth muscle relaxation. Low levels of  $\text{TxA}_2$  which are formed even in non-activated platelets serve as the major counterplayer of  $\text{PGI}_2$  under resting conditions. This equilibrium favors a tightly closed endothelium and disaggregation of platelets without adhesion of white blood cells passing with the blood stream. It becomes out of balance under pathophysiological conditions like endotoxemia, ischemia-reperfusion or diabetes (Curzen 1994; Hink 2001; Cosentino 1997; Buttery 1996; Beckman 1994). Such conditions are associated with a highly elevated production of  $\bullet\text{O}_2^-$  from xanthine oxidase, NADPH oxidase, mitochondria or uncoupled NO-synthases leading to an efficient trapping of  $\bullet\text{NO}$ .

### 1.11.2. Endothelial Cell Activation

The endothelium can be regarded as a vital part of the host's immune system. It orchestrates a complex series of events enabling leukocytes a directed movement from the blood to the site of an infection. For these purposes the endothelium has to alter from a resting to an "activated" state which is accompanied by a partial loss of its regulatory properties. This well



**Figure 1.10. Endothelial Cell Activation (ECA).** Under physiological conditions, the endothelium maintains an equilibrium of vascular tone by a basal formation of endothelial NO-synthase (NOS-3)-derived nitric oxide (NO) and prostaglandin endoperoxide H<sub>2</sub> synthase-1/2 (PGHS-1/2)-derived prostacyclin (PGI<sub>2</sub>) (left). NO directly enters the smooth muscle cell and activates soluble guanylyl cyclase (sGC) yielding elevated intracellular levels of cGMP. PGI<sub>2</sub> activates its receptor (IP) on the surface of the smooth muscle cell which in a G-protein-mediated way activates adenyl cyclase to form cAMP. By several intermediate pathways, cGMP and cAMP trigger a decline of intracellular free Ca<sup>2+</sup> and therefore smooth muscle relaxation.

known clinical observation can now be explained on the molecular level by previous findings of our group. The initial event in the conversion from a resting to an activated state is an increased intracellular formation of •O<sub>2</sub><sup>-</sup> leading to the following three events:

First, •NO released by activated NOS-3 is trapped by its reaction with •O<sub>2</sub><sup>-</sup> resulting in the formation of peroxynitrite. Peroxynitrite was demonstrated to effectively nitrate and inactivate PGI<sub>2</sub> synthase at concentrations of only 50nM in a heme-thiolate catalyzed

mechanism. As a consequence of PGI<sub>2</sub> synthase nitration, an effective reduction of endothelial PGI<sub>2</sub> formation is observed. Since PGHS-1 is constantly active under such conditions, PGH<sub>2</sub> formation is maintained but fails to serve as substrate for the inhibited PGI<sub>2</sub> synthase. Therefore, as a third effect, unconverted PGH<sub>2</sub> accumulates and binds to the thromboxane (TP) receptor on smooth muscle cells exerting the same vasoconstricting properties as TxA<sub>2</sub> does.

Taken together, upregulation of endothelial  $\bullet\text{O}_2^-$  reduces the levels of both vasodilators PGI<sub>2</sub> and  $\bullet\text{NO}$  and additionally provokes vasoconstriction by a PGH<sub>2</sub>-mediated activation of the TP-receptor.

All these events can be observed within the first hour of stimulation and require no *de novo* protein synthesis. As a physiological consequence, presentation of preformed P-selectin as well as an opening of the endothelial barrier initiates the transmigration of activated leukocytes. These conditions, which are characterized by vascular constriction, were defined by clinicians as “endothelial dysfunction”. This term is rather misleading and should be replaced by “endothelial cell activation” since all the processes are involved in the host immune response and fulfill beneficial functions. In contrast, clinicians are aware of the fact that for instance after inflammatory states or in some cases after vaccination, the vascular endothelium exerts a reduced responsiveness to agonists for several weeks. This state is sometimes designated as “endothelial stunning” (Bhagat 1996; Vallance 1997), however in this case the term “endothelial dysfunction” should be favored.

To further clarify the events during endothelial cell activation we would like to separate the procedures in the activated vasculature into two phases. “**Phase I**” occurs within the first hour of activation and requires no *de novo* protein synthesis. “**Phase II**” is initiated after roughly 2h and includes the induction of early immediate genes like ICAM’s and VCAM’s or PGHS-2 and NOS-2. Phase II is not limited to the endothelium and also includes the vascular smooth muscle which is the major subject of this thesis.

## 2. Materials and Methods

### 2.1. Materials

#### **Smooth muscle experiments:**

Aspirin, LPS (*E. coli*; Serotype 026:B26), IL-1 $\beta$ , TNF- $\alpha$ , glutathione (GSH), hematin, hypoxanthine and glutathione-peroxidase were from Sigma, St. Louis, USA. DuP-697, SC-560, purified PGHS-2 and Spermine-NONOate were from Cayman Chemicals, Ann Arbor, USA. SIN-1 was purchased from Calbiochem/Merck, Darmstadt, Germany. <sup>14</sup>C-AA (0.1mCi/ml) from ARC, St. Louis, MO, USA. Catalase from Roche Diagnostics, Mannheim, Germany. M199, FCS, penicillin, streptomycin and collagenase Type CLS were from Biochrom, Berlin, Germany.

#### **Macrophage experiments:**

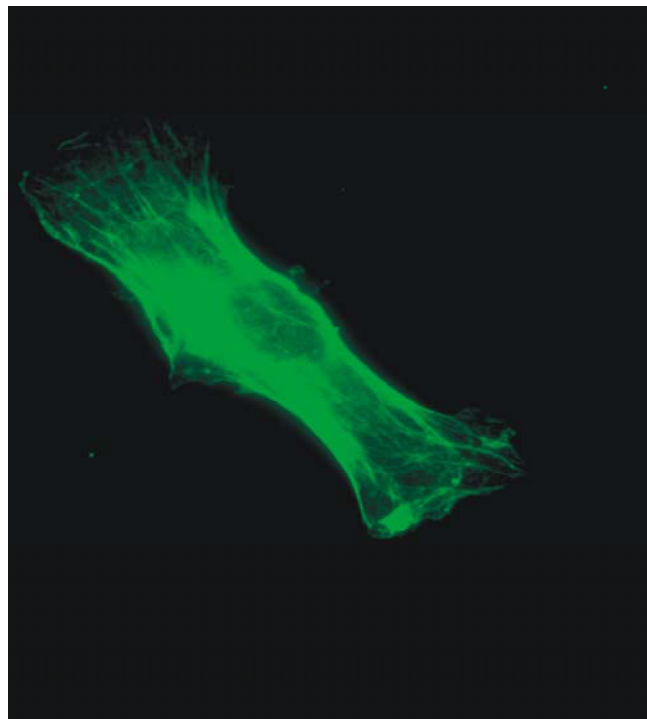
Arachidonic acid, purified PGHS-2, NDGA and Spermine-NONOate were obtained from Cayman Chemicals Co. (Ann Arbor, MI, USA). SIN-1, MPO and catalase were purchased from Calbiochem-Novabiochem (Schwalbach, Germany). Allopurinol, gliotoxin, protein A & G sepharose, DMP, pronase and LPS (*E. coli*; Serotype 026:B26) were purchased from Sigma (St. Louis, MO, USA). AMT was a product of Alexis Biochemicals (San Diego, USA). Anti-PGHS-2 and anti-NOS-2 monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA) and the monoclonal and polyclonal antibodies against 3-nitrotyrosine were from HBT (Uden, The Netherlands) and Upstate Biotechnology (Lake Placid, USA). The secondary antibody GAM-POX (goat anti mouse, HRP-labeled) was obtained from Pierce Biotechnology (Rockford, IL, USA). Cell culture medium DMEM and the additions FCS, penicillin, streptomycin and amphotericin B were supplied by Biochrom AG (Berlin, Germany). Thin layer chromatography plates were purchased from Merck (Darmstadt,

Germany) and  $^{14}\text{C}$ -arachidonic acid from American Radiolabeled Chemicals (St. Louis, MO, USA).

## 2.2. Smooth Muscle Cell Culture

### **Bovine smooth muscle cells:**

Primary cultures of bovine aortic smooth muscle cells (SMC) were obtained by collagenase digestion (Type CLS) of endothelium-denuded aortic media strips. Cells were grown in M199-medium supplemented with 10% FCS and 100U/ml penicillin/streptomycin. Experiments were performed with cells of passage 1, maintained in a 37°C humidified incubator in an atmosphere of 95% air and 5% CO<sub>2</sub>.



Representative staining of smooth muscle cell actin in bovine smooth muscle cells which is routinely performed for the detection of contamination with other cell types. (400 fold magnification)

For all experiments, primary cultures of bovine aortic SMC in passage 1 were used. With higher passages the amount of PGI<sub>2</sub>-synthase decreased whereas the content of PGHS-2 increased probably due to serum stimulation. Therefore cells were incubated without serum for 24h prior to experiments. For a single experiment, cells from the same batch were used. Differences in the total amount of 6-keto-PGF<sub>1α</sub> detected between different experiments can originate from differences in total cell numbers since shape and growth pattern of SMC does not allow reliable counts.

**Human, and rat smooth muscle cells:**

In pre-studies whole endothelium-denuded aortic segments from rats and segments of human arteria mammaria and vena saphena were incubated with LPS, however such incubations were lacking sufficient oxygen supply when incubated for several hours. Therefore primary cultures of human and rat aortic smooth muscle cells (SMC) were obtained by collagenase digestion (Type CLS; Biochrom, Berlin, Germany) of endothelium-denuded aortic vessel segments. Cells were grown in M199-medium (Biochrom, Berlin, Germany) supplemented with 10% FCS and 100U/ml penicillin/streptomycin. SMC were allowed to rest in serum-free medium 24 h prior to experiments which were performed with cells of passage 3-4. During passage, SMCs were pooled to achieve identical cell numbers since shape and growth pattern of the cells made it difficult to obtain reliable counts for standardization. SMCs were utilized for experiments when confluence was reached. This method allowed a comparison between different species.

### **2.3. Preparation of Rat Alveolar Macrophages**

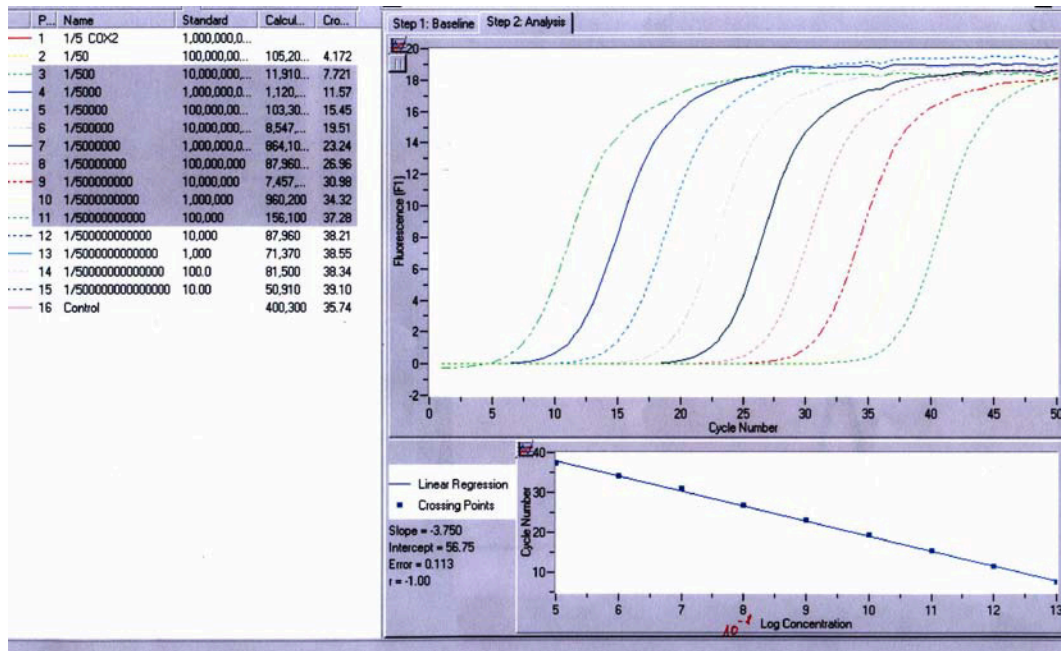
Male Wistar rats (400-500g, ex-breeder) were anesthetized by inhalation of Isofluran (Essex, Munich, Germany), lungs were excised and lavaged 3-times with sterile ice-cold 0.9% NaCl solution (Braun, Melsungen, Germany). Cells were pooled, resuspended in RPMI 1640 medium containing 10% FCS, 100U/ml Pen/Strep and plated at  $5 \times 10^6$  cells/dish in 4ml medium. After incubation for 4h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, medium was changed and the cells were allowed to rest overnight since the procedure of isolation turned out as sufficient for the induction and nitration of PGHS-2. Animal housing and treatment was conducted in accordance with the German Institutional Animal Care and Use Committee.

### **2.4. Western-Blot Analysis**

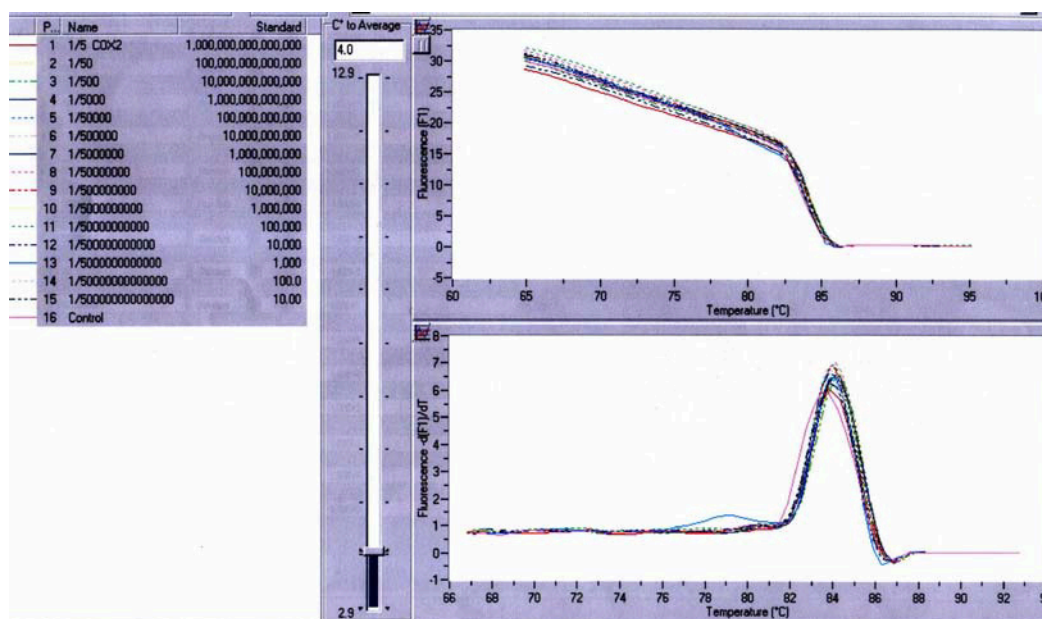
Proteins were separated electrophoretically by 8% SDS-PAGE and then transferred to nitrocellulose membranes (Hybond<sup>TM</sup>-C extra, Amersham, GB) by semidry blotting. The membrane was blocked for 2h in 5% milk-powder. It was then incubated with the primary antibodies for 2h at room temperature or at 4 °C overnight and for 45 min with a peroxidase conjugated secondary antibody at room temperature. Bands were visualized using the ECL Western blot system (Interchim, Montlucon, France). Anti-PGHS-2, anti-NOS-2 and anti-SMC-actin monoclonal antibodies were purchased from Transduction Laboratories, Lexington, USA. Anti-PGI<sub>2</sub>-synthase polyclonal antibody was a kind gift from Prof. Tanabe, Osaka, Japan.

## 2.5. Quantitative PCR Analysis

Total RNA isolation was performed according to the guanidine isothiocyanate/phenol method described in the manufacturers instructions (Peqlab, Erlangen, Germany). For reverse transcription, murine leukemia virus reverse transcriptase (Superscript, Invitrogen, California, USA) and oligo dT15 primers (Promega, Mannheim, Germany) were used. The reaction was performed at 42 °C for 60 min. PCR amplification was carried out in a LightCycler™ Instrument (Roche Diagnostics, Mannheim, Germany) using the PGHS-1 specific primer: 5'-GAC GAG CAG CTC TTC CAG AC-3'; and the antisense primer: 5'-GGC GAT GCG GTT GCG TAC TGG-3'; For PGHS-2 specific sense primer: 5'-ATC TTT GGG GAG ACC ATG GTA GA-3' and antisense-primer: 5'-ACT GAA TTG AGG CAG TGT TGA TG-3'; For bovine/human NOS-2 sense-primer: 5'-GGC TGG CGG GCG AGG CGT TTC-3' and anti-sense primer: 5'-GCT GCT TCA GGG TGG GGG CCA-3'; For rat NOS-2 sense-primer: 5'-AGT GTC AGT GGC TTC CAG CTC-3' and anti-sense primer: 5'-AGT GTC AGT GGC TTC CAG CTC-3'; For human/bovine GAPDH sense primer: 5'-ACC CTC AAG ATT GTC AGC AAT GC-3' and anti-sense primer 5'-GTC GTC ATA AGT CCC TCC ACG AT-3'; For rat GAPDH sense primer: 5'-TCC ATG ACC GTT GTC AGC AAT GC-3' and anti-sense primer: 5'-GTG GTC ATT AGC CCT TCC ACG AT-3'. Settings were used as follows: Denaturation at 95 °C for 15 s, annealing at 65 °C for 5 s, amplification at 72 °C for 19 s. 50 cycles were run before the reaction was stopped. Amplification was followed online, its crossing-points were used for evaluation. After PCR, a melting curve analysis for checking primer specificity was carried out. GAPDH served for standardization.



**Representative standard curve analysis for PGHS-2.** The quantitative information in a PCR reaction comes from those cycles with a logarithmic growth between background and the plateau. The higher the number of template copies in the reaction mix is, the earlier the log-linear phase is reached. For all RT-PCR experiments in this work SYBR Green I was used, which binds to the minor groove of dsDNA. In the unbound state of ssDNA, SYBR Green I has a lower fluorescence. As the amount of dsDNA increases, the fluorescence increases proportionally. For the standard curve demonstrated above, a PGHS-2 amplicon of a previous reaction was used in a 10-fold serial dilution and the amplification of this series was monitored. With decreasing template copy number, the curves are shifted to increasingly higher cycle numbers (top). The crossing points were used for calculation of a standard curve (bottom).



After the PCR, a **melting curve analysis** was performed ranging from 65°C to 95°C. When the  $T_m$  of the PGHS-2 amplicon was reached (84°C), a steep decrease in fluorescence can be observed as the product denatures to single strands. Values in the bottom window represent the first derivation of the melting curve above. The fluorescence in the control sample comes from a mix of non-specific products (primer-dimers).

## 2.6. PGHS Activity

Purified PGHS-2 (5units/100 $\mu$ l) was incubated at 37°C with 5units glutathione (GSH)-peroxidase, 0.2mM GSH, 325units/100 $\mu$ l catalase and 500 $\mu$ M phenol in 0.1M Tris-HCl pH 8.0.  $^{14}$ C-AA (25 $\mu$ M; 100nCi/100 $\mu$ l) was added for 10s followed by SIN-1, Spermine-NONOate or xanthine oxidase/hypoxanthine. The reaction was terminated after 2min by the addition of ethyl acetate/citric acid (2M) (30:1). The organic phase was spotted by glass capillaries onto silica TLC plates (Silica 60, Merck, Darmstadt, Germany) and subjected to chromatography in a solvent of ethyl acetate : 2,2,4-trimethylpentane : acetic acid : water (110 : 50 : 20 : 100). Plates were exposed to a PhosphorImager<sup>TM</sup> screen overnight. For quantification, a PhosphorImager<sup>TM</sup> system from Molecular Dynamics (Sunnyval, CA, USA) was used. Quantification was performed by the detection of total PGHS metabolites relative

to unmetabolized  $^{14}\text{C}$ -AA by utilizing “ImageQuant”<sup>TM</sup> software. One unit of PGHS-2 is defined as the consumption of one nmol oxygen per minute at 37°C in 0.1M Tris-HCl buffer, pH 8.0, containing 100 $\mu\text{M}$  AA, 5mM EDTA, 2mM phenol, and 1 $\mu\text{M}$  hematin.

## 2.7. NOS Activity Assay

Cells were homogenized by sonication in PBS. Samples were centrifuged at 20 000g for 20 min. NOS activity was measured by the conversion of  $^{14}\text{C}$ -arginine to  $^{14}\text{C}$  citrulline. 20 $\mu\text{l}$  of supernatant was incubated for 30 min at 37°C with a mixture of 80 $\mu\text{l}$  assay buffer containing 32.3 mM HEPES with 0.8% glycerol, 6 $\mu\text{M}$  FAD (Sigma), 6 $\mu\text{M}$  FMN (Sigma), 100 $\mu\text{g/ml}$  CaM (BioMol), 3mM DTT (Lancaster), 5 $\mu\text{M}$   $^{12}\text{C}$ -arginine (Sigma), 5 $\mu\text{M}$   $^{14}\text{C}$ -arginine (Amersham) and 250 $\mu\text{M}$  NADPH (BioMol). After 30 min, the reaction was stopped by the addition of 100 $\mu\text{l}$  methanol. 20 $\mu\text{l}$  of the mixture were spotted by glass capillaries onto a silica TLC plate (Silica 60, Merck) and subjected to chromatography. The solvent consisted of ammonium hydroxide, chloroform, methanol and water in a 2 : 0.5 : 4.5 : 1 mixture. Plates were exposed to a PhosphorImager<sup>TM</sup> screen overnight. For reading the screen, a PhosphorImager<sup>TM</sup> system from Molecular Dynamics, USA was used, quantification was performed by using “ImageQuant”<sup>TM</sup> software.

## **2.8. Measurement of Prostanoids and •NO-Products in Cell Culture Supernatants**

6-keto-PGF<sub>1α</sub>, the stable hydrolysis product of PGI<sub>2</sub>, was determined by using commercially available EIA-kits (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's instructions. During passage, cells for a single experiment were pooled to achieve identical cell numbers since shape and growth pattern of the cells made it difficult to obtain reliable counts for standardization. Therefore, prostanoid release was not presented in absolute concentrations but as relative amounts standardized to a basal value in each single experiment. This method allowed comparison between different experiments. Nitrite (NO<sub>2</sub><sup>-</sup>) as the stable oxidation product of •NO formation was measured by the Griess assay. Briefly, 30μl 12.5μM sulfanilamide and 30μl 6M HCl were mixed with a 300μl sample at room temperature and incubated for 5min. Absorbance was measured before and after the addition of 25μl *N*-(1-naphthyl)ethylenediamide (12.5μM) at 560nm using a microtiter plate reader. Nitrite concentrations were calculated from a NaNO<sub>2</sub> standard curve in the range of 0.5-50μM. Nitrate was quantified after conversion to nitrite by nitrate reductase (Roche, Mannheim, Germany) prior to the Griess reaction.

## 2.9. Immunoprecipitation

To prepare covalent protein G/antibody complexes, 40 µg of PGHS-2 antibody was added to 1 ml of protein G sepharose beads and incubated at room temperature for 2 h. The beads were washed with phosphate buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5), 6 mg/ml DMP (approximately 40 mM) were added and the mixture was incubated for 2 h at room temperature. Beads were washed with 0.2 M ethanolamine (pH 8.2) and incubated in this buffer for further 2 h. Finally they were washed with PBS, before being resuspended in PBS/0.05 % Na-azide for further use.

For immunoprecipitation, whole cell lysates were incubated with 50 µl of protein A sepharose beads in a 1.5 ml tube for 1 h. Beads and membrane fraction were removed by centrifugation for 25 min at 12000g. The supernatant was incubated with 100µl of antibody/ protein G-complex at 4°C overnight. Beads were sedimented and washed with PBST. PBST was removed, samples were mixed with 40 µl Laemmli buffer containing 5% 2-mercaptoethanol and boiled for 5 min. 100 µl of the antibody / protein G complex were run as a control. Samples were allowed to cool to room temperature and subjected to SDS-PAGE and Western blot as described.

## 2.10. HPLC Analysis of Protein-bound 3-NT

For nitration, purified PGHS-2 was incubated as described above. Inhibitors (aspirin 200 µM; diclophenac 10, 100 µM; NDGA 10 µM; KCN 1mM; phenol 50, 200µM) were added before initiating the nitration and incubated for 1 h. The reaction was terminated by adding digestion solution (phosphate buffer containing 1 mM calcium, 5% acetonitrile, 2 mg/ml pronase).

After 24 h cells were harvested in PBS and lysed. After pronase digestion (24 h, 37°C) samples were evaporated to dryness and resuspended in HPLC buffer.

The nitrite content of the cell culture medium was routinely determined to exclude any influence of the PGHS inhibitors on the induction of NOS-2.

Samples were analyzed by using a HPLC system (Jasco) equipped with a ternary low pressure gradient system and a variable-wavelength UV detector. Separation was achieved by a Nucleosil column (125/4.6; 100-3-C<sub>18</sub> Macherey-Nagel, Düren, Germany), isocratically eluted with potassium phosphate buffer (50 mM, pH 7.4) containing 3% acetonitrile at a flow rate of 0.8 ml/min. 3-nitrotyrosine was identified by spiking the samples with authentic nitrotyrosine and by reduction of nitrotyrosine with sodium dithionite. Concentrations were calculated by integration of the peak area and comparison with a standard curve of authentic 3-nitrotyrosine. In addition, HPLC-Electrochemical Detection (ECD) analysis was used with an ESA (Bedford, MA, USA) Coulochem II detector. 3-nitrotyrosine was separated on a C<sub>18</sub> Nucleosil-100-5 250x4.6 reversed phase column from Macherey-Nagel (Düren, Germany) by isocratic elution with 10 % (v/v) methanol in phosphate buffer pH 6.0 at a flow rate of 0.8 ml/min and detected at a potential of +825 mV.

## 2.11. GC-MS

Purified PGHS-2 was incubated with 10  $\mu$ M nitrite and 20  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min at 37°C. Briefly, sample extracts were spiked with deuterated internal standards. Prostaglandins and isoprostanes were extracted by 3 volumes ethyl acetate and solvent was removed. The methoxime was obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted and the pentafluorobenzylesters were formed. Samples were purified by TLC and two broad zones with  $R_f$  0.03-0.39 and 0.4-0.8 were eluted. After evaporation of the organic layers, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis.

A Finnigan MAT TSQ700 GC/MS/MS equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler were employed. Gas chromatography of prostanoid derivatives was carried out on a J&W DB-1 (20m, 0.25mm i.d., 0.25 $\mu$ m film thickness) capillary column (Analyt, Mühlheim, Germany) in the splitless mode. Products gained from zone 1 were analyzed for PGE<sub>2</sub>, TxB<sub>2</sub>, 6-keto-PGF<sub>1 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> , and isoprostanes, products from zone 2 for PGD<sub>2</sub>. For the quantification of PGF<sub>2 $\alpha$</sub> , and isoprostanes, [P-3 (CH<sub>3</sub>)<sub>3</sub>SiOH]- daughter ions (m/z=299) were used.

### **2.12. Statistical Analysis**

Results were expressed as means  $\pm$ SD and were compared by one-way analysis of variance (ANOVA) using  $P < 0.05$  as the significance level.

### 3. Aims of the Study

Previous publications of our group demonstrated tyrosine (Tyr)-nitration and inhibition of prostacyclin (PGI<sub>2</sub>)-synthase in LPS-exposed endothelial cells. Since the endothelium is regarded as a major source of systemic PGI<sub>2</sub> production, one could assume a declined release in patients undergoing endotoxemia or septic shock. In contrast, several *in vivo* observations in animals and man indicated a significant upregulation of plasma 6-keto-PGF<sub>1α</sub> levels (the stable hydrolysis product of PGI<sub>2</sub>) under septic conditions. The present study was initiated by this discrepancy and sought to solve the following questions:

1. Can vascular smooth muscle cells (SMC) be activated to produce high amounts of PGI<sub>2</sub> as found in the plasma of septic patients under conditions of a dysfunctional endothelium. Smooth muscle was reported as a rich source of PGI<sub>2</sub> synthase but is nearly devoid of prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) activity. This raises the question whether PGHS-2 could be induced to provide the constitutively expressed PGI<sub>2</sub> synthase with substrate.
2. If vascular SMC essentially contribute to PGI<sub>2</sub> formation, the problem arises why PGI<sub>2</sub>-synthase becomes nitrated and inhibited in vascular endothelial cells exposed to LPS whereas the same treatment even elevates PGI<sub>2</sub> formation in SMC. To further elaborate this discrepancy, the impact of endogenously formed as well as exogenously added peroxynitrite on the formation of PGI<sub>2</sub> by SMC will be investigated in the second part of this thesis.

3. A widely used model to study the effects of endotoxemia is the rat. However, from several *in vivo* and *in vitro* studies, distinct discrepancies between rodents and man with respect to the release of PGI<sub>2</sub> and •NO by vascular cells seem to exist. Therefore, it was investigated whether species-dependent variations in the induction pattern of inducible PGHS-2 and NOS-2 in vascular SMC of man, cattle and rat exposed to LPS exist.
  
4. Since PGHS catalyzes the rate-limiting step in prostanoid biosynthesis, a posttranslational control affecting its activity would have fundamental impact on prostanoid formation during several pathophysiological events. A variety of reports in literature indicated that Tyr-nitrations can be observed by the peroxidatic activation of inorganic nitrite catalyzed by myeloperoxidase. As catalysis of PGHS includes a peroxidase activity, it is hypothesized that in the presence of elevated intracellular levels of nitrite which may originate mainly from the induction of NOS-2, the concomitantly induced PGHS-2 could become inhibited by an autocatalytic activation of nitrite and the subsequent nitration of the enzyme. As a model system which is known to induce both PGHS-2 and NOS-2 in response to LPS, RAW 264.7 macrophages were chosen for the mechanistic studies on PGHS-2 nitration.
  
5. To elaborate a physiological role of nitrite-dependent PGHS-2 nitration and inhibition, rat alveolar macrophages were investigated. These cells are largely involved in orchestrating the inflammatory response in the lung by releasing bronchoconstrictive thromboxane A<sub>2</sub> in a PGHS-2-dependent manner. An autocatalytic inhibition of PGHS-2 could therefore represent a potent endogenous mechanism to terminate the inflammatory cascade in the lung and is investigated in the last part of this work.

## **4. Results and Discussions**

### **4.1. Prostacyclin Release by LPS Exposed**

#### **Vascular Smooth Muscle Cells**

##### **Introduction**

Since the discovery of prostaglandin endoperoxide H<sub>2</sub> synthase-2 (PGHS-2) as the inflammation-induced isoform of prostaglandin H synthase (O'Bannion 1991; Simmons 1993), it has become a preferred pharmacological target resulting in a new generation of non-steroidal anti-inflammatory drugs (NSAIDs) (Kurumbail 1996; Vane 1998). These compounds were developed to avoid the interference with the physiological PGHS-1-dependent prostaglandin actions in gastroprotection and hence are supposed to avoid such adverse effects on normal cellular regulation (Masferrer 1994; Warner 1999). However, PGHS-2 can also be involved in physiological processes or may counterregulate pathophysiological conditions by prostacyclin (PGI<sub>2</sub>) or prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) formation (Morita 2002; Parente 2003). Likewise, both PGHS-2 deficient mice as well as PGI<sub>2</sub> and PGI<sub>2</sub>-receptor knock outs showed deficiencies in kidney development (Harris 1994; Cheng 2002). Thus it appears that some physiological processes with beneficial actions of PGI<sub>2</sub> seem to depend on the presence of PGHS-2 (Brock 1999).

Concerning the vascular system, each cyclooxygenase isoform has a clearly defined role: Under physiological conditions, a balanced equilibrium between prothrombotic mediators like platelet-derived thromboxane A<sub>2</sub> (TxA<sub>2</sub>) and antithrombotic mediators like PGI<sub>2</sub> released by

the endothelium exists (Scheme 4.1/A) (Vane 2002). Under these circumstances, both eicosanoids originate from a PGHS-1 dependent pathway, because it was found that endothelial PGI<sub>2</sub> synthase is coupled to PGHS-1 whereas PGHS-2 was not detectable (Liou 2000). PGHS-2 can be induced in the endothelium by shear stress and cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interleukin-1 $\beta$  (IL-1 $\beta$ ) at the site of infections (Ristimäki 1994) or it can be present in atherosclerotic plaques (Schonbeck 1999). A shift of PGI<sub>2</sub> formation from PGHS-1 to PGHS-2 (Caughey 2001) has led to some cautionary notes that endothelial PGI<sub>2</sub> synthesis under such conditions could be influenced by PGHS-2 inhibitors (Mukherjee 2002). The resulting prothrombotic conditions could explain the outcome of the Vioxx Gastrointestinal Outcomes Research (VIGOR) (Bombardier 2000) clinical trial showing fivefold higher incidences of myocardial infarction with the selective PGHS-2 inhibitor rofecoxib as compared with the unspecific inhibitor naproxen for patients at risk (Wright 2002). The Celecoxib Long-term Arthritis Safety Study (CLASS) (Silverstein 2000) has revealed a similar tendency which has stimulated a discussion on the molecular basis of such adverse effects (Wright 2002; Bing 2002).

One explanation is the uninhibited PGHS-1 dependent TxA<sub>2</sub> formation in platelets which tips the balance towards a prothrombotic state (Mukherjee 2001). Another one could be the inhibition of PGHS-2-dependent PGI<sub>2</sub> formation in the endothelium. However, previous work in our laboratory has established that at an early stage of the inflammatory response seen with endotoxin (LPS) in bovine coronary arteries, PGI<sub>2</sub> synthesis was strongly impaired by nitration of endothelial PGI<sub>2</sub> synthase after  $\sim$  1h of endotoxin exposure (Zou 1996). The inhibition was initiated by increased superoxide production leading to an imbalance of three mediators: In the beginning of Phase I vasodilating  $\bullet$ NO is trapped by its almost diffusion-limited reaction with  $\bullet$ O<sub>2</sub><sup>-</sup> to form peroxynitrite (Koppenol 1992). In a subsequent step, peroxynitrite was shown to nitrate and inactivate PGI<sub>2</sub> synthase (Zou 1999). Finally, the remaining prostaglandin endoperoxide H<sub>2</sub> (PGH<sub>2</sub>) can activate the TxA<sub>2</sub>/PGH<sub>2</sub> receptor (TP)

on smooth muscle cells, leading to a sustained vasospasm (Scheme 4.1/B) (Mais 1985). Thus, under such conditions, PGI<sub>2</sub> synthesis should be impaired.

However patients in endotoxic shock release high amounts of 6-keto-PGF<sub>1α</sub> which then may have originated from non-endothelial sources (Halushka 1985; Eldor 1981). In contrast to high amounts of PGI<sub>2</sub> synthase, PGHS-activity was found low in smooth muscle of unstimulated vessels (DeWitt 1983). Our laboratory has previously confirmed by isolation and immunohistochemistry the smooth muscle layer as a rich source of PGI<sub>2</sub> synthase (Zou 1996; Zou 1999) and it could be hypothesized that PGHS-2 induction may provide PGH<sub>2</sub> as a substrate for this otherwise silent pool of enzyme as suggested earlier (Ullrich 2001).

## Results

### Prostaglandin synthesis and induction of PGHS-2

In pre-studies whole endothelium-denuded bovine aortic segments were incubated with LPS (10μg/ml) for 8h resulting in an increase of 6-keto-PGF<sub>1α</sub> in the supernatant measured together with an increase of PGHS-2 protein levels (data not shown). Such ex vivo incubations however were lacking a sufficient supply of oxygen to the tissue and thus only could give qualitative results. Therefore primary smooth muscle cell cultures were prepared from this tissue and used from passage 1 since with higher passages the amount of PGI<sub>2</sub> synthase decreased and the content of PGHS-2 increased; the latter probably due to stress effects (Neubert 1997). In order to study the effect of inflammatory conditions on the PGHS-pathway, we therefore used passage 1 for all further experiments. When such confluent cells were exposed to 10ng/ml IL-1β or TNF-α, a 2-3-fold increase in PGI<sub>2</sub> production (measured as 6-keto-PGF<sub>1α</sub>) over controls were monitored in the supernatant whereas incubation with

LPS resulted in a massive increase of about 300 fold (Table 4.1) reaching values of up to 30ng/ml after 8 h. For comparison, the levels of PGE<sub>2</sub> were increased only 3-5 fold for all three stimuli.

Comparison of different proinflammatory stimuli on 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> release by smooth muscle cells

| 6-keto-PGF <sub>1α</sub> release |         |                    |                    |                  |
|----------------------------------|---------|--------------------|--------------------|------------------|
| h                                | Control | IL-1β<br>(10ng/ml) | TNF-α<br>(10ng/ml) | LPS<br>(10μg/ml) |
| 0                                | 100 ± 3 | 100 ± 7            | 100 ± 7            | 100 ± 28         |
| 2                                | 111 ± 6 | 94,26 ± 7          | 109,49 ± 15        | 3667,52 ± 289    |
| 4                                | 82 ± 7  | 171,04 ± 13        | 164,16 ± 21        | 20322,28 ± 1304  |
| 6                                | 94 ± 6  | 174,74 ± 13        | 221,47 ± 22        | 26066,02 ± 1675  |
| 8                                | 112 ± 7 | 222,19 ± 19        | 333,91 ± 27        | 23777,12 ± 1209  |

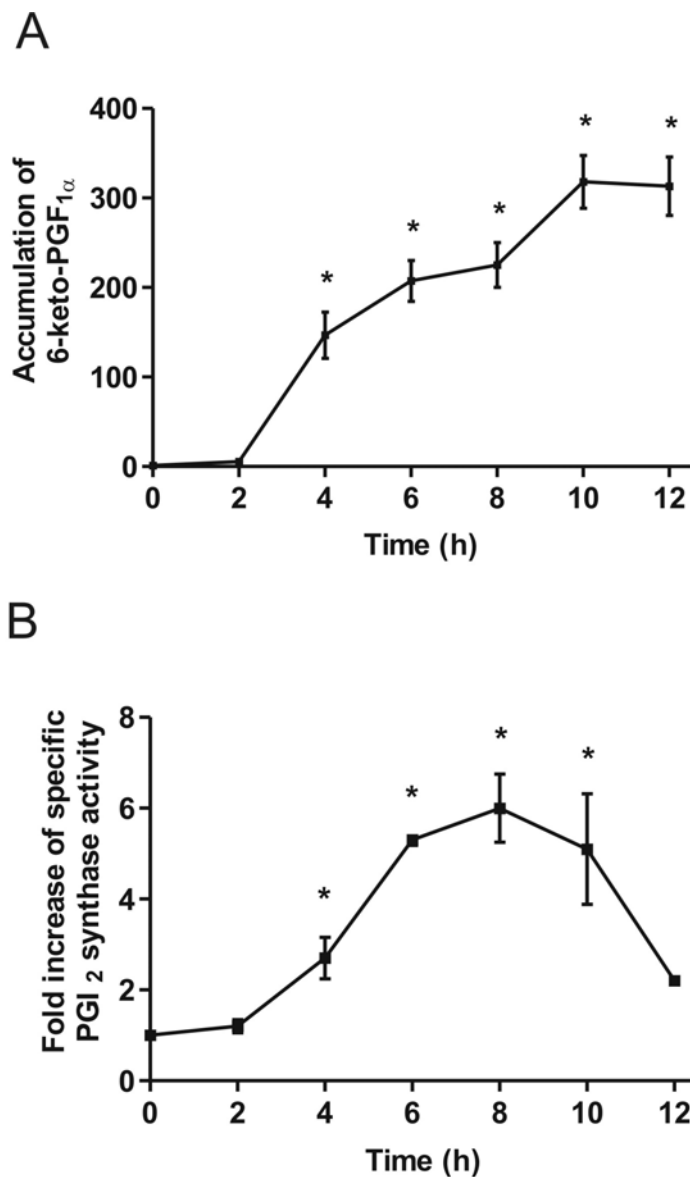
  

| PGE <sub>2</sub> release |          |                    |                    |                  |
|--------------------------|----------|--------------------|--------------------|------------------|
| h                        | Control  | IL-1β<br>(10ng/ml) | TNF-α<br>(10ng/ml) | LPS<br>(10μg/ml) |
| 0                        | 100 ± 12 | 100 ± 7,62         | 100 ± 40,6         | 100 ± 3,74       |
| 2                        | 133 ± 18 | 63,71 ± 1,69       | 98,34 ± 5,12       | 110,23 ± 3,27    |
| 4                        | 105 ± 6  | 187,61 ± 30,18     | 170,08 ± 59,37     | 202,83 ± 10,14   |
| 6                        | 122 ± 12 | 258,39 ± 57,72     | 253,66 ± 5,9       | 394,25 ± 87,02   |
| 8                        | 87 ± 10  | 457,71 ± 67,31     | 268,59 ± 9,5       | 330,81 ± 84,41   |

Table 4.1. **Comparison of different proinflammatory stimuli on prostaglandin release.** Confluent cultures of smooth muscle cells were treated with IL-1β (10ng/ml), TNF-α (10ng/ml) or LPS (10μg/ml) for the time periods indicated. Accumulation of prostanoid release was measured by EIA. Formation of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> were standardized to values at t=0. 6-keto-PGF<sub>1α</sub> level was 100pg/ml, PGE<sub>2</sub> level was 200pg/ml at t=0 and defined as 100%. Release of 6-keto-PGF<sub>1α</sub> into serum-free medium increased only moderately when IL-1β or TNF-α were used as stimuli compared with the high increase by LPS. In contrast, PGE<sub>2</sub> release shows no specificity for one of the three different stimuli. Data are expressed as mean ± SD of four separate experiments. *P* < 0.05 vs. t=0.

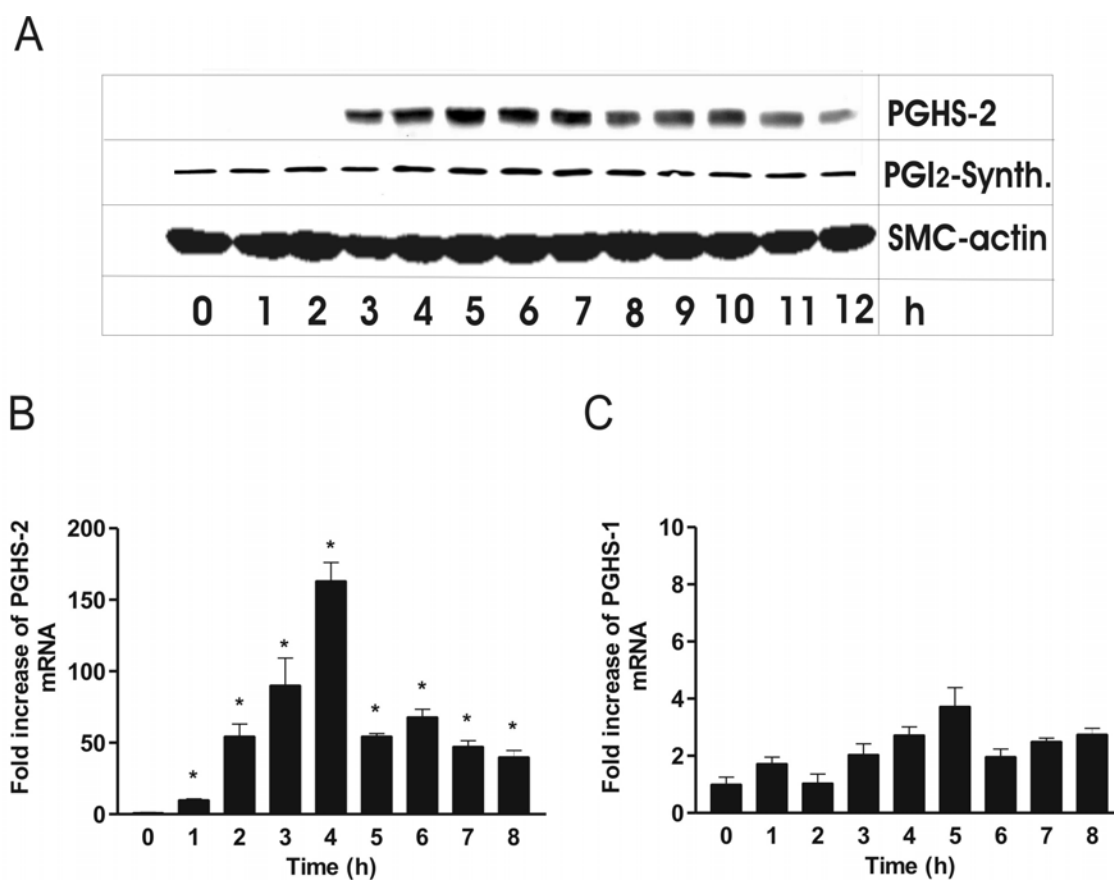
Fig. 4.1.1A shows PGI<sub>2</sub> release by smooth muscle cells with and without addition of LPS after increasing incubation times. Values without LPS cannot be distinguished from the abscissa, excluding significant formation of PGI<sub>2</sub> under resting conditions. Since the observed 6-keto-PGF<sub>1α</sub> concentrations represent accumulated metabolites over time, they could have been subject to considerable degradation activity. Therefore, in a second set of experiments, at the indicated time points medium was removed and discarded, cells were washed and then newly stimulated with LPS for 30 min in order to assess biosynthesis of PGI<sub>2</sub> directly from

the given time point after LPS (Fig. 4.1.1B). As a result, qualitatively the same time course as in Fig. 4.1.1A was observed.  $\text{TxB}_2$  levels were also analyzed but were undetectable over the whole time period (data not shown).



**Figure 4.1.1. Formation of 6-keto-PGF<sub>1α</sub> by smooth muscle cells.** (A) Time course of LPS-stimulated 6-keto-PGF<sub>1α</sub> release (end point detection). Smooth muscle cells were treated with and without LPS (10μg/ml; E.coli) for the indicated periods of time. Without LPS, no formation of 6-keto-PGF<sub>1α</sub> was observed (time course coincides with abscissa). When LPS was added, the first two hours show nearly no 6-keto-PGF<sub>1α</sub> formation. Following this period, a large increase was observed exceeding levels up to 30ng/ml after 10 h. (B) **Activity of prostacyclin synthase** (relative formation). LPS (10μg/ml) was added to smooth muscle cells at t=0. Medium was removed at the times indicated, cells were washed twice and new medium + LPS was added for another 30 min. Data reflect formation of PGI<sub>2</sub> within these 30 min. A maximum in activity was observed at 8h. Formation of 6-keto-PGF<sub>1α</sub> was standardized to values at t=0. 6-keto-PGF<sub>1α</sub> level at t=0 was 100pg/ml and defined as 1. Prostanoid formation was analyzed by EIA. Values are mean SD (n=4).  $P < 0.05$  vs. t=0.

Fig. 4.1.2A+B show the induction of PGHS-2 on both the protein and mRNA level. The dramatic increase of PGI<sub>2</sub> after exposure to LPS correlated with the increase in PGHS-2 protein as indicated by Western blotting. PGHS-2 protein became visible after 3h LPS incubation time when probed with a monoclonal antibody. The staining intensified at 5 to 6h after LPS addition and reproducibly decreased from 8 to 12h (Fig. 4.1.2A). At the mRNA level,



**Figure 4.1.2. (A): Protein expression of PGHS-2 and prostacyclin synthase in bovine smooth muscle cells.**

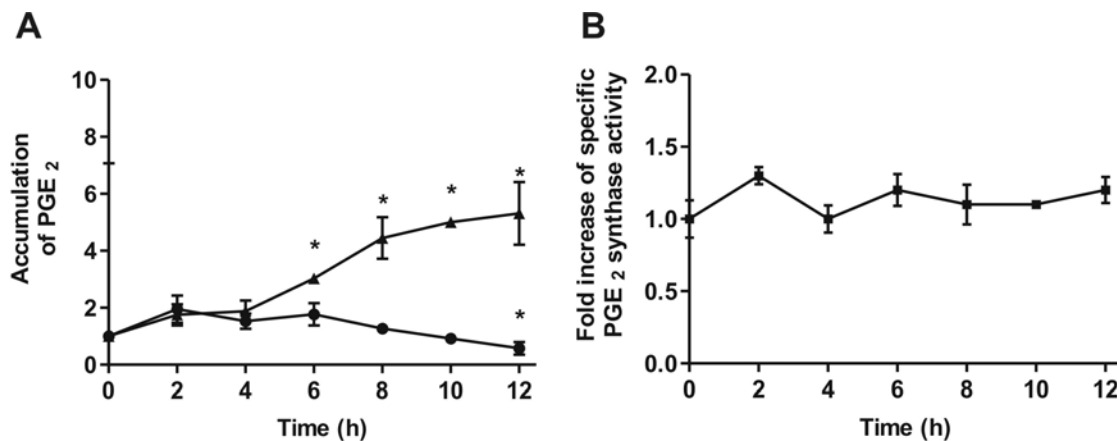
Cells were stimulated with LPS (10 $\mu$ g/ml) for the time periods indicated. Levels of PGI<sub>2</sub> synthase (52kDa) remained on the same constant level all over the time. Within the first two hours, PGHS-2 protein (72kDa) was not detectable. A peak in protein expression can be observed at 5-6h. From 8-12h, a weaker steady-state expression was detected. The blot shows representative results of four independent experiments.

**(B): Induction of cyclooxygenase-2 (PGHS-2) mRNA in smooth muscle cells** as a function of time after exposure to LPS (10 $\mu$ g/ml). Without stimulation, PGHS-2 mRNA was under detection limit. The message peaks at 4h and rapidly declines afterward to a weaker constant expression.

**(C): Expression of cyclooxygenase-1 (PGHS-1) mRNA** in smooth muscle cells as a function of time. Expression of PGHS-1 did no change significantly. Note the small changes of expression compared with PGHS-2. Data were normalized with respect to GAPDH. Data are mean  $\pm$  SD from three independent experiments.  $P < 0.05$  vs.  $t=0$ .

PGHS-2 increased with a maximum at 4h and then rapidly decreased to a steady-state level with approximately a 50-fold PGHS-2-mRNA content compared with the levels of unstimulated cells. PGHS-2 protein expression therefore followed its mRNA with a delay of about 1h as could be expected (Fig. 4.1.2B). PGHS-1 mRNA as measured by RT-PCR showed no significant alteration following stimulation with LPS (note the different scales; Fig. 4.1.2C). PGHS-1 protein was under detection limit with the antibodies used.

Fig. 4.1.3A illustrates accumulation of PGE<sub>2</sub> with and without stimulation by LPS. Compared with the formation of approximately 30ng/ml PGI<sub>2</sub> after an incubation period of 12h, release of nearly 1ng/ml PGE<sub>2</sub> was relatively weak. Interestingly, levels of PGE<sub>2</sub> already present in the medium even declined without LPS, suggesting an increased degradation over time as was separately shown by incubation of smooth muscle cells with <sup>14</sup>C-labeled PGE<sub>2</sub> (data not shown). Therefore, to minimize a potential degradation of PGE<sub>2</sub>, the medium was removed at given time points after initiation of LPS treatment and cells were treated with both LPS and

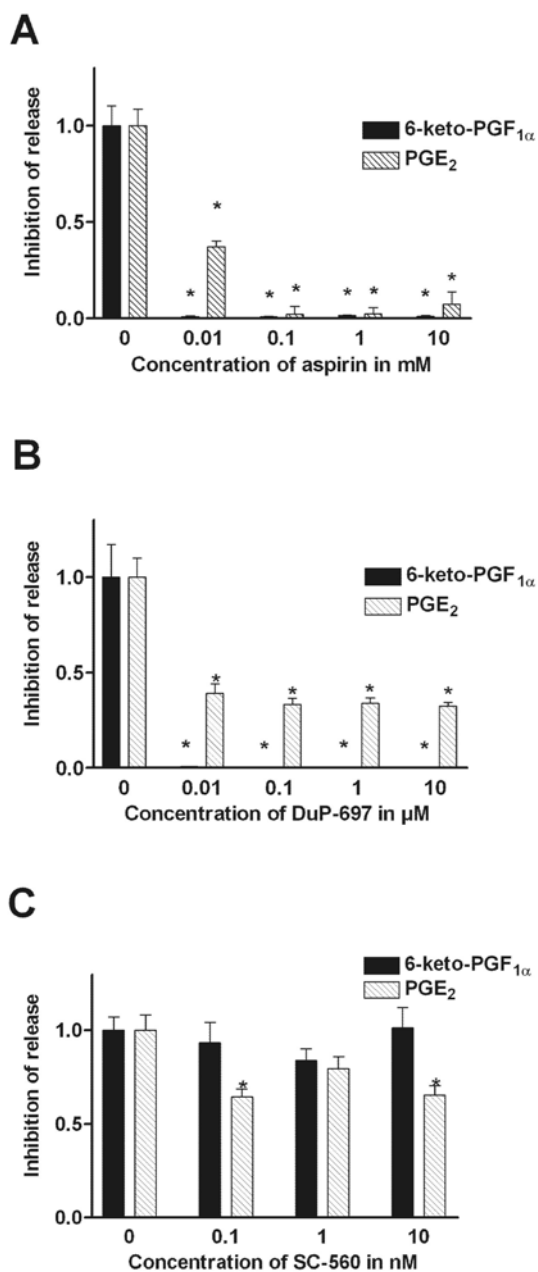


**Figure 4.1.3. Formation of PGE<sub>2</sub> by smooth muscle cells.** (A): Time course of LPS stimulated PGE<sub>2</sub> release (end point detection). Smooth muscle cells were treated with (▲) and without (●) LPS (10µg/ml) for the indicated periods of time. Without LPS, a decline of PGE<sub>2</sub> already present in the medium was shown. The increase of PGE<sub>2</sub> after stimulation with LPS revealed relatively weak. (B): **Activity of PGE<sub>2</sub> synthases** (relative formation). LPS (10µg/ml) was added to cultured smooth muscle cells at time 0. Medium was removed at the times indicated, cells were washed twice and new medium + LPS was added for 30 min. Data reflect the formation of PGE<sub>2</sub> within these 30 min. No significant alteration in the relative formation of PGE<sub>2</sub> was observed. Formation was standardized to the values at t=0. PGE<sub>2</sub> level at t=0 was nearly 200pg/ml and defined as 1. Prostanoid formation was analyzed by EIA. Values are mean ± SD of four experiments. *P* < 0.05 vs. t=0.

A23187 ( $10^{-5}$ M) for 30 min. (Fig. 4.1.3B). Interestingly, relative formation of PGE<sub>2</sub> did not change over time. Even within the first two hours, in which PGI<sub>2</sub> showed nearly no new synthesis, PGE<sub>2</sub> formation remained at the same level as in progressed stages.

### **Effects of PGHS inhibitors**

The aim of our work consisted in the proof and biochemical confirmation that PGI<sub>2</sub> synthesis in inflammation is PGHS-2 dependent and therefore should be suppressed by specific PGHS-2 inhibitors. The unspecific PGHS inhibitor acetylsalicylic acid (aspirin) blocked release of PGI<sub>2</sub> even at relatively low concentrations (10 $\mu$ M). The IC<sub>50</sub> values for ovine PGHS-1 and -2 were 0.75 and 1.25 $\mu$ M respectively (Kalgutkar 1998). PGE<sub>2</sub> was only partially suppressed at 10 $\mu$ M aspirin but was also effectively blocked at higher levels (Fig. 4.1.4A). In agreement with literature data (Sengupta 1999), 6-keto-PGF<sub>1 $\alpha$</sub>  production was completely inhibited at 10nM by the PGHS-2 specific inhibitor DuP-697 with an IC<sub>50</sub> of 2.1nM, whereas PGE<sub>2</sub> levels remained at about one third (Fig.4.1.4B). The PGHS-1 specific inhibitor SC-560 with an IC<sub>50</sub> of 9nM for PGHS-1 (Smith 1998) revealed no consistent inhibition of PGI<sub>2</sub> while PGE<sub>2</sub> was reduced by roughly 25% (Fig. 4.1.4C). To this point, we have shown that release of PGI<sub>2</sub> by smooth muscle cells completely depends on expression of PGHS-2 whereas PGE<sub>2</sub> formation seems to involve different PGHS isoforms.



**Figure 4.1.4 (A): Inhibition of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> formation by the unspecific inhibitor aspirin in smooth muscle cells.** Cells were incubated with LPS (10 μg/ml) and aspirin in the concentrations indicated for 3h. Formation of 6-keto-PGF<sub>1α</sub> was completely inhibited even at very low concentrations (10 μM) of aspirin. Formation of PGE<sub>2</sub> was also effectively inhibited.

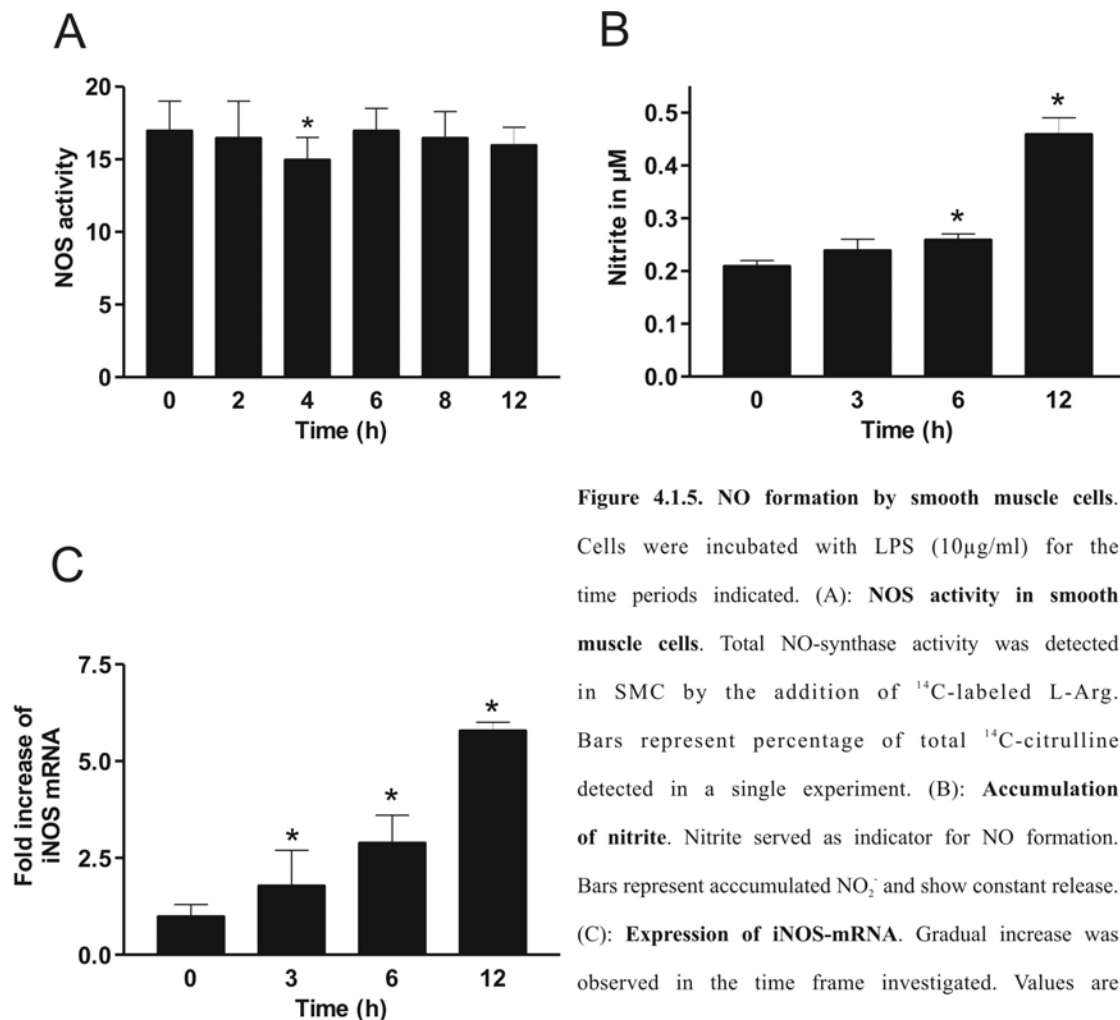
**(B): Inhibition of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> formation by the PGHS-2 selective inhibitor DuP-697.** 6-keto-PGF<sub>1α</sub> formation was completely inhibited by low concentrations (10 nM) of DuP-697. PGE<sub>2</sub> values remained at about one third.

**(C): Inhibition of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> formation by the PGHS-1 specific inhibitor SC-560.** No significant reduction in 6-keto-PGF<sub>1α</sub> formation was observed both at incubation times of 3h and 7h. When cells were incubated for 3h as shown in (C), PGE<sub>2</sub> levels were reduced by roughly 25%. When cells were incubated for 7h (not shown), SC-560 showed no inhibitory effect. Prostanoid formation without inhibitors was defined as 1. Values are mean ± SD of four experiments. \**P* < 0.05 vs. values without inhibitor.

### •NO formation by smooth muscle cells

In previous work, we have shown that under inflammatory conditions, endothelial PGI<sub>2</sub> synthase was nitrated and inactivated by peroxynitrite in early stages of inflammation (~1h LPS incubation). Since our data now show an abundant generation of PGI<sub>2</sub> by smooth muscle cells, we assayed formation of •NO as a potential precursor of peroxynitrite generation. NOS activity assay (Fig. 4.1.5A) showed no change in the activity of NO synthases in bovine

smooth muscle cells. Accumulated nitrite levels as indicator for  $\bullet$ NO formation did not exceed  $0.5\mu\text{M}$  after 12h of incubation with LPS (Fig. 4.1.5B). This indicates a constant and not induced activity over time. For comparison, plasma levels can easily reach  $5\text{-}10\mu\text{M}$  in infected tissue after NOS-2 induction (Ersoy 2002). On the other hand, gradual increase of iNOS mRNA levels (Fig. 4.1.5C) were observed but it should be noted that the induction detected was low compared to PGHS-2 (Fig. 4.1.2B) and to the induction of iNOS in mesangial cells (Zou 1998).



**Figure 4.1.5. NO formation by smooth muscle cells.** Cells were incubated with LPS ( $10\mu\text{g/ml}$ ) for the time periods indicated. (A): **NOS activity in smooth muscle cells.** Total NO-synthase activity was detected in SMC by the addition of  $^{14}\text{C}$ -labeled L-Arg. Bars represent percentage of total  $^{14}\text{C}$ -citrulline detected in a single experiment. (B): **Accumulation of nitrite.** Nitrite served as indicator for NO formation. Bars represent accumulated  $\text{NO}_2^-$  and show constant release. (C): **Expression of iNOS-mRNA.** Gradual increase was observed in the time frame investigated. Values are mean  $\pm$  SD of four experiments. \* $P < 0.05$  vs.  $t=0$ .

## Discussion

Purpose of this study was to examine the contribution of the tunica media smooth muscle to the formation of vasoactive mediators during septic shock. The results refer to a situation of endotoxemia where LPS levels above the microgramme level per ml are acting on the vessel wall (Deitch 1998; Schultz 2002). As we have shown previously, such conditions cause endothelial dysfunction with a loss of  $\bullet$ NO and PGI<sub>2</sub> by the superoxide-triggered formation of peroxynitrite and its inhibiting effect on PGI<sub>2</sub> synthase (Zou 1996; Zou 1999). In connection with endothelial dysfunction usually an opening of the endothelial barrier (Vallance 1997; Berman 1993) or even a partial destruction of the endothelium occurs which will directly expose smooth muscle cells to endotoxin like in the Shwartzman reaction, where detached endothelial cells can be observed (Maeda 1995; Gaynor 1970). In contrast, smooth muscle cells in culture reacted strongly to LPS, but did not undergo cell death as endothelial cells do. We herein provide a biochemical basis for the hypothesis proposed by Bishop-Bailey (Bishop-Bailey 1999) that LPS-triggered PGHS-2 induction could initiate PGI<sub>2</sub> synthesis from smooth muscle which then together with  $\bullet$ NO from NOS-2 induction could replace the function of the intact endothelium (Vane 1994; Stoclet 1999). However, our results point to a more sophisticated regulation of vessel function in developing septic shock. In agreement with earlier data on cytokine effects on rat smooth muscle cells (Rimarachin 1994), TNF- $\alpha$  and IL-1 $\beta$  caused a only minor and equal increase in both PGI<sub>2</sub> and PGE<sub>2</sub> synthesis together with PGHS-2 induction after about 3h with further increase up to 8h. High levels of PGI<sub>2</sub> synthase were present constitutively in smooth muscle cells of passage 1. But this resting pool became activated only when PGHS-2 was expressed. With LPS as a strong proinflammatory stimulus, our results clearly differed from those with cytokines since the observed PGI<sub>2</sub> synthesis became much more pronounced by LPS and was strongly suppressed by low concentrations

of a specific PGHS-2 inhibitor. This suggested a tighter coupling of PGI<sub>2</sub> synthase with PGHS-2 in contrast to PGE<sub>2</sub> synthesis. Thus, LPS exerted a selective effect on PGI<sub>2</sub> release compared to TNF- $\alpha$  and IL-1 $\beta$  which enhanced both prostaglandins but only moderately. A comparison of the time courses for PGHS-2 induction and activity reveals a significant difference of about 2-3h with regard to the maxima. Since all our data can be interpreted in terms of a tight coupling of PGHS-2 and PGI<sub>2</sub> synthase which has been found located to the caveolae, there may be a posttranslational process involved for directing the newly synthesized PGHS-2 to the constitutively present PGI<sub>2</sub> synthase in the caveolar compartment.

Another new and unexpected result was the virtually absent release of  $\bullet$ NO as measured by both the activity of NO synthases and by the formation of nitrite in the time frame investigated. Neither  $\bullet$ NO or nitrite in such concentrations will affect the PGHS-pathway, only with 20 fold higher levels (nitrite > 10 $\mu$ M) inhibitory effects were observed (Bachschmid; unpublished results). Usually iNOS protein follows PGHS-2 induction with a delay of 1-2h. We argue that the absence of  $\bullet$ NO may be a reason why PGI<sub>2</sub> synthase did not undergo massive nitration as reported for LPS-exposed mesangial cells (Zou 1998). Otherwise this could have caused inactivation of the enzyme and would have antagonized the beneficial actions of PGI<sub>2</sub> with regard to vasorelaxation, inhibition of platelet aggregation or suppression of SMC proliferation. The observation that SMC upon LPS exposure synthesize extracellular Cu/Zn-SOD (Oury 1996) may be an additional factor that prevents sufficient peroxynitrite formation from  $\bullet$ NO and  $\bullet$ O<sub>2</sub><sup>-</sup> (Beckman 1996). Since it had been reported that PGI<sub>2</sub> can block NOS-2 induction in a macrophage cell line (Marotta 1992), the mechanism of NOS-2 suppression may be linked to a rise of cAMP formation which suppresses PGHS-2 induction. Due to the thickness of the smooth muscle layer compared to the endothelium, the output of PGI<sub>2</sub> after PGHS-2 induction became massive and could explain the high 6-keto-PGF<sub>1 $\alpha$</sub>  serum

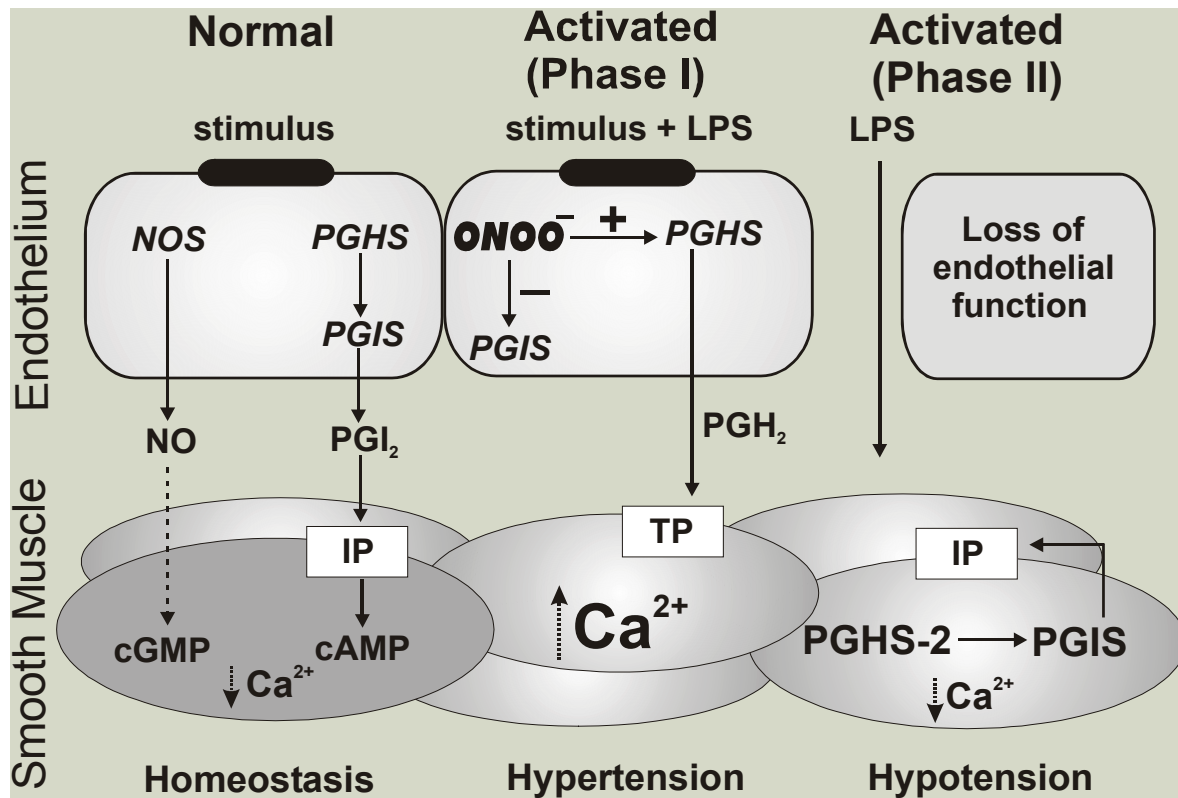
levels of shock patients and their extreme hypotension (Halushka 1985). In literature, most of such studies were performed with rats, in which •NO dominates as a vasodilator (Sirso 1994). As mentioned before, the relative contributions of PGI<sub>2</sub> and •NO to hypotension may vary and be species-dependent. Human coronary arteries with this respect may be closer to bovine vessels whereas rat seems to rely more on •NO as a counterregulator to endothelial dysfunction (Griffiths 1995; Knowles 1994).

The most relevant result of our study for practical medicine could be that PGI<sub>2</sub> synthesis in SMC is completely abolished by specific PGHS-2 inhibitors which on one hand could ameliorate the extreme hypotension in shock but eventually could become lethal due to hemorrhagic events. A different situation arises with drugs like aspirin directing unmetabolized arachidonic acid into alternative counterregulatory directions like the so-called “aspirin-triggered lipoxin” pathway (Fierro 2001; Serhan 1997). Since aspirin additionally blocks PGHS-1, the risk of infarction by platelet aggregation would also be reduced (Catella-Lawson 2001). Due to the different molecular inhibition mechanism of the specific PGHS-2 inhibitors such alternative pathways may not become evoked.

The unusual behavior of PGE<sub>2</sub> formation under the influence of LPS was rather unexpected. In contrast to PGI<sub>2</sub>, there was no significant increase in PGE<sub>2</sub> after LPS exposure but rather accumulation of PGE<sub>2</sub> from a constant activity. The PGHS-2 inhibitor used blocked about 70% of the activity but even higher concentrations could not bring it down further (Fig. 4.1.4B). A similar behavior was also described in a recent report of Uracz et al. (Uracz 2002). In this case, PGE<sub>2</sub> synthesis showed an unusual inhibition pattern with acetaminophen being most active. Ongoing investigations are concerned with the properties of the PGHS enzyme and the PGE<sub>2</sub> synthase isoform possibly coupled to it (Chandrasekharan 2002).

The strong action of LPS on smooth muscle may essentially be due to a direct interaction after the endothelium is disrupted, since the endothelial barrier would otherwise limit access of LPS and other PGHS-2 inducing factors from which serum is another example. It also

corresponds to the situation after removal of the endothelium by a balloon catheter (Connolly 2002). Obviously, the LPS levels determine whether endothelial cells are dying or convert to an activated state. The latter would correspond to a normal inflammatory response of the endothelium as elicited by cytokines and characterized also by P-selectin release from the Weibel-Palade bodies (Davenpeck 1994). At this stage of endothelial dysfunction without execution of cell death, it is not yet known how much PGI<sub>2</sub> formation in the endothelium is coupled to PGHS-2 induction and what the effects of PGHS-2 inhibitors would be. It is therefore suggested that the cytokine-dependent action on the vessel is a reversible response and also elicited by low LPS levels whereas the high levels applied here correspond to endotoxemia with progression to shock leading to a much stronger output of PGI<sub>2</sub> from smooth muscle. This sequence of events is depicted in Scheme 4.1:



**Scheme 4.1. Vascular smooth muscle in the development of septic shock.**

Under physiological conditions, the endothelium maintains an equilibrium of vascular tone by a basal formation of eNOS derived <sup>•</sup>NO and PGHS-1 derived PGI<sub>2</sub> among a variety of other mediators.

Exposure to endotoxin leads to an activation of the endothelium. Increased formation of <sup>•</sup>O<sub>2</sub><sup>-</sup> traps <sup>•</sup>NO to form peroxynitrite which in turn nitrates and inactivates prostacyclin synthase. The lack of <sup>•</sup>NO and PGI<sub>2</sub> favors vasoconstriction which is further triggered by the activation of the TxA<sub>2</sub>/PGH<sub>2</sub> receptor. This Phase I of endothelial activation (1-2 h after LPS) is followed by the induction of PGHS-2 leading to PGI<sub>2</sub> synthesis in smooth muscle and vessel relaxation (Phase II).

High concentrations of endotoxin present during sepsis may lead to a loss of endothelial barrier integrity or even complete loss of the endothelium. Exposure of the smooth muscle layer to LPS and cytokines results in the induction of PGHS-2 and PGI<sub>2</sub> release to provide antiaggregatory and antiadhesive properties.

NOS= NO-synthase; PGHS= prostaglandin endoperoxide synthase; PGI<sub>2</sub>= prostacyclin; PGH<sub>2</sub>= prostaglandin H<sub>2</sub>; PGIS= prostacyclin synthase; IP= prostacyclin receptor; TP= PGH<sub>2</sub>/TxA<sub>2</sub> receptor; ONOO<sup>-</sup>= peroxynitrite.

## **4.2. Peroxynitrite Provides the Peroxide Tone for PGHS-2 Dependent Prostacyclin Synthesis in Vascular Smooth Muscle Cells**

### **Introduction**

Prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) catalyzes the committed step in the synthesis of prostanoids and constitutes of two heme-dependent catalytic functions. First, the cyclooxygenase activity catalyzes the cyclization of arachidonate to 15-hydroperoxy-prostaglandin-9,11,-endoperoxide (PGG<sub>2</sub>) which in a second reaction is reduced by the peroxidase activity to the 15-hydroxy derivative (PGH<sub>2</sub>) (Smith 2000; Vane 1998). PGHS exists as at least two isoenzymes from which PGHS-1 is expressed constitutively (Hemler 1976; Miyamoto 1976) whereas PGHS-2 belongs to the family of early immediate genes and is induced by cytokines or endotoxin (lipopolysaccharide, LPS) (Fu 1990; O'Banion 1991; Rimarachin 1994). Initiation of the PGHS reaction requires a peroxide (Smith 1972) to convert the resting ferric enzyme into its active form in which the ferric heme together with a neighboring Tyr-residue are converted to a ferryl species and a Tyr-radical (Dietz 1988; Landino 1997). The latter abstracts a hydrogen atom from arachidonate at C-13 which starts the cyclization to PGG<sub>2</sub> under incorporation of two molecules of dioxygen (Tsai 1995). The peroxidase site of PGHS then transfers two electrons to PGG<sub>2</sub> in an as yet ill-defined fashion to finally yield PGH<sub>2</sub>. During catalysis the Tyr-radical is

regenerated and hence the enzyme in principle would require only one initial activation but since side-reactions, resulting from the cellular reductive potential, can abort radical formation, a permanent low level of peroxides is required to preserve activity over time (Capdevila 1995; Margalit 1998). It was of interest to find the endogenous “peroxide tone” different for the two isoenzymes with 21nM for PGHS-1 and only 2nM for PGHS-2 (Kulmacz 1995). The chemical basis of this difference has been elucidated by the seminal work of Kulmacz et al., but only speculations exist for its physiological significance. One is that dependent on the peroxide tone only PGHS-2 could be activated and PGHS-1-dependent prostanoid formation would follow only when the cellular peroxide levels increase by tenfold in a given cell (Kulmacz 1998; Reddy 1997; Kuwata 1998). Since peroxide levels could limit PGHS turnover, the peroxide tone is therefore one of the earliest examples how redoxregulation can affect prostanoid biosynthesis. Low intracellular peroxide levels are characteristic for resting cells and are maintained by cellular peroxidases together with the cellular reductive potential (Sies 1997; Nordberg 2001). Which peroxides form the peroxide tone under cellular conditions was not known but  $\text{H}_2\text{O}_2$ ,  $\text{PGG}_2$  or other arachidonate hydroperoxides were considered as likely candidates to initiate the cyclization and provide the tone as the reaction proceeds (Smith 2000; Vane 1998; Marshall 1987).

In the past we have studied prostacyclin ( $\text{PGI}_2$ ) biosynthesis in the endothelium which is regarded to be mainly dependent on PGHS-1 (Stanfield 2001). Under influence of a strong inflammatory stimulus like LPS, inhibition of  $\text{PGI}_2$  synthesis was observed to be caused by the nitration of a Tyr-residue at the active site of  $\text{PGI}_2$ -synthase (Zou 1997). Inhibition of the constitutive endothelial NO-synthase (NOS-3) as well as trapping of  $\bullet\text{O}_2^-$  prevented nitration indicating formation of peroxynitrite as the nitrating agent (Zou 1996; Bachschmid 2003). From bolus additions of peroxynitrite, half-inhibition of 6-keto- $\text{PGF}_{1\alpha}$  formation as the stable  $\text{PGI}_2$  hydrolysis product occurred at 50nM with the purified

enzyme (Zou 1997; Zou 1996; Schmidt 2003). This nitration of PGI<sub>2</sub>-synthase represents an early and essential step in endothelial cell activation which is a hallmark of various vascular pathophysiological states (Zou 1999a; Zou 1999b).

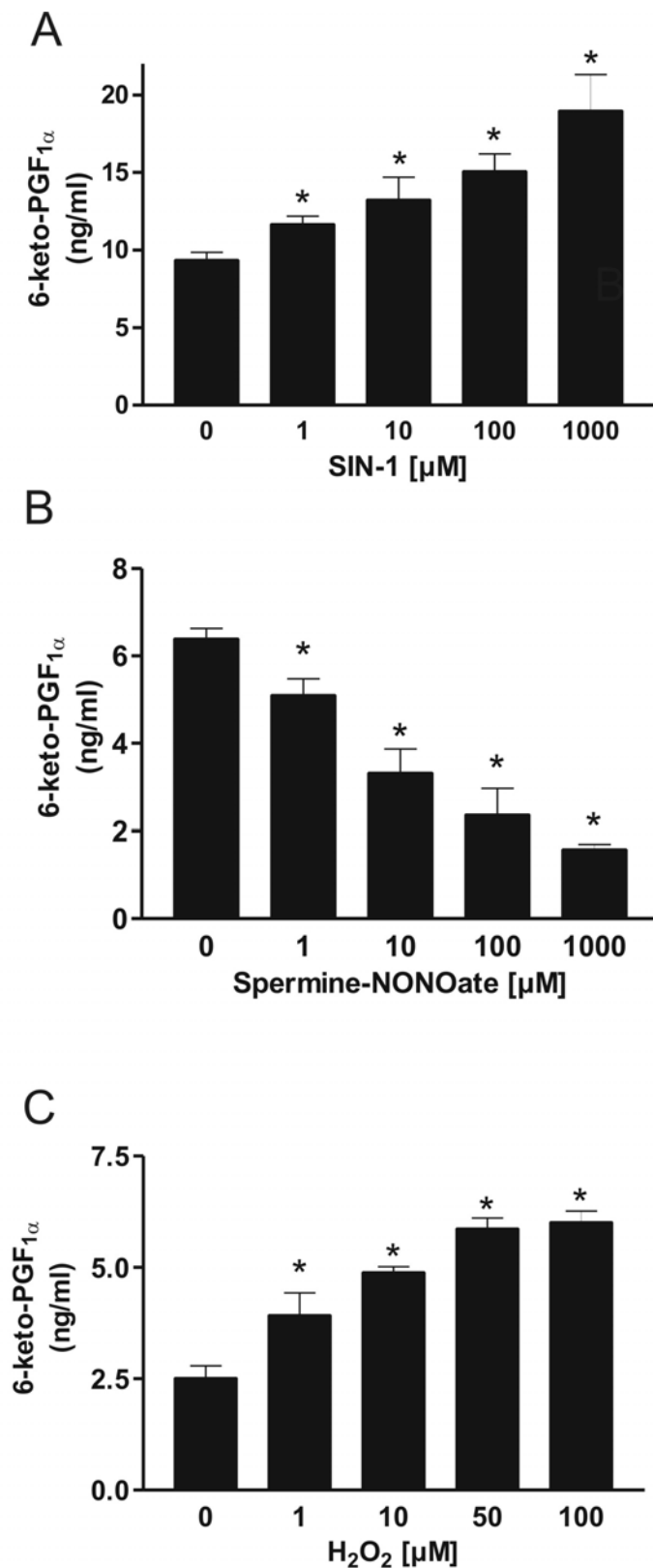
The apparent discrepancy that in septic patients high 6-keto-PGF<sub>1α</sub> levels were observed (Halushka 1985) could be solved by the finding of a high output of PGI<sub>2</sub> from LPS-treated bovine aortic smooth muscle cells (SMC). This synthesis strictly depends on the induction of PGHS-2 which exclusively provides PGH<sub>2</sub> to the constitutively present PGI<sub>2</sub>-synthase and could counterregulate the observed vasoconstriction resulting from an impaired endothelial PGI<sub>2</sub> synthesis (Schildknecht 2004). To our surprise, no nitration of PGI<sub>2</sub>-synthase in stimulated SMC was observed even not by the exogenous addition of SIN-1 as a peroxynitrite generating compound.

In the present investigation, when trying to find a mechanistic explanation of this unexpected behavior, we even noticed an increase of PGI<sub>2</sub> synthesis after addition of SIN-1. We found evidence that peroxynitrite acted by increasing the peroxide tone for PGHS-2 which was estimated being at only half-saturating levels. Of even more importance was the finding that the basal peroxide tone in LPS-treated SMC required simultaneous generation of low levels of  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  providing peroxynitrite for the endogenous peroxide tone. Literature is divided with respect to whether  $\bullet\text{NO}$  activates (Davidge 1995; Salvemini 1993) or inhibits (Doni 1988; Upmacis 1999) prostaglandin biosynthesis. The proposed model which includes both  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  in the regulation of PGHS activity may serve to explain a variety of such reports concerning the interaction of the NO-synthase with the PGHS-pathway.

## Results

The optimal conditions for PGHS-2 induction and PGI<sub>2</sub> synthesis in SMC had been established previously (Schildknecht 2004). The standard assay used 5h LPS (10µg/ml) stimulated cells which after washing were again incubated with LPS and different agents for another 60min. It should be mentioned that the formation of 6-keto-PGF<sub>1α</sub> proceeded linearly with time which appears quite remarkable in view of the known autoinactivation (Song 2001; Egan 1976) of PGHS and the instability of PGI<sub>2</sub> synthase in the presence of oxidants (Moncada 1976; Weaver 2001). The latter refers in particular to the high sensitivity against peroxynitrite which leads to nitration of an active site-located Tyr-residue and inhibition of activity as reported in endothelial cells (Bachschnid 2003; Zou 1999). However, unlike in endothelial cells, only a very low generation of nitrite as a stable oxidation product of •NO or nitrate originating from the formation of peroxynitrite was detected in SMC. When SIN-1 as a peroxynitrite-generating compound was added at increasing concentrations, the activity of PGI<sub>2</sub> formation was not inhibited but even increased by almost 100% compared to controls (Figure 4.2.1A). Western blots probed with 3-nitrotyrosine antibodies did not indicate any nitration of the enzyme even at the highest concentrations (1mM SIN-1) (not shown). In contrast incubation with Spermine-NONOate, a pure •NO-donor, caused a concentration-dependent decrease in PGI<sub>2</sub> synthesis (Figure 4.2.1B). This excluded •NO and favored peroxynitrite as the activating principle. To exclude an impact of SIN-1 or Spermine-NONOate on PGHS-2 expression, cells were treated as described and PGHS-2 mRNA was detected by quantitative PCR. Under the conditions employed, PGHS-2 mRNA was not affected by SIN-1 or Spermine-NONOate. •NO was suggested to directly interact with the Tyr-radical of activated PGHS-2 (Gunther 1997). However, •NO-levels of around 1mM were required which excludes a significance of this reaction for biological systems (Tsai 1994). For peroxynitrite it was

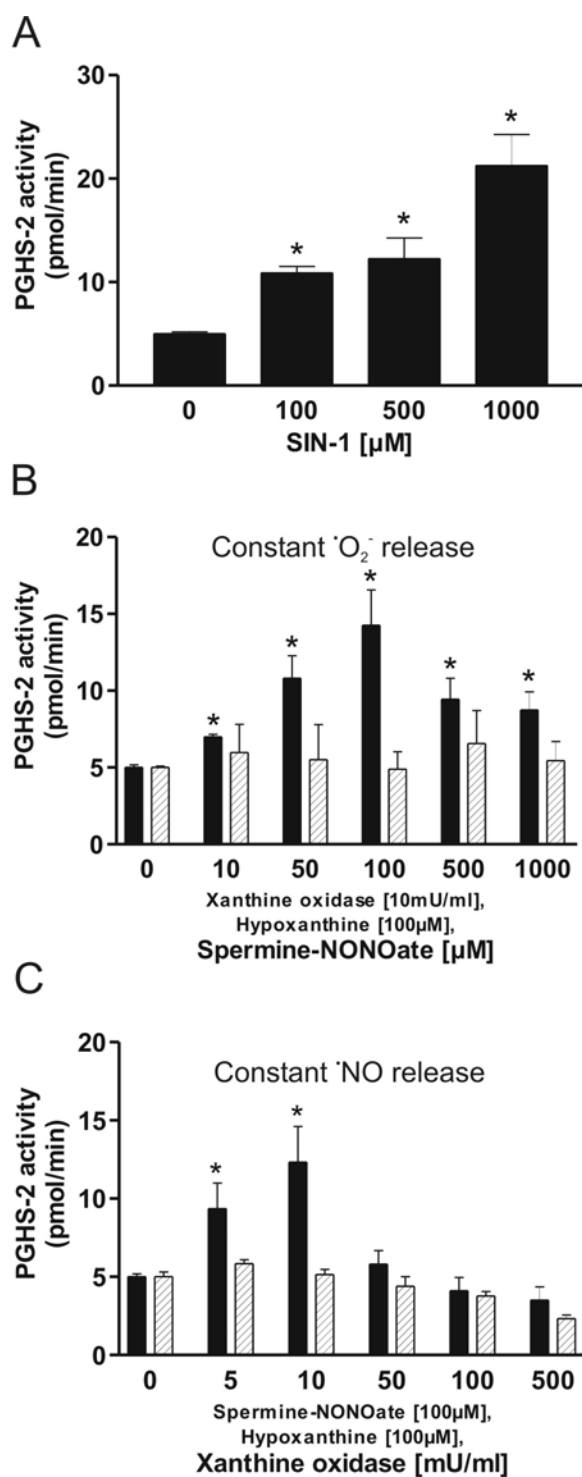
known from the work of Landino et al. (Landino 1996) that it can stimulate PGHS-1 activity by providing the peroxide tone for generating the Tyr-radical at the active site as reported for  $H_2O_2$  or arachidonic acid peroxides.



**Figure 4.2.1.** Bovine SMC were incubated with LPS (10 $\mu$ g/ml) for 5h to allow induction of PGHS-2. Medium was discarded, cells were washed twice and incubated with new medium containing LPS (10 $\mu$ g/ml) and the respective compounds in the concentrations indicated. 6-keto-PGF<sub>1 $\alpha$</sub>  release measured by EIA was stimulated by the peroxynitrite-generating compound SIN-1 (A) as well as by  $H_2O_2$  (C) in a concentration-dependent manner. Since 1mM SIN-1 releases only 10nmolml<sup>-1</sup>min<sup>-1</sup> peroxynitrite under the conditions employed, peroxynitrite can be regarded as a far more potent activator than  $H_2O_2$ . (B) 6-keto-PGF<sub>1 $\alpha$</sub>  release was concentration-dependently inhibited by the NO-donor Spermine-NONOate. Values are means  $\pm$  SD (n=4). \* $P$  < 0.05 vs. SIN-1,  $H_2O_2$ , or Spermine-NONOate = 0.

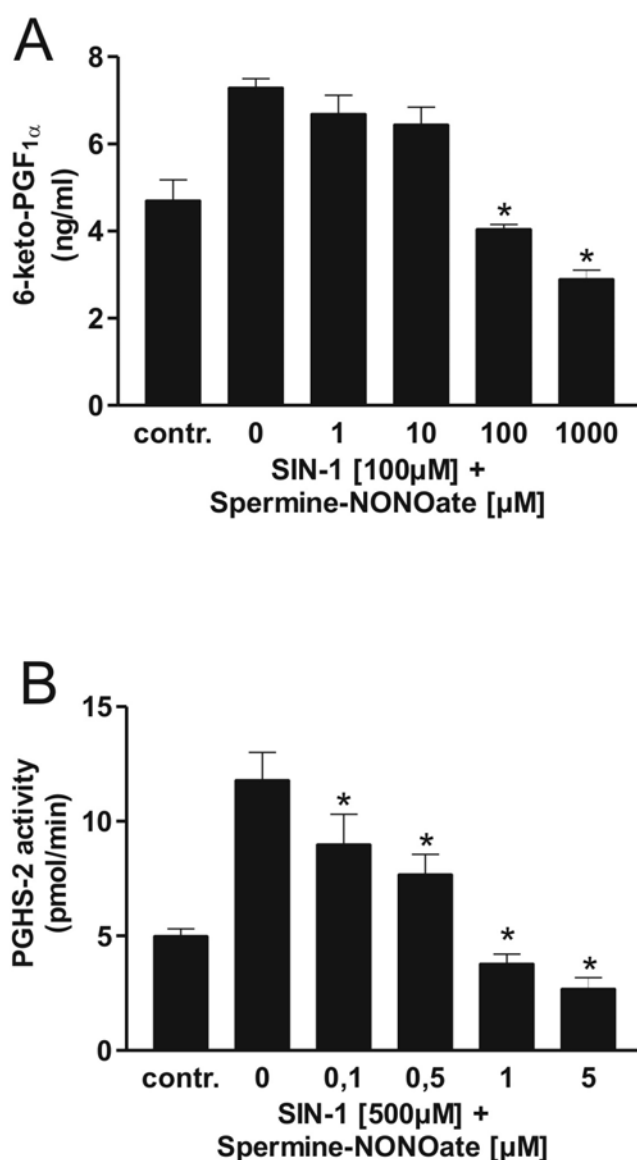
Therefore  $\text{H}_2\text{O}_2$  was added to the cells resulting also in a stimulation of activity by roughly 100% (Figure 4.2.1C). This strengthened the hypothesis that in whole cells the peroxide tone was limiting and could be saturated by further additions of peroxides.

The assumption of peroxynitrite as an efficient physiological peroxide tone provider was then verified for purified PGHS-2 using  $^{14}\text{C}$ -AA as a substrate and measuring the total amount of PGHS metabolites. To avoid activation of purified PGHS-2 by contaminating peroxides, glutathione and glutathione-peroxidase were added. Under such conditions SIN-1 caused a fourfold increase in a concentration-dependent manner (Figure 4.2.2A). Due to the inhibitory action of glutathione peroxidase/glutathione, activation of purified PGHS-2 required higher concentrations of SIN-1 compared with SMC. Using the isolated PGHS-2 enzyme, the peroxide tone was then provided by separate generation of  $\bullet\text{O}_2^-$  and  $\bullet\text{NO}$  with constant 10mU/ml xanthine oxidase, 100 $\mu\text{M}$  hypoxanthine and increasing concentrations (0-1mM) of the  $\bullet\text{NO}$ -donor Spermine-NONOate (Figure 4.2.2B). The activity changed in a bell-shaped curve indicating that an excess of  $\bullet\text{NO}$  could lower peroxynitrite levels. The complementary experiment with a fixed  $\bullet\text{NO}$  release (100 $\mu\text{M}$  Spermine-NONOate) and variable  $\bullet\text{O}_2^-$  generation caused the same stimulatory and then inhibitory effect (Figure 4.2.2C). Kinetic calculations on the rates of the respective  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  formation under the buffer conditions employed confirmed that the maximal stimulation occurred at about equal rates of  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  generation (100 $\mu\text{M}$  Spermine-NONOate + 10mU/ml xanthine oxidase  $\approx 1.5\text{nmolml}^{-1}\text{min}^{-1}$  respectively).



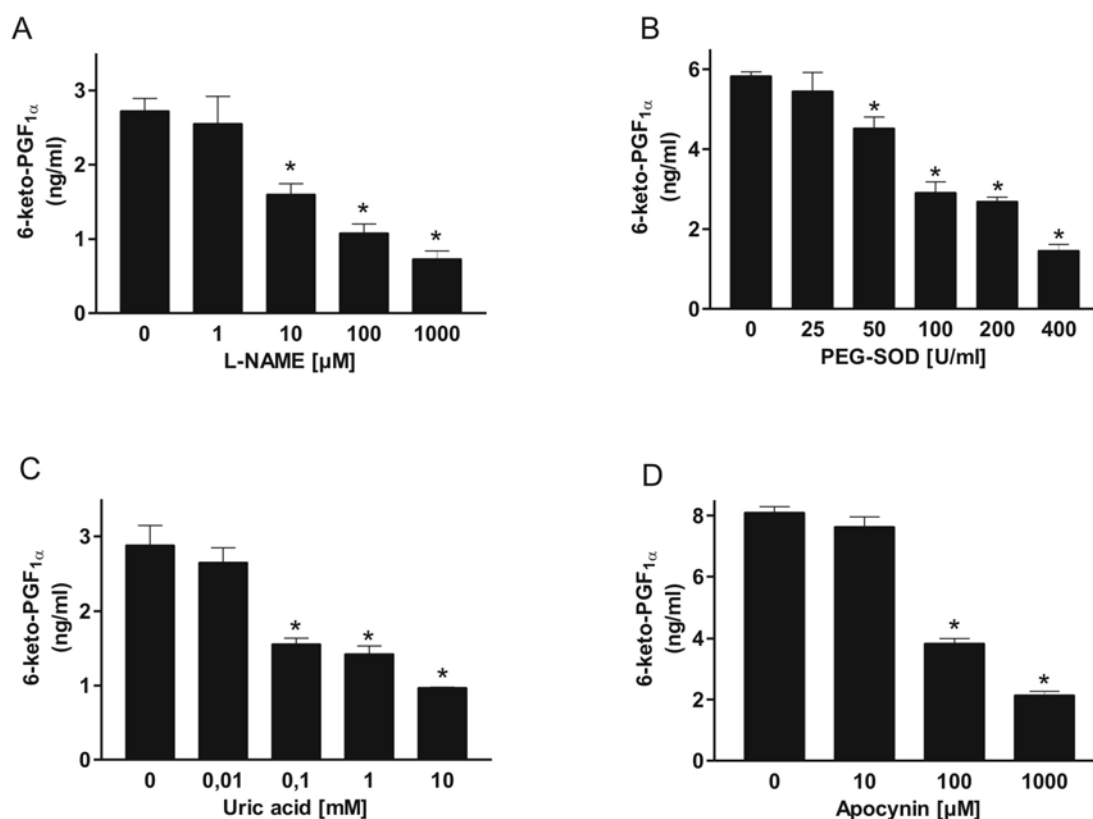
**Figure 4.2.2.** Activation of purified PGHS-2 by peroxynitrite. PGHS-2 (5units/100 $\mu\text{l}$ ) was incubated at 37°C with 5 units glutathione peroxidase, 0.2mM glutathione, 325units/100 $\mu\text{l}$  catalase, and 500 $\mu\text{M}$  phenol in 0.1M Tris-HCl pH 8.0. *A*) Activity wo. SIN-1 was defined as 100% and elevated by nearly fourfold. *B*) PGHS-2 was incubated with 10munits/ml xanthine oxidase, 100 $\mu\text{M}$  hypoxanthine and variable concentrations of the NO-donor Spermine-NONOate (black bars). *C*) PGHS-2 was incubated with 100 $\mu\text{M}$  Spermine-NONOate, 100 $\mu\text{M}$  hypoxanthine, and variable activities of xanthine oxidase (black bars). Open bars (control) represent activity of PGHS-2 incubated only with Spermine-NONOate (*B*) or xanthine oxidase (*C*) in the concentrations indicated. The reaction was terminated after 2min by the addition of ethyl acetate/citric acid (2M) (30:1). Values are means  $\pm$  SD (n=4). \* $P < 0.05$  vs. Spermine-NONOate or xanthine oxidase =0 respectively.

Thus, the data with isolated PGHS-2 isozyme supported the response of cellular PGHS-2 to peroxynitrite in LPS-treated SMC. Further proof for a suppressive effect of  $\bullet\text{NO}$  on peroxynitrite levels could be obtained by a coincubation of SMC with  $100\mu\text{M}$  SIN-1 and increasing concentrations (0-1mM) of the  $\bullet\text{NO}$ -donor Spermine-NONOate (Figure 4.2.3A). These data are consistent with an oxidation of  $\bullet\text{NO}$  by peroxynitrite and could be an explanation for the results in Figure 4.2.1B with the implication that the endogenous peroxide tone in LPS-treated SMC is provided by peroxynitrite from a simultaneous generation of endogenous  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$ .



**Figure 4.2.3.** A) Inhibition of peroxynitrite-mediated stimulation of 6-keto-PGF<sub>1 $\alpha$</sub>  release by nitric oxide. SMC were incubated with LPS ( $10\mu\text{g/ml}$ ) for 5h. Medium was discarded, cells were washed twice and new medium + LPS ( $10\mu\text{g/ml}$ ) + SIN-1 ( $100\mu\text{M}$ ) and Spermine-NONOate in the concentrations indicated were added for 60min. Control = wo. SIN-1, wo. Spermine-NONOate. 6-keto-PGF<sub>1 $\alpha$</sub>  formation was analyzed by EIA. Values are mean  $\pm$  SD (n=4) \* $P < 0.05$  vs. Spermine-NONOate = 0. B) Activation of purified PGHS-2 by peroxynitrite is reversed by the NO-donor Spermine-NONOate. PGHS-2 was incubated with  $500\mu\text{M}$  SIN-1 and Spermine-NONOate in the concentrations indicated. Excess of NO over peroxynitrite concentration-dependently decreased PGHS-2 activity. Contr. = wo. SIN-1, wo. Spermine-NONOate. Values are means  $\pm$  SD (n=4). \* $P < 0.05$  vs. SIN-1 = 0.

The same experiment was performed with purified PGHS-2 (Figure 4.2.3B) and qualitatively led to the same results. This new and exciting hypothesis was further tested by the addition of L-NAME as an effective inhibitor of NO-synthases (Figure 4.2.4A). There was a distinct inhibition of PGI<sub>2</sub>-synthesis and by employing polyethylene-glycol-superoxide dismutase (PEG-SOD) as a membrane-permeable form of SOD, a similar decrease in activity was found (Figure 4.2.4B). The spin-trap 4-oxo-TEMPO which is active in converting  $\cdot\text{O}_2^-$  to H<sub>2</sub>O<sub>2</sub> had the same effect (not shown). Uric acid is a quite selective reactant for peroxynitrite and caused a concentration-dependent suppression of 6-keto-PGF<sub>1 $\alpha$</sub>  formation (Figure 4.2.4C).



**Figure 4.2.4.** Inhibition of endogenous peroxynitrite formation lowers release of 6-keto-PGF<sub>1 $\alpha$</sub> . SMC were incubated with LPS (10μg/ml) for 5h. Medium was discarded, cells were washed twice and new medium + LPS (10μg/ml) + L-NAME (A), polyethylene glycol-superoxide dismutase (PEG-SOD) (B), uric acid (C) and apocynin (D) in the concentrations indicated were added for 60min. Inhibition of endogenous  $\cdot\text{O}_2^-$  formation by PEG-SOD-catalyzed dismutation or inhibition of the NADPH-oxidase complex by apocynin resulted in a concentration-dependent inhibition of 6-keto-PGF<sub>1 $\alpha$</sub>  release. The NO-synthase isoenzyme-unspecific inhibitor L-NAME as well as uric acid as a scavenger of endogenously formed peroxynitrite also yielded a concentration-dependent inhibition of 6-keto-PGF<sub>1 $\alpha$</sub>  generation by SMC. Values are means  $\pm$  SD (n=4). \**P* < 0.05 vs. values = 0.

It was also intended to identify the NOS-isozyme for  $\bullet$ NO-generation. It is one of the characteristics of LPS-treated bovine SMC that the induction of PGHS-2 is accompanied by only a very weak induction of NOS-2. NOS-3 was not present but in literature SMC were reported to contain NOS-1 and a  $\mu$ -splice variant of NOS-1 (Schwarz 1999; Brophy 2000). In view of the very low formation of nitrite close to the detection limit in accordance to reports in literature, (0.2 $\mu$ M over 10h) no conclusive experiments on the contribution of either one of these enzymes were successful. With regard to the  $\bullet$ O<sub>2</sub><sup>-</sup> source it is well known that LPS can activate cellular oxidases such as NADPH-oxidases, xanthine oxidase, or mitochondria. Apocynin had been proposed as a selective inhibitor of NADPH-oxidase and indeed proved to be concentration-dependent inhibitory (Figure 4.2.4D). These results altogether allow the conclusion that peroxynitrite serves as the dominating endogenous provider of the peroxide tone in LPS-treated SMC. A possible involvement of cGMP on 6-keto-PGF<sub>1 $\alpha$</sub>  formation could be excluded on the basis of no detected effects of 8-bromo-cGMP on 6-keto-PGF<sub>1 $\alpha$</sub>  formation. Also PGHS-2 mRNA and protein expression remained constant over the 60min incubated with the different components.

## Discussion

The results demonstrated in this work provide a mechanistic explanation for the previously observed differences in PGI<sub>2</sub>-biosynthesis between native endothelial and LPS-treated vascular smooth muscle cells (Bachschmid 2003; Schildknecht 2004). Peroxynitrite readily caused inhibition of PGI<sub>2</sub>-synthase by Tyr-nitration in endothelial cells whereas in SMC rather a doubling of PGI<sub>2</sub> release was observed in presence of the •NO and •O<sub>2</sub><sup>-</sup> forming compound SIN-1. Since H<sub>2</sub>O<sub>2</sub> was able to cause the same increase one can conclude the peroxide tone as the affected parameter in the modulation of PGHS-2 activity. Thus, not only the LPS-dependent expression of PGHS-2 acts as a regulator of PGI<sub>2</sub> output but also the posttranslational regulation via the peroxide tone turned out to control this release. Being at half-maximal saturation the peroxide tone is set at optimal conditions to allow regulation in both directions.

This finding was rather unexpected due to the reported extremely low saturation of PGHS-2 with peroxides at already 2nM in contrast to that for PGHS-1 with 21nM (Kulmacz 1995). Working at half-saturation implies that in bovine SMC PGHS-2 is exposed to peroxide levels of around 1nM which allows to conclude on a high peroxidase activity in these cells. GSH-dependent peroxidases as well as the intrinsic peroxidase of PGHS-2 could contribute to keep the intracellular levels of peroxynitrite, H<sub>2</sub>O<sub>2</sub> or PGG<sub>2</sub> at the low nanomolar level. 1mM SIN-1 equals a peroxynitrite generation of roughly 100nM/sec with a t<sub>1/2</sub> of 1sec for peroxynitrite. On this background it becomes understandable that peroxynitrite generation even at 1mM SIN-1 does not lead to a nitration of PGI<sub>2</sub>-synthase for which bolus additions of 50nM are required for half-inhibition (Zou 1997; Zou 1996). Our data also allow to conclude that PGG<sub>2</sub> or other arachidonic acid hydroperoxides as products of the PGHS-2 reaction do not contribute essentially to the peroxide tone and

hence must be either readily reduced or not able to compete with arachidonic acid for binding at the active site of PGHS-2.

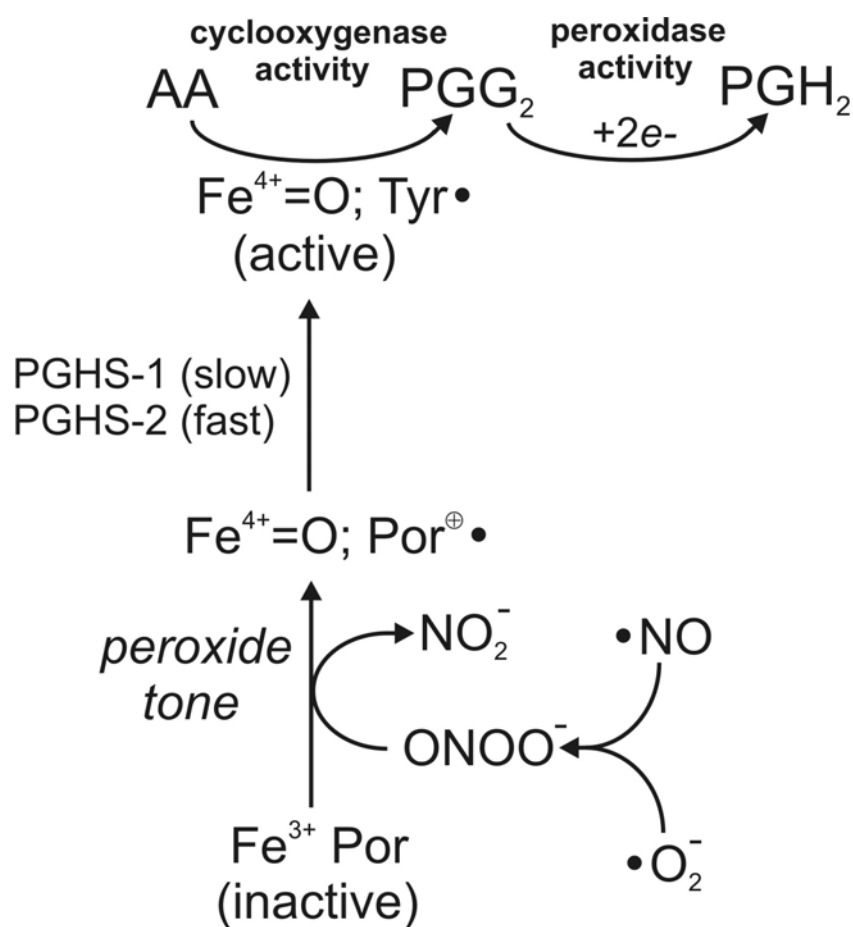
The second novel finding also came unexpected when the inhibition of PGI<sub>2</sub> formation by L-NAME, PEG-SOD, uric acid and apocynin clearly indicated the generation of peroxynitrite as a provider of the endogenous peroxide tone. This must be derived from an equal rate of •NO and •O<sub>2</sub><sup>-</sup> formation since additional •NO as well as •O<sub>2</sub><sup>-</sup> caused a decrease in PGI<sub>2</sub> formation in accordance with the known chemistry of peroxynitrite (Jourdeuil 2001; Daiber 2002; Goldstein 2000):

- 1) •NO + •O<sub>2</sub><sup>-</sup> → ONOO<sup>-</sup>
- 2) ONOO<sup>-</sup> + H<sup>+</sup> → ONOOH (pK 6.6)
- 3) ONOOH + •NO → •NO<sub>2</sub> + NO<sub>2</sub><sup>-</sup> + H<sup>+</sup>
- 4) ONOOH + •O<sub>2</sub><sup>-</sup> → O<sub>2</sub> + OH<sup>-</sup> + •NO<sub>2</sub>

That this chemistry is valid also under conditions of low levels of peroxynitrite generated by SIN-1 in presence of glutathione and glutathione peroxidase was shown with isolated PGHS-2. It remains to be investigated how a coordinated •NO and •O<sub>2</sub><sup>-</sup> production to yield peroxynitrite is balanced out in the cells.

Since the required levels of both radicals seem to be extremely low, the enzymatic sources for both are difficult to assess. We had previously ruled out that NOS-2 is induced to a significant extent however basal levels of NOS-2 mRNA were present (Schildknecht 2004). NOS-3 also was not detectable but according to literature the μ-splice variant of NOS-1 could be constitutively expressed (Schwarz 1999; Brophy 2000; Boulanger 1998). As judged from the accumulating nitrite/nitrate levels only minimal NOS activity seems to be present (Zehetgruber 1993). With regard to the source of •O<sub>2</sub><sup>-</sup> the inhibitory effect of apocynin would point to NADPH-oxidase which has been detected in SMC (Griendling 2000). The addition of PEG-SOD (Figure 4.2.4B) resulted in a decline of 6-keto-PGF<sub>1α</sub>

formation. It could be expected that  $\text{H}_2\text{O}_2$  originating from the SOD activity would increase the peroxide tone and 6-keto-PGF $_{1\alpha}$  release. In Figure 4.2.1C however we have demonstrated that  $\text{H}_2\text{O}_2$  concentrations in the micromolar range were necessary to evoke such an increase whereas  $\text{H}_2\text{O}_2$  levels originating from dismutation of endogenously formed  $\cdot\text{O}_2^-$  can be expected in the low nanomolar range. At present, the conditions of the generation of the endogenous “peroxynitrite tone” require further studies also with respect



**Figure 4.2.5. Peroxynitrite as mediator of the peroxide tone.**

PGHS comprises a cyclooxygenase activity in which arachidonate (AA) is oxygenated to yield the 15-hydroperoxy-prostaglandin-9,11-endoperoxide ( $\text{PGG}_2$ ) which is reduced to its hydroxy derivate ( $\text{PGH}_2$ ) by the peroxidase activity of the enzyme. Peroxynitrite ( $\text{ONOO}^-$ ) initially reacts with  $\text{Fe}^{3+}\text{Por}$  of the resting enzyme to form the ferryl-porphyrin  $\pi$  cation radical intermediate ( $\text{Fe}^{4+}\text{Por}^{\oplus\bullet}$ ). By intramolecular redox reactions, a ferryl Tyr-radical ( $\text{Fe}^{4+}\text{Tyr}^{\bullet}$ ) is formed that interacts with AA to form an arachidonate radical ( $\text{AA}^{\bullet}$ ) which incorporates two molecules of  $\text{O}_2$  yielding  $\text{PGG}_2$ . The porphyrin cation radical or the Tyr-radical can become reduced sporadically to end in the ferric resting state and the cycle again requires an initiation by peroxides. Conversion of  $\text{Fe}^{4+}\text{Por}^{\oplus\bullet}$  to  $\text{Fe}^{4+}\text{Tyr}^{\bullet}$  was reported faster in PGHS-2 than in PGHS-1 which explains the lower peroxide requirement of PGHS-2 (2nM) compared with that of PGHS-1 (21nM). Peroxynitrite originates from the interaction of  $\cdot\text{NO}$  and  $\cdot\text{O}_2^-$ , however excess of one radical results in increased decomposition of peroxynitrite and therefore decreased PGHS-2 activity.

to the localization and compartmentation of the enzymes involved (Spisni 2001; Sato 2004; Liou 2001). It also should be emphasized that SMC can acquire NOS-2 after about 24h of LPS-exposure with still unknown effects on PGI<sub>2</sub>-synthesis. In case of LPS-treated rat mesangial cells, a high induction of NOS-2 indeed causes partial nitration of PGI<sub>2</sub>-synthase (Zou 1998). In summary, a sophisticated mechanism of PGHS-2 regulation in LPS-induced SMC evolves for which our main findings are incorporated in a model based on the work of Kulmacz et al. (Figure 4.2.5) (van der Dink 2002; Chen 1999; Lu 1999; Kulmacz 1994; Tsai 2000; Tsai 1999).

With reference to the tenfold lower peroxide tone required for PGHS-2 compared to PGHS-1 (Kulmacz 1995) it becomes obvious that nitration of PGI<sub>2</sub>-synthase is unlikely to occur in SMC since about 50nM peroxyxynitrite was found as the IC<sub>50</sub> value for the nitration of isolated PGI<sub>2</sub>-synthase (Zou 1997; Zou 1996). If, however, PGHS-1 would operate with a peroxyxynitrite-provided peroxide tone (21nM at saturation) even at half-saturation partial nitration of PGI<sub>2</sub>-synthase could be reached. It was beyond the aims of this study to identify the peroxide tone in endothelial cells, but peroxyxynitrite also is a likely candidate and half-saturation may also apply as can be concluded from a rise in PGI<sub>2</sub>-release under oxidative stress conditions (Matthews 1995). This would then be in agreement with the observed nitration of endothelial PGI<sub>2</sub>-synthase under LPS-treatment. Meanwhile, experiments with redox cyclers applied to LPS-exposed SMC have succeeded in a nitration of PGI<sub>2</sub>-synthase (Zimmerling, unpublished) in accordance with the assumption of a strong peroxidase activity in these cells. Also PGHS-2 profits from such high activities of cellular peroxidases since an autocatalytic oxidative self-inactivation (Markey 1987; Wu 1999; Song 2001; Egan 1976) seems to be suppressed in SMC as judged from the linear release of PGI<sub>2</sub> over time (Schildknecht 2004).

In view of such strictly and elegantly regulated PGI<sub>2</sub>-synthesis the physiological role of PGI<sub>2</sub> release deserves a second consideration. In endothelial cells the regulation seems to rely mainly on the nitration of PGI<sub>2</sub>-synthase which can be achieved by LPS-triggered •O<sub>2</sub><sup>-</sup> generation since an endogenous NOS-3 activity is present (Bachschmid 2003). After •O<sub>2</sub><sup>-</sup> has caused endothelial dysfunction by eliminating •NO and PGI<sub>2</sub> release, the subsequent PGHS-2 induction allows SMC to counteract by massive PGI<sub>2</sub> synthesis (Wu 1999; Schildknecht 2004). In order to guarantee a sustained release, the redox milieu seems to be optimally adjusted for PGHS-2 and PGI<sub>2</sub>-synthase activity. It has to be noted that this regulation of vascular tone in sepsis is species dependent and applies for the bovine and human vasculature whereas in SMC of the rat mainly •NO instead of PGI<sub>2</sub> is released (Schildknecht 2005).

In summary, the conditions in LPS-exposed bovine SMC appear to be optimized for PGHS-2 dependent sustained PGI<sub>2</sub> synthesis in the progressive stages of sepsis. The unsaturated peroxide tone allows immediate regulation at the PGHS-2 level in addition to the up and downregulation of the PGHS-2 protein. The low peroxide requirement of PGHS-2 together with an efficient peroxide-reducing capacity of SMC are prerequisite for an undisturbed PGI<sub>2</sub> releasing function of SMC under inflammatory conditions. Conflicting reports in literature on stimulating or inhibiting interactions of •NO with the PGHS pathway may now find new mechanistic interpretations by these novel findings.

## **4.3. Endotoxin Elicits Nitric Oxide Release in Rat but Prostacyclin Synthesis in Human and Bovine Vascular Smooth Muscle Cells**

### **Introduction**

Septic shock is associated with an imbalance of the finely tuned homeostatic network in the vasculature (Bone 1991; Wort 1999). Understanding of the biochemical events occurring in sepsis is essential for a rational pharmacological treatment and hence animal and cellular models are being investigated to study the sequence of events from the first inflammatory responses to endothelial dysfunction and finally death which still occurs in a high percentage of patients (Parrillo 1993; Vallance 1997; Martin 1991). The rat model has been intensively used for many studies although the higher resistance of rats and their lower mortality upon endotoxin (lipopolysaccharide, LPS) exposure is well known (Schultz 2002).

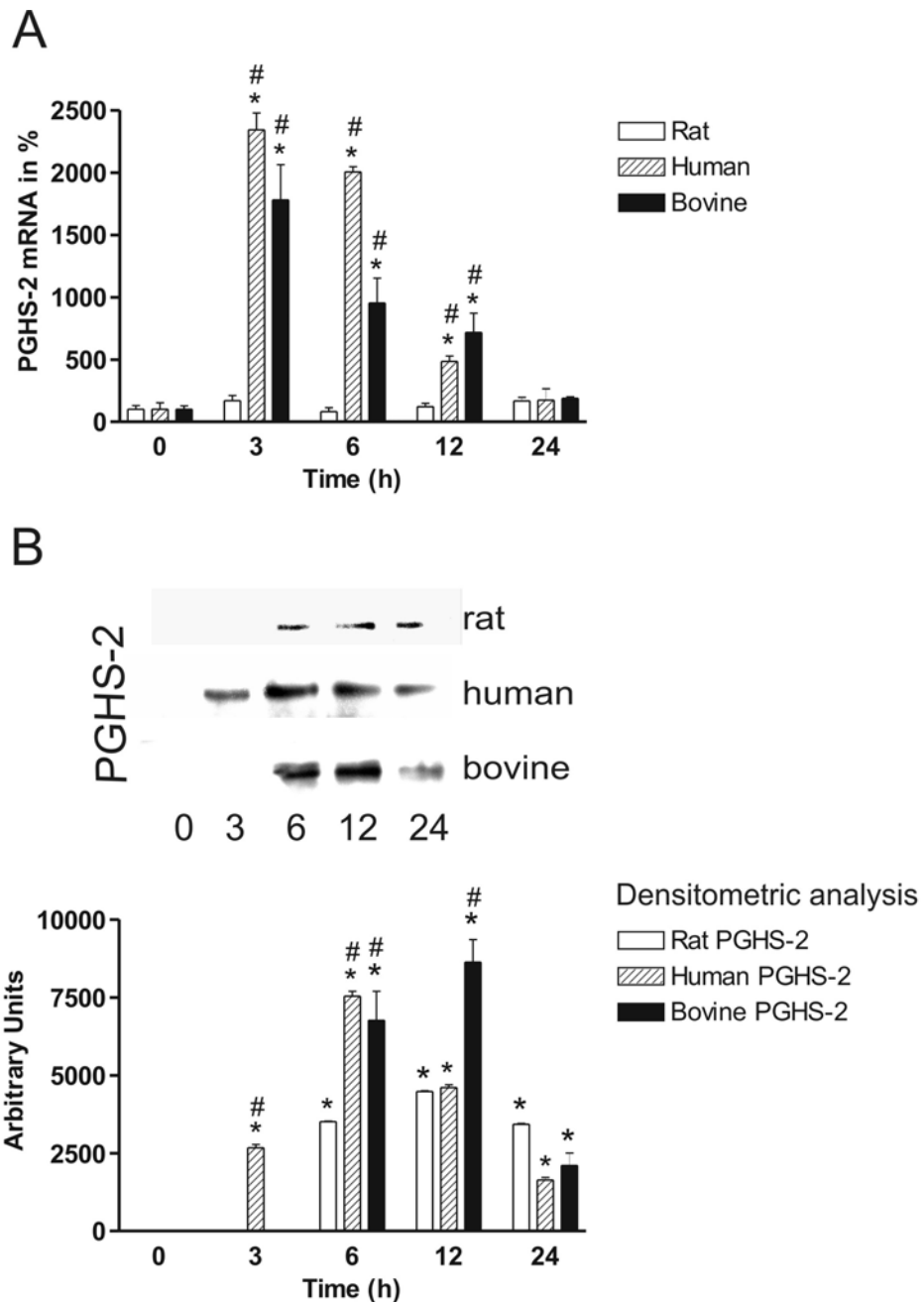
Bovine vascular endothelial cells under resting conditions release basal amounts of prostacyclin (PGI<sub>2</sub>) and nitric oxide ( $\bullet$ NO) which both exert anti-aggregatory, anti-adhesive and vasodilatory properties (Noll 1998; Furchgott 1980; Moncada 1979). Exposure to LPS results in increased endothelial superoxide ( $\bullet$ O<sub>2</sub><sup>-</sup>) formation which interacts with  $\bullet$ NO in a nearly diffusion-limited reaction to form peroxynitrite (Bachschnid 2003; Huie 1993). This reactive intermediate was found capable to inhibit endothelial PGI<sub>2</sub>-synthase by nitration of a tyrosine-residue near the active site (Zou 1997; Schmidt 2003). An upregulation of  $\bullet$ O<sub>2</sub><sup>-</sup> therefore inhibits the release of both vasodilators PGI<sub>2</sub> and  $\bullet$ NO. Since endothelial

prostaglandin endoperoxide synthase-1 (PGHS-1) which supplies PGI<sub>2</sub>-synthase with substrate remains active under such conditions, 15-hydroxy-prostaglandin 9,11 endoperoxide (PGH<sub>2</sub>) is still produced and can activate the thromboxane A<sub>2</sub> (TxA<sub>2</sub>)/ PGH<sub>2</sub> receptor on vascular smooth muscle cells resulting in additional vasoconstriction (Mais 1985; Ogletree 1985). These events which require no de novo protein synthesis can be observed within the first hour of stimulation and prepare the stage for leukocyte adhesion and emigration (Gumina 1997). A second phase in the activation of the vasculature is initiated after roughly 2h and involves the induction of early immediate genes in vascular smooth muscle cells. Under such conditions, PGHS-2 was observed upregulated in bovine aortic smooth muscle cells (SMC) to supply the so far dormant constitutive PGI<sub>2</sub> synthase with substrate (Schildknecht 2004). It was further deduced that this PGHS-2-dependent PGI<sub>2</sub> generation vitally contributes to the highly elevated 6-keto-PGF<sub>1α</sub> levels and the extreme hypotension observed in patients with septic shock syndromes (Halushka 1985).

Notably, no significant nitration of PGI<sub>2</sub> synthase was observed in these bovine SMC which was explained by the lack of NOS-2 induction in this tissue. This appeared to be at variance with reports of intense NOS-2 induction in the rat aorta (Fries 2003; Rees 1990) and led us to investigate potential differences in the response of rat and bovine aortic SMC towards LPS. In order to compare with the situation in humans we included SMC from human aortic segments. As an essential result it was verified that rat aortic SMC responded to LPS by an elevated output of •NO as a consequence of NOS-2 induction whereas human and bovine SMC responded mainly by a PGHS-2 dependent PGI<sub>2</sub> release and only marginal formation of •NO.

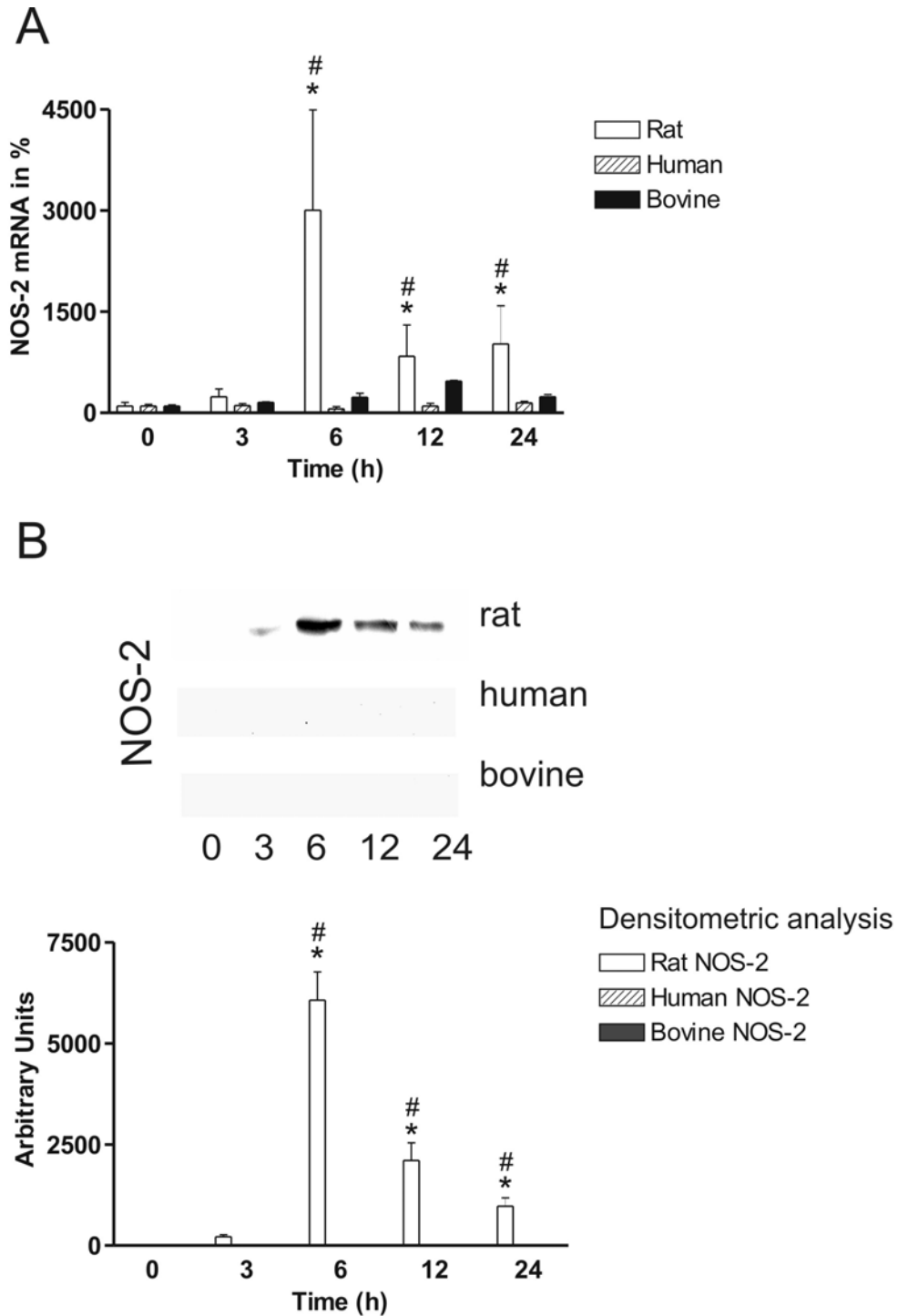
## Results

Smooth muscle cells from rat, bovine and human aortae were cultivated and used from passage 1-3. As described earlier, LPS addition (10 $\mu$ g/ml) to bovine SMC caused a rapid

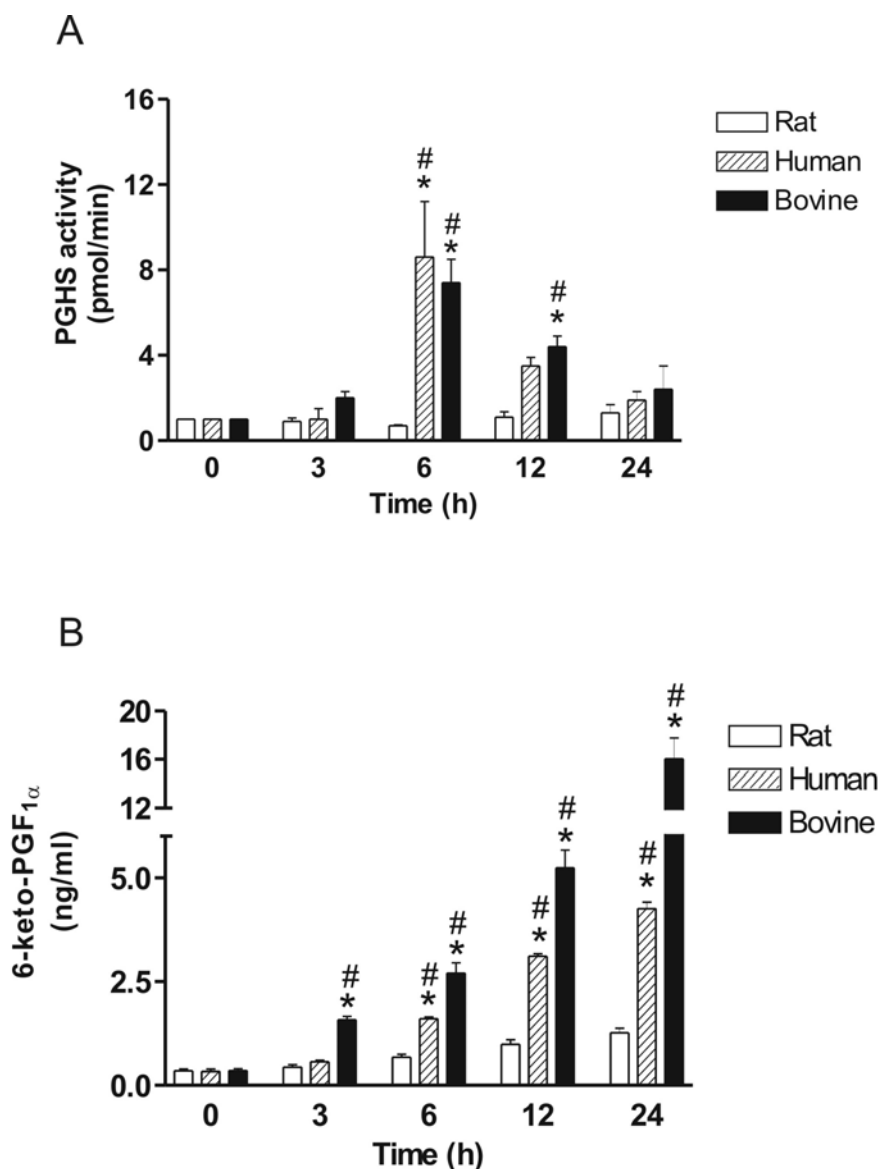


**Figure 4.3.1** PGHS-2 mRNA induction in aortic SMC (A). LPS (10 $\mu$ g/ml) was added at t=0, cells were incubated for the time periods indicated. Human and bovine SMC exhibited a distinct induction of PGHS-2 whereas induction in rat SMC was nearly negligible. PGHS-2 protein expression followed its corresponding mRNA in human and bovine SMC whereas expression in rat was detected much weaker (B). Note that incubation time of X-ray detection film was three times longer in case of rat PGHS-2 blot. Western blots are representative for three separate experiments which were evaluated by densitometry as demonstrated. Values are mean  $\pm$  SD (n=4 in A; n=3 in B). (\*): inner-group differences  $P < 0.05$  vs. values at t=0. (#): inter-group differences  $P < 0.05$  of human and bovine vs. rat values.

increase of prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA after 3h followed by a steady decline until 24h after LPS exposure (Fig. 4.3.1A).



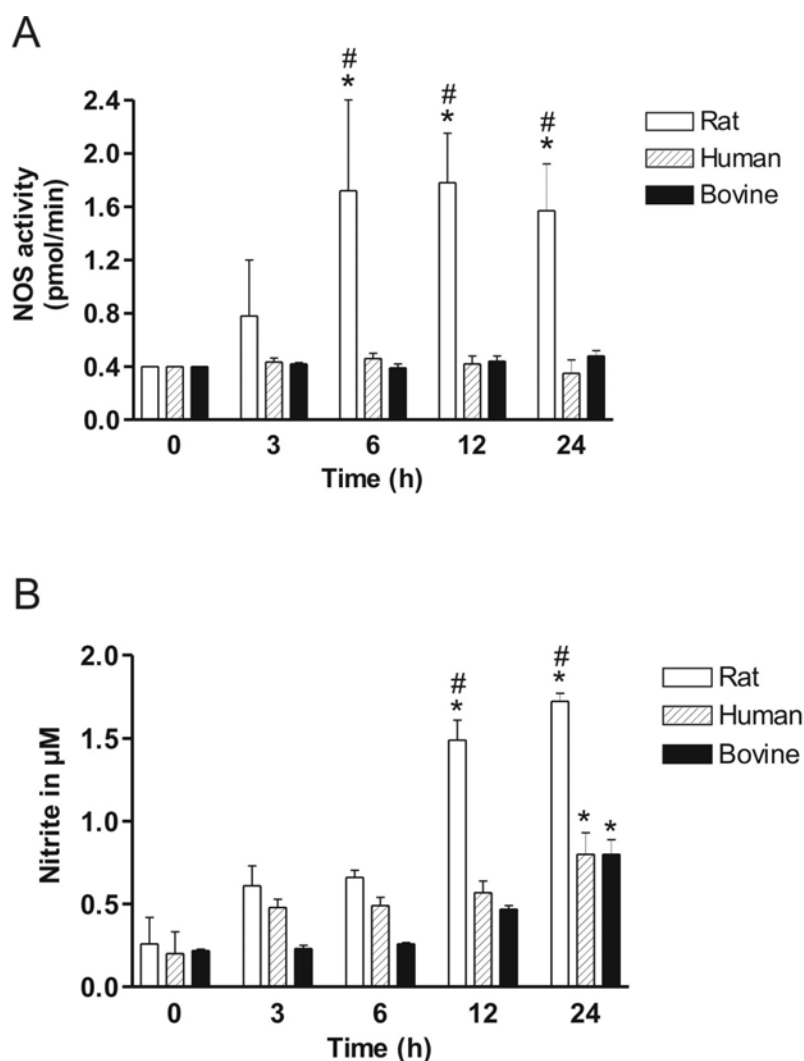
**Figure 4.3.2** NOS-2 induction in aortic SMC. Cells were treated as in Fig. 1. Rat SMC demonstrated a clear induction of NOS-2 mRNA (A) as well as NOS-2 protein (B) while induction in human and bovine cells was only minimal. Densitometric evaluation of NOS-2 protein expression (B) underlined a maximal induction after 6h of LPS exposure followed by a gradual decline in rat SMC. Values are mean SD (n=4 in A; n=3 in B), (\*): inner-group differences  $P < 0.05$  vs. values at t=0. (#): inter-group differences  $P < 0.05$  of rat vs. human values.



**Figure 4.3.3** Total PGHS activity of SMC homogenates (A) revealed an upregulation only in human and bovine cells. 6-keto-PGF<sub>1α</sub> as the stable hydrolysis product of PGI<sub>2</sub> was profoundly upregulated in human and bovine SMC while rat SMC exhibited a constant but moderate release (B). 6-keto-PGF<sub>1α</sub> release by bovine SMC between 12-24h was definitely higher than in human cells. Values are mean ± SD (n=4). (\*): inner-group difference  $P < 0.05$  vs. values at t=0. (#): inter-group difference  $P < 0.05$  of human and bovine vs. rat values.

Primary SMC from human aortic segments showed the same time course, whereas cells from rat aortae were lacking significant PGHS-2 induction in the time frame investigated. For PGHS-2 protein the western blots indicated qualitatively the same trend (Fig. 4.3.1B) with a slightly earlier peak at 6h for human cells and at about 12h for bovine cells. For rat SMC only a small band appeared after 6h which remained low at 12h and 24h. For NOS-2 mRNA and

protein mass the induction pattern between the three species was just reverse: a rapid NOS-2 mRNA increase after 6h (Fig. 4.3.2A) with a parallel expression of NOS-2 protein (Fig. 4.3.2B) in rat SMC and no detectable staining in western blots for human and bovine NOS-2 although the sensitivity of the antibody was in the same range (data not shown). When measuring enzymatic activities of PGHS, the conversion of  $^{14}\text{C}$ -arachidonate did not result in any significant values in the first 3h after LPS treatment in line with the absence of PGHS-2



**Figure 4.3.4** Total NOS activity of SMC homogenates (A) indicate an upregulation only in rat SMC while NOS activity in human and bovine cells remained constant over time. Nitrite detection (B) as indicator of  $\cdot\text{NO}$  formation illustrates distinct higher rates released by rat SMC. Human and bovine SMC demonstrated a far weaker but constant basal formation of  $\cdot\text{NO}$ . Values are mean  $\pm$  SD (n=4). (\*): inner-group difference  $P < 0.05$  vs. values at t=0. (#): inter-group difference  $P < 0.05$  of rat vs. human values.

(Fig.4.3.1B). Human and bovine SMC greatly increased this conversion of  $^{14}\text{C}$ -arachidonate to prostanoids after 6h which decreased at 12h and 24h (Fig. 4.3.3A). As an apparent discrepancy, the activity of 6-keto-PGF $_{1\alpha}$  formation increased from 3-24h continuously (Fig. 4.3.3B) at variance with the declining total PGHS activity from 6 to 24h. This interesting observation seems to be connected with a different time-dependent coupling of PGHS-2 to PGI $_2$  synthase but was not further investigated in the context of the present study. In agreement with the data in Fig. 4.3.1, rat SMC did not display significant upregulation of cyclooxygenase activities but elevated NOS-activity after 3h and maintained an about 4-fold increase between 6 and 24h (Fig. 4.3.4A). As a consequence, nitrite accumulated about 6-fold in rat SMC over 24h whereas human and bovine SMC showed a basal activity which led to only modest nitrite levels after 24h (Fig. 4.3.4B). It should be taken into account that such levels arise from a constant basic activity and hence the steady increase with time results from an unchanged basal NOS activity.

## Discussion

Knowledge of the biochemical events in developing sepsis and septic shock is crucial for a therapeutic approach of this still highly lethal pathophysiological situation. Sepsis and its associated syndromes is accompanied by severe hypotension which mainly arises from an PGI<sub>2</sub> or •NO-mediated elevation of intracellular cAMP or cGMP in vascular smooth muscle cells and the subsequent downregulation of free Ca<sup>2+</sup> (Wort 1999; Furchgott 1983). Since in progressive stages of endotoxin exposure the vascular endothelium becomes dysfunctional and fails to serve as a regulator of vessel tone (Wort 1999), autocrine stimulation of the smooth muscle mainly accounts for the extreme hypotension observed under these conditions. Several studies described in literature were performed with rats as the model of choice, however our results revealed dramatic species-specific differences in the response of vascular smooth muscle cells to LPS. In contrast to human and bovine SMC, cells from the rat responded by the distinct induction of NOS-2 and only moderate elevation of PGHS-2. Although •NO in principal can replace PGI<sub>2</sub> and vice versa, there was obviously no combined action of these two mediators in the smooth muscle within a species in contrast to the situation in the endothelium which was demonstrated to release •NO and PGI<sub>2</sub> synchronously (Akarasereenont 1995a; Akarasereenont 1995b). Furthermore it appears rather unusual that the early immediate genes PGHS-2 and NOS-2 are not induced in parallel since both depend on NF-κB signaling (Xie 1994; Lin 2004). With respect to the biochemical events leading to endothelial dysfunction, the species-dependent exclusive release however could be regarded as an effective mechanism to allow a sustained autocrine dilation of the smooth muscle in response to prolonged endotoxin exposure. In endothelial cells, proinflammatory stimuli like angiotensin II or LPS cause an elevation of •O<sub>2</sub><sup>-</sup> formation which interacts with •NO to form peroxynitrite (Bachschnid 2003). In the following step, peroxynitrite nitrates and inhibits

endothelial PGI<sub>2</sub>-synthase and therefore the release of both •NO and PGI<sub>2</sub> becomes inhibited. Interestingly in bovine SMC exposed to LPS up to 24h no nitration and inhibition of PGI<sub>2</sub>-synthase was observed (Schildknecht 2004). This can be explained partially by the higher antioxidative capacity of SMC compared with endothelial cells, but the most intriguing explanation for this lack of inhibition may arise from the observed lack of significant •NO formation since •O<sub>2</sub><sup>-</sup> generation is also upregulated in SMC in response to LPS (Patterson 1999). In rat mesangial cells for example, induction of both NOS-2 and PGHS-2 was detected and about half of PGI<sub>2</sub>-synthase became nitrated and inhibited under such conditions (Zou 1998). It has to be noted that the mutual exclusion in smooth muscle cells is limited to the time window between 2 and 24h. Stimulation of rat aortic vessel for more than 24h revealed induction of PGHS-2 (Bishop-Bailey 1997) whereas bovine and human SMC in later stages demonstrated an upregulation of NOS-2 (unpublished). It could be speculated that in humans the late induction of NOS-2 may provide a mechanism to guarantee anti-thrombotic and dilatory properties up to roughly 24h of vascular activation. A rise of •NO could then facilitate nitration of PGI<sub>2</sub>-synthase and yield the pro-aggregatory and vasoconstrictive state observed in the final stages of septic shock patients. In contrast, rat SMC responded mainly by the induction of NOS-2. The resistance of NOS-2 against oxidative stress e.g. by peroxynitrite is much higher (≈10μM) (Pasquet 1996) compared with PGI<sub>2</sub>-synthase (≈50nM) (Schmidt 2003). PGI<sub>2</sub>-synthase is also more vulnerable against certain fatty acid hydroperoxides which may arise during oxidative stress conditions (Weaver 2001). This difference may contribute to the known higher resistance of rats against bacterial infections compared with other species and should be considered when data obtained in the rat model are transferred to the situation in humans.

## **4.4. Autocatalytic Tyrosine Nitration of Prostaglandin Endoperoxide Synthase-2 in LPS-Stimulated RAW 264.7 Macrophages**

### **Introduction**

Activated macrophages release large quantities of prostanoids as well as nitric oxide ( $\bullet\text{NO}$ ) and superoxide ( $\bullet\text{O}_2^-$ ) (Stuehr 1985; Rosen 1995). Next to their role in the innate immune system (Linares 2001) these radicals may also interact with the prostanoid pathway on different levels. Such interactions range from the regulation of mRNA and protein expression (Perkins 1999; Habib 1997) to posttranslational modifications of the enzymes involved in the prostanoid biosynthetic pathway (Goodwin 1999).

The key enzymatic step in prostanoid biosynthesis is catalyzed by prostaglandin endoperoxide  $\text{H}_2$  synthase (PGHS). While the constitutively expressed PGHS-1 maintains house-keeping functions at various sites of the body, PGHS-2 is usually induced in response to pathophysiological stimuli like endotoxin (lipopolysaccharide, LPS) cytokines or mechanical stress (Vane 1998; Smith 2000).

Both isoenzymes of PGHS possess two distinct but interconnected catalytic sites, the cyclooxygenase domain located in a hydrophobic channel near the core of the enzyme and a peroxidase domain located close to the surface (Picot 1994). Both domains share a single heme prosthetic group which initially reacts with a peroxide like  $\text{H}_2\text{O}_2$  or peroxyntirite to yield a ferryl species ( $\text{Fe}^{4+}=\text{O}$  PPIX) and a tyrosyl radical ( $\text{Tyr}^{385}$  in PGHS-1;  $\text{Tyr}^{371}$  in PGHS-2) (Dietz 1988; Kulmacz 1994; Ogino 1978; Shimokawa 1990; Tsai 2000). This

initiation and maintenance of the PGHS catalytic cycle by peroxides is summarized by the term “peroxide tone” (Cleland 1984). The tyrosyl radical then initiates the cyclooxygenase reaction by abstracting a hydrogen atom at C13 of arachidonic acid. The resulting arachidonate radical subsequently reacts with a first molecule of O<sub>2</sub> to form a C9-C11 endoperoxide bond and with a second molecule of O<sub>2</sub> to form the 15-hydroperoxy-endoperoxide PGG<sub>2</sub> which is reduced by the peroxidase activity to finally yield PGH<sub>2</sub>. PGH<sub>2</sub> can then be further converted by downstream synthases to PGE<sub>2</sub>, PGD<sub>2</sub>, prostacyclin (PGI<sub>2</sub>), PGF<sub>2α</sub> or thromboxane A<sub>2</sub> (TxA<sub>2</sub>).

Nitric oxide ( $\bullet$ NO) activates guanylyl cyclase but also represents the precursor for a variety of reactive nitrogen species (Grisham 1999). It can react with  $\bullet$ O<sub>2</sub><sup>-</sup> in a nearly diffusion-limited reaction to form peroxynitrite (ONOO<sup>-</sup>) (Huie 1993). Its acid form (ONOOH) can easily decompose to the highly reactive oxidants  $\bullet$ NO<sub>2</sub> and  $\bullet$ OH. In absence of other paramagnetic species or when present excessively,  $\bullet$ NO can also undergo autoxidation with O<sub>2</sub> yielding nitrite (NO<sub>2</sub><sup>-</sup>) as the stable end product (Ford 1993). In healthy subjects, plasma NO<sub>2</sub><sup>-</sup> levels can be detected between 0.5-3μM whereas in septic patients plasma levels of more than 50μM were observed which originate mainly as a consequence of NO-synthase-2 (NOS-2) induction in various cell types (Mitaka 2003; Ersoy 2002; Chauhan 2003).

Several reports have indicated an increase of nitrated tyrosine residues (3-NT) in cells, tissues and organs exposed to severe stress conditions (Ischiropoulos 1998; Bian 1999; Fries 2003) but examples for physiological or pathophysiological roles are scarce and difficult to approach experimentally. For prostacyclin (PGI<sub>2</sub>)-synthase nitration and inhibition by peroxynitrite has been described in association with endotoxemia, ischemia-reperfusion, atherosclerosis and diabetes (Bachschnid 2003; Zou 1999a; Zou 1999b; Zou 2002). Another reported example for a regulatory role of Tyr-nitration is manganese superoxide dismutase (Mn-SOD) (Pittman 2002; MacMillan-Crow 1999). Doubts had been raised in literature about

the ability of physiological levels of peroxynitrite in the low micromolar range to perform Tyr-nitrations (Pfeiffer 2001a; Pfeiffer 2001b; Espey 2002; Pfeiffer 1998) but such criticisms do not consider the role of metal centers during catalysis which certainly applies for PGI<sub>2</sub>-synthase and other heme proteins and possibly also for Mn-SOD (Cassina 2000; Alayash 1998; Daiber 2000).

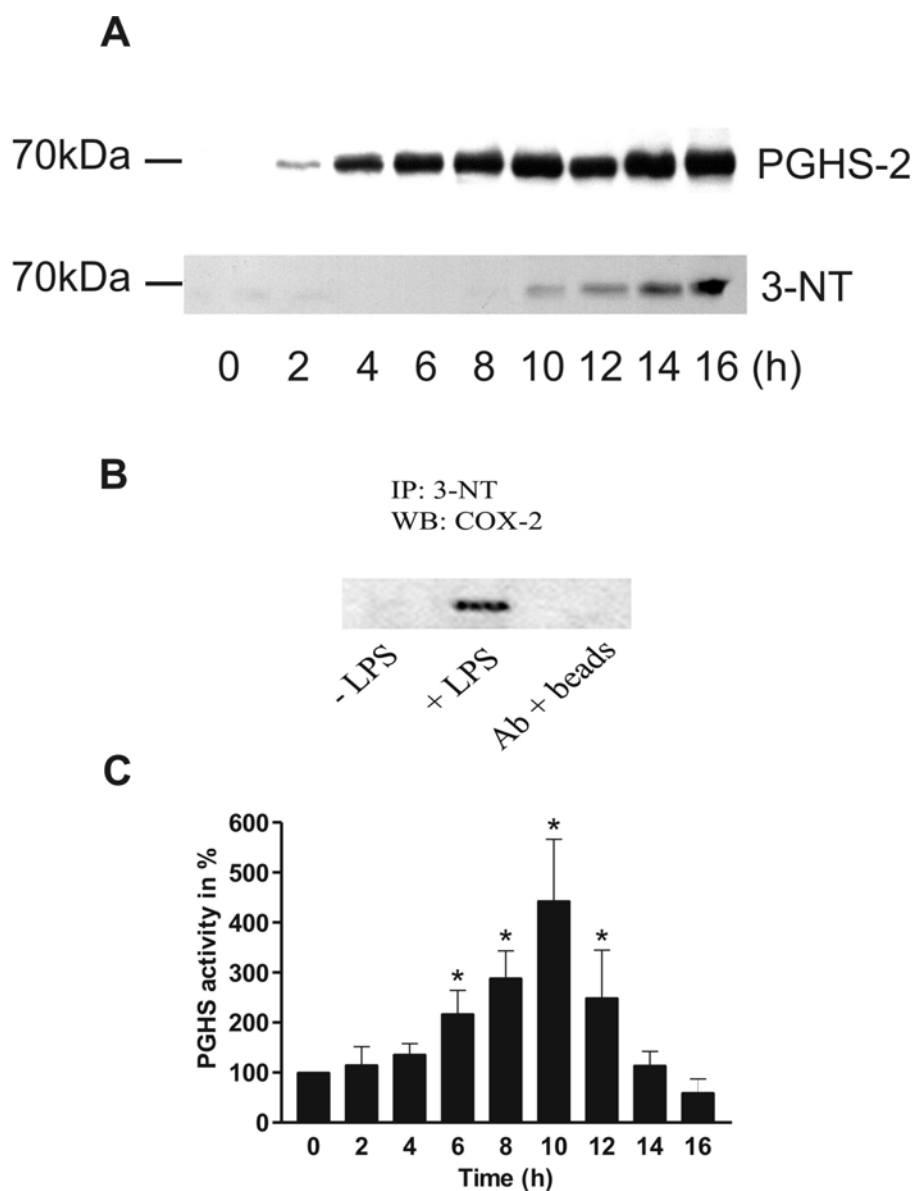
An alternative pathway leading to Tyr-nitration can be observed with nitrite in the presence of peroxidatic and pseudo-peroxidatic activities as exemplified by myeloperoxidase (MPO) (Baldus 2002; Sampson 1998) or myoglobin (Bourassa 2001; Kilinc 2001; Herold 2001). MPO has been associated with host defence mechanisms of activated granulocytes. It uses H<sub>2</sub>O<sub>2</sub> to oxidize its primary substrate chloride to generate the highly reactive hypochlorous acid but can alternatively also oxidize NO<sub>2</sub><sup>-</sup> to yield the •NO<sub>2</sub> radical (Eiserich 1998). Recently the MPO-mechanism was proposed for Tyr-nitration of unidentified proteins in LPS-stimulated macrophages (Pfeiffer 2001a; Pfeiffer 2001b; Eiserich 1998). The presence of MPO, nitrite and H<sub>2</sub>O<sub>2</sub> in these cells and an inhibition of the nitration process by catalase was taken as an argument that this nitration proceeded through an activation of nitrite by MPO and endogenous peroxides. In contrast, Galinanes and coworkers (Galinaes 2002) demonstrated that the MPO mechanism does not account for Tyr-nitration in human leukocytes. Thus, the issue of nitrite as a physiological nitrating agent is still open for speculation.

During our work on PGI<sub>2</sub>-synthase nitration we noticed a dominating Tyr-nitrated band at about 72 kDa in LPS-stimulated RAW 264.7 macrophages. We herein demonstrate this protein as prostaglandin endoperoxide synthase-2 (PGHS-2) and provide evidence that its nitration *in vitro* originates mainly from an autocatalytic activation of nitrite by the intrinsic peroxidase activity of PGHS-2. Thus, at least for stimulated macrophages the nitrite/peroxide-driven nitration pathway seems to depend mostly on the heme containing active site of PGHS-2 and not on the catalytic action of MPO.

## Results

Exposure of RAW 264.7 macrophages to 10 $\mu$ g/ml lipopolysaccharide (LPS) resulted in a time-dependent induction of PGHS-2 (Figure 4.4.1A). When such blots were stripped and incubated with an anti-3-nitrotyrosine (3-NT) antibody, a main positive band at around 72kDa was observed suggesting the nitration of PGHS-2 after 10h with a maximum at 16h. The increase of 3-NT between 10 and 16h was further confirmed by GC-MS analysis of a total hydrolysate of RAW 264.7 macrophages (not shown). In order to verify a specific nitration of PGHS-2, immunoprecipitation with an anti-3-NT antibody followed by western-blotting and incubation with an anti-PGHS-2 antibody confirmed the observed nitrated band as PGHS-2 (Figure 4.4.1B). The reversed experiment failed because of insufficient precipitation with the available PGHS-2 antibody. Total PGHS activity of cell homogenates was detected by the conversion of <sup>14</sup>C-labeled arachidonate (Figure 4.4.1C). PGHS activity increased with time until a maximum at 10h was reached. From 12-16h, in spite of high PGHS-2 protein expression, PGHS activity rapidly declined.

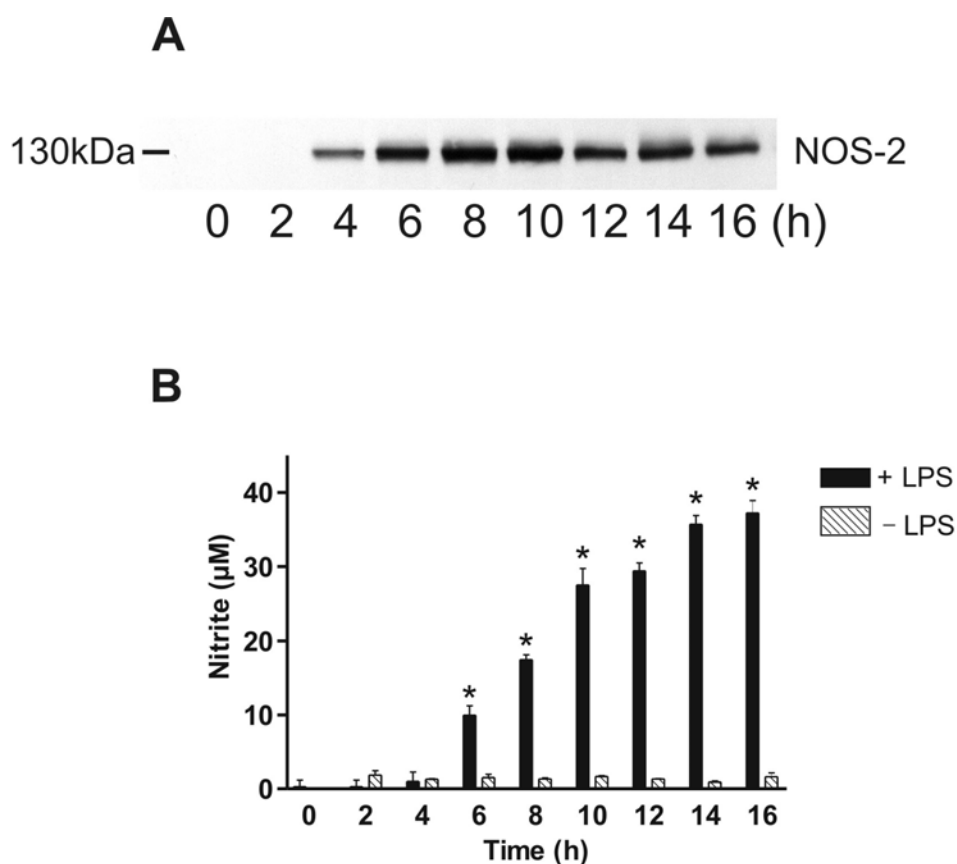
The presence of 3-NT in PGHS-2 implies the activity of NO-synthases in stimulated RAW 264.7 macrophages. Inducible NOS-2 was not detectable without stimulation but was clearly upregulated between 4-10h followed by a decline between 12-16h (Figure 4.4.2A). Nitrite accumulation as indicator for total NOS activity paralleled NOS-2 protein expression with a significant increase between 6-10h followed by a reduced increment from 12-16h (Figure 4.4.2B). Thus nitrite release closely followed NOS-2 expression.



**Figure 4.4.1. Nitration of PGHS-2 in RAW 264.7 macrophages.** **A:** RAW 264.7 macrophages were exposed to LPS (10 $\mu$ g/ml) for the time periods indicated. After staining of PGHS-2, the membrane was stripped and probed for 3-NT. PGHS-2 protein was under detection limit at  $t=0$  and gradually increased until a maximum was reached after 8-10h. The same band was stained positive for 3-NT after 10h with a gradual increase up to 16h. **B:** Homogenates of LPS-exposed RAW 264.7 macrophages (24h) were immunoprecipitated with an anti-3-NT antibody, staining against PGHS-2 indicated nitration of PGHS-2. **C:** Total PGHS activity of RAW 264.7 homogenates incubated with LPS for various time intervals. A maximum was reached after 10h followed by a decline between 12-16h. Values are mean  $\pm$  SD (n=4). \* $P < 0.05$  vs.  $t=0$

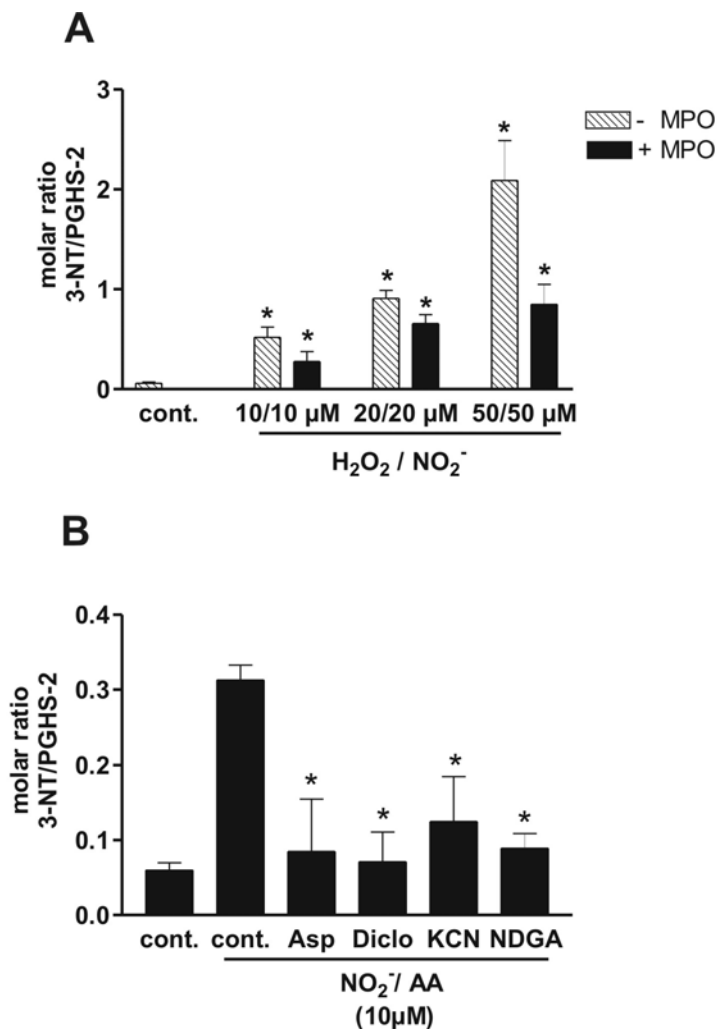
Without LPS, no significant basal release of  $\bullet$ NO was detected which indicates NOS-2 as the major source of  $\bullet$ NO in LPS-treated RAW 264.7 macrophages.

Since PGHS-2-nitration was initiated when maximal NOS-2 activity was already transgressed, it seems rather plausible that the stable end product nitrite, but not the short-lived  $\bullet$ NO or peroxynitrite, mainly accounts for the observed inhibition of the enzyme. To exclude a possible involvement of MPO present in the cells, these conclusions were further substantiated by experiments with isolated PGHS-2.



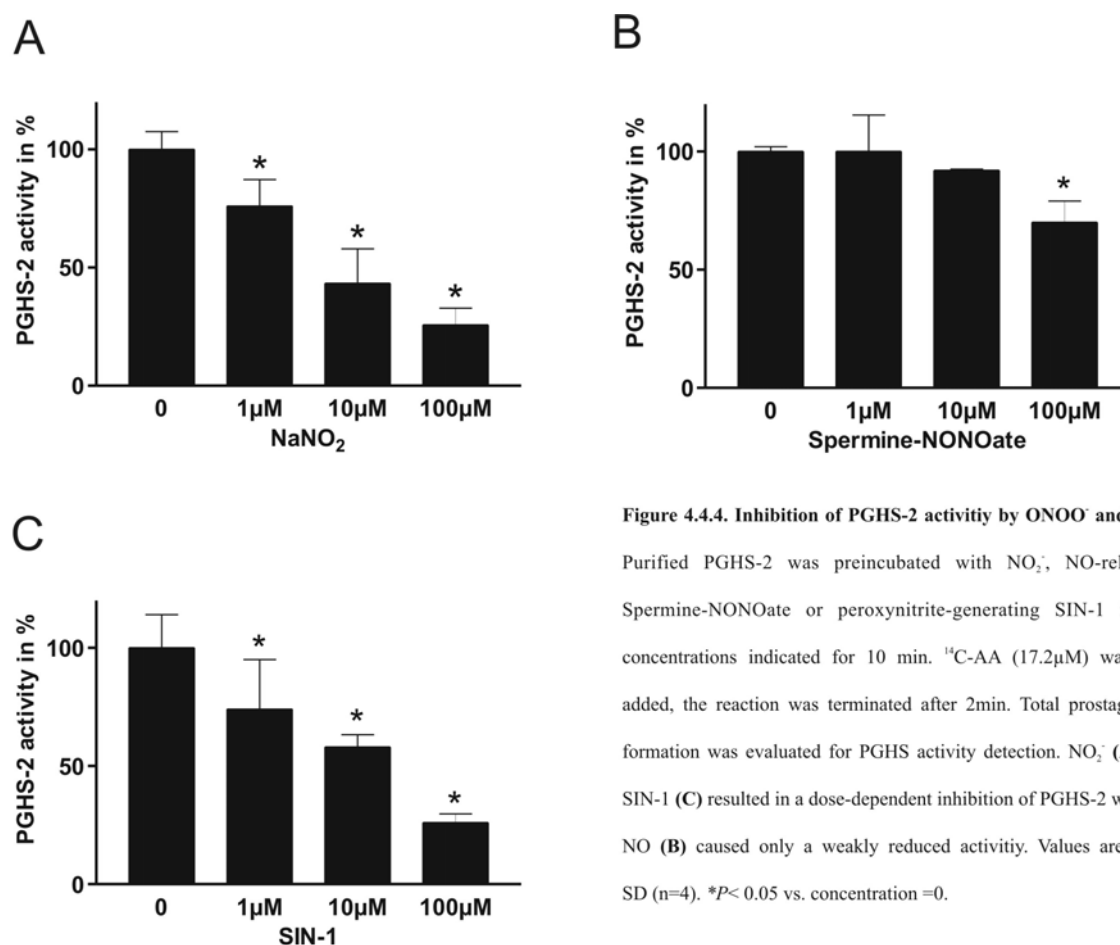
**Figure 4.4.2. NOS-2 dependent nitrite formation in RAW 264.7 macrophages.** **A:** NOS-2 protein expression of LPS (10 $\mu$ g/ml) exposed RAW 264.7 macrophages was first visualized after 4h with an increase up to 10h. From 12-16h, a decline in protein expression was detected. **B:** Nitrite formation in cell culture supernatants was observed as indicator for total NOS activity. In the absence of LPS nearly no generation of nitrite was detected. The presence of LPS caused significant upregulation after 6h. From 10-16h de novo synthesis of nitrite declined. Values are mean SD (n=4). \* $P < 0.05$  vs. t=0.

Figure 4.4.3A demonstrates the nitration of isolated PGHS-2 by  $\text{NO}_2^-/\text{H}_2\text{O}_2$  in the absence or presence of MPO. Surprisingly, without MPO 3-NT was massively formed in PGHS-2 up to a molar ratio of 2 whereas in the presence of MPO 3-NT-formation was only about half yielding a molar ratio of about 1:1. These observations support a peroxidatic activation of nitrite by the intrinsic peroxidase activity of PGHS-2. Not only  $\text{H}_2\text{O}_2$  but also the primary



**Figure 4.4.3. Involvement of peroxides in PGHS-2 nitration by  $\text{NO}_2^-$ .** **A:** Purified PGHS-2 enzyme was incubated with increased concentrations of  $\text{NO}_2^-/\text{H}_2\text{O}_2$ . Nitrotyrosine formation increased in a concentration-dependent manner. The presence of myeloperoxidase (MPO, 1 $\mu\text{M}$ , black bars) even reduced PGHS-2 nitration by  $\text{NO}_2^-/\text{H}_2\text{O}_2$ . **B:** Purified PGHS-2 was incubated with 20 $\mu\text{M}$   $\text{NO}_2^-$ , 20 $\mu\text{M}$  AA and different inhibitors. Inhibition of the cyclooxygenase activity of PGHS-2 by aspirin (Asp, 500 $\mu\text{M}$ ) or diclofenac (Diclo, 10 $\mu\text{M}$ ) as well as inhibition of the catalytic heme center by cyanide (KCN, 1mM) or inhibition of the peroxidase activity by NDGA (10 $\mu\text{M}$ ) resulted in a significant inhibition of PGHS-2 nitration. Values are mean  $\pm$  SD (n=5). \* $P < 0.05$  vs. cont.

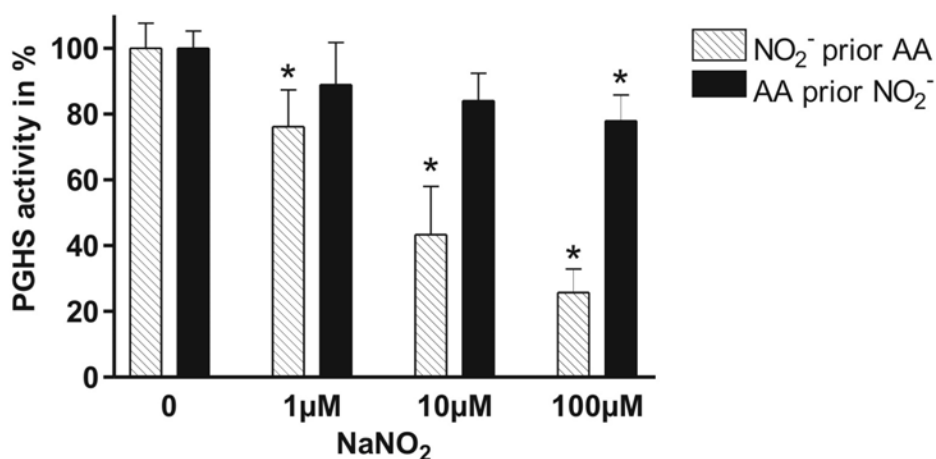
PGHS-2 product 15-hydroperoxy-prostaglandin endoperoxide (PGG<sub>2</sub>) formed by the cyclooxygenase activity of the PGHS enzyme is supposed to provide the peroxide tone. Blocking the cyclooxygenase function by aspirin or diclofenac and also a 1mM concentration of the heme-ligand KCN as well as the phenolic antioxidant NDGA caused an inhibition of PGHS-2-nitration (Fig. 4.4.3B). This underlines the role of the heme active site of PGHS-2 for the activation of nitrite to result in Tyr-nitration. To better compare the efficacy of nitrite as a Tyr-nitrating agent for PGHS-2 against  $\bullet$ NO and peroxynitrite, the activity of purified enzyme was determined after an incubation with NO<sub>2</sub><sup>-</sup>, the  $\bullet$ NO-releasing compound Spermine-NONOate or the peroxynitrite-generating compound SIN-1 for 15min (Figure 4.4.4). Both NO<sub>2</sub><sup>-</sup> and SIN-1 demonstrated a dose-dependent inhibition whereas incubation with Spermine-NONOate resulted only in a moderate inhibition at high concentrations of  $\bullet$ NO.



**Figure 4.4.4. Inhibition of PGHS-2 activity by ONOO<sup>•</sup> and NO<sub>2</sub><sup>-</sup>.**

Purified PGHS-2 was preincubated with NO<sub>2</sub><sup>-</sup>, NO-releasing Spermine-NONOate or peroxynitrite-generating SIN-1 in the concentrations indicated for 10 min. <sup>14</sup>C-AA (17.2μM) was then added, the reaction was terminated after 2min. Total prostaglandin formation was evaluated for PGHS activity detection. NO<sub>2</sub><sup>-</sup> (A) and SIN-1 (C) resulted in a dose-dependent inhibition of PGHS-2 whereas NO (B) caused only a weakly reduced activity. Values are mean SD (n=4). \*P<0.05 vs. concentration=0.

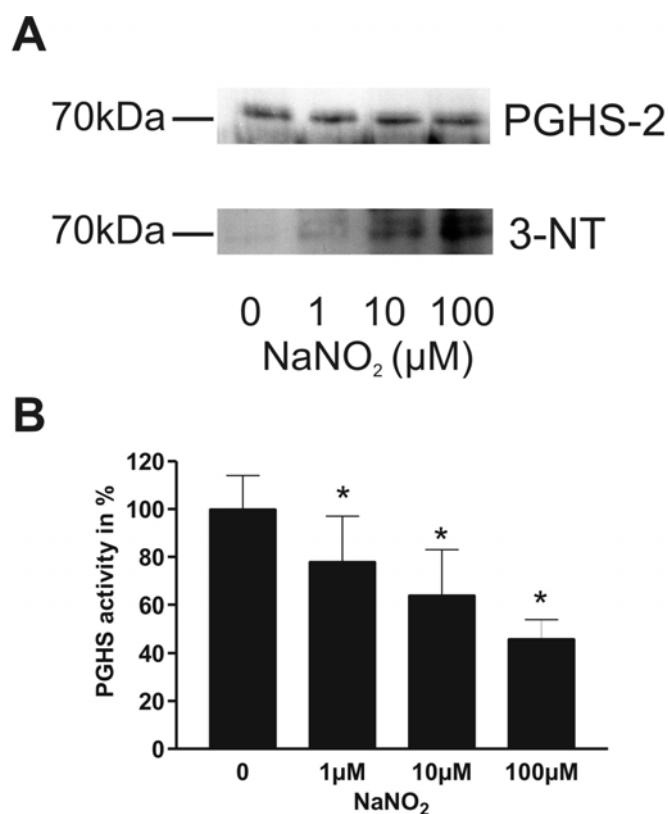
Since autoxidation of  $\bullet\text{NO}$  could occur under the conditions employed, inhibition at  $100\mu\text{M}$  Spermine-NONOate may have indirectly occurred via formation of  $\text{NO}_2^-$ . It was puzzling to find  $\text{NO}_2^-$  alone sufficient for PGHS-2 inhibition but catalase released both the inhibition as well as 3-NT formation thus indicating the presence of low levels of  $\text{H}_2\text{O}_2$  formed by autoxidation. This would be in agreement with the low levels of peroxides ( $2\text{nM}$ ) as the saturating peroxide tone for PGHS-2. According to these results, the simplest interpretation would be a competitive reaction of nitrite and arachidonate with the ferryl-Tyr-radical enzyme intermediate. This assumption could be verified by preincubation with unlabeled AA for 1min followed by 1min  $\text{NO}_2^-$  or vice versa (Figure 4.4.5). Preincubation of purified PGHS-2 with AA prevented the enzyme from nitration by  $\text{NO}_2^-$ . In contrast, when PGHS-2 was incubated with  $\text{NO}_2^-$  first, a dose-dependent inhibition could be observed.



**Figure 4.4.5. Competition between AA and  $\text{NO}_2^-$ .** Purified PGHS-2 was preincubated with  $\text{NO}_2^-$  for 1 min followed by addition of  $10\mu\text{M}$  unlabeled AA for 1min and  $17.2\mu\text{M}$   $^{14}\text{C}$ -AA for 2min (open bars). Alternatively, PGHS-2 was incubated with  $10\mu\text{M}$  unlabeled AA for 1min followed by 1 min  $\text{NO}_2^-$  and 2min  $17.2\mu\text{M}$   $^{14}\text{C}$ -AA (black bars). Preincubation with nitrite significantly prevented nitrite-dependent inhibition of PGHS-2 activity. Values are mean  $\pm$  SD (n=4).

\* $P < \text{vs. NaNO}_2=0$

To confirm that nitration of PGHS-2 by nitrite can also be observed under cellular conditions, RAW 264.7 macrophages were stimulated with LPS in the presence of the NOS-inhibitors AMT or L-NMMA. This experiment however lead to a significant inhibition of PGHS-2 expression in the LPS-stimulated RAW 264.7 macrophages by the NOS-inhibitors. Although an involvement of  $\bullet$ NO-synthesis in the induction process for PGHS-2 represents an interesting new aspect of interactions between the  $\bullet$ NO and the prostanoid pathway, this topic was not further studied here. Therefore, homogenates of RAW 264.7 macrophages treated with LPS for 5h for PGHS-2 induction were supplied with increasing levels of nitrite and analyzed for PGHS-2 nitration. A Tyr-nitration of PGHS-2 could be achieved at 10 $\mu$ M



**Figure 4.4.6. Nitration of PGHS-2 by NO<sub>2</sub><sup>-</sup> in RAW 264.7 homogenates.** **A:** Cells were incubated with LPS for 5h for PGHS-2 induction. Aliquots of a pooled homogenate were incubated with NO<sub>2</sub><sup>-</sup> in the concentrations indicated for 10 min followed by the addition of 17.2 $\mu$ M AA. 3-NT staining intensified with elevated NO<sub>2</sub><sup>-</sup> concentrations. **B:** PGHS activity of RAW 264.7 homogenates is inhibited with increasing concentrations of NO<sub>2</sub><sup>-</sup>. Samples were treated as described in A. After preincubation with NO<sub>2</sub><sup>-</sup> for 10 min, <sup>14</sup>C-AA (17.2 $\mu$ M) was added for 2 min. Total prostaglandin formation was integrated for evaluation. Values are mean  $\pm$  SD (n=4). \**P* < 0.05 vs. NO<sub>2</sub><sup>-</sup> = 0.

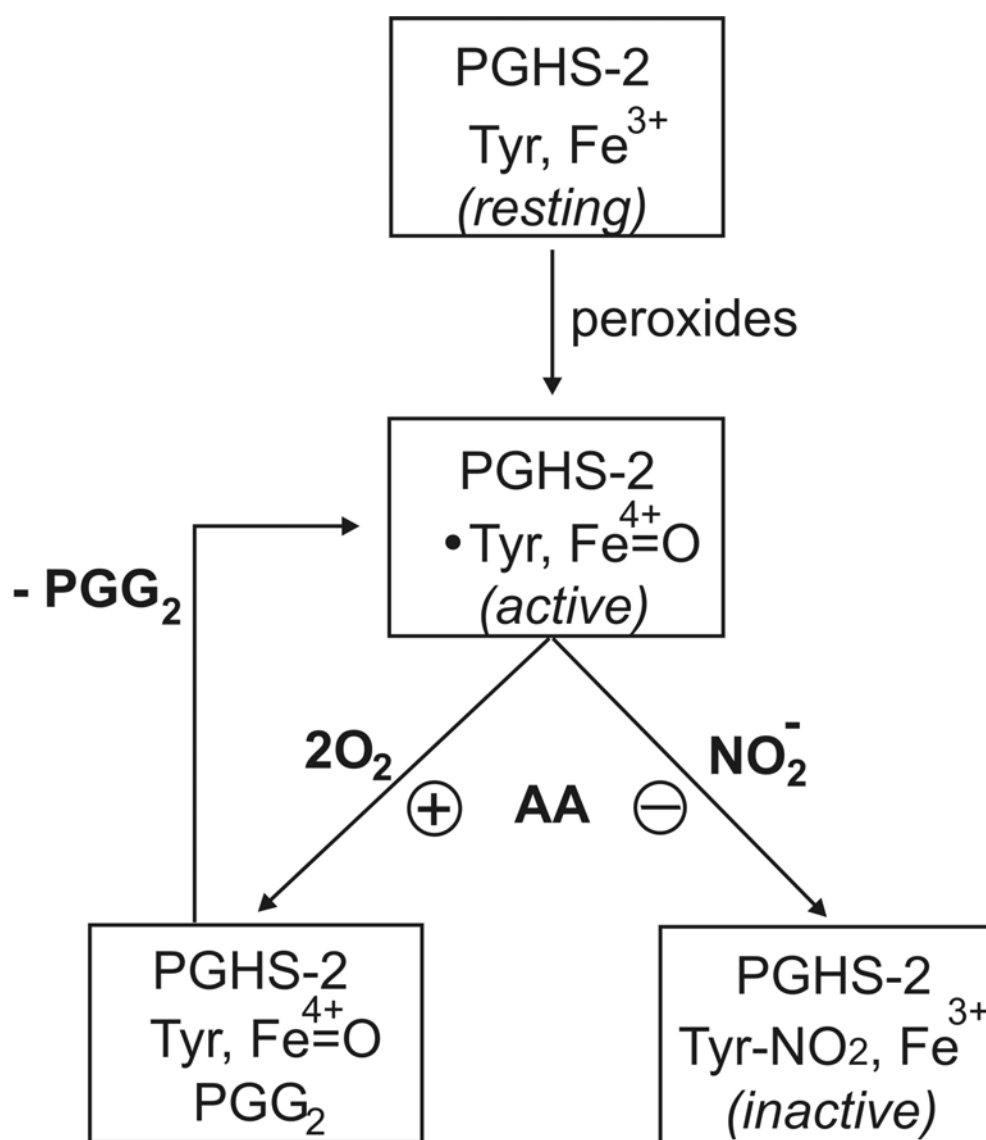
nitrite which was further enhanced at 100 $\mu$ M nitrite (Figure 4.4.6A). PGHS-2 activity declined only to about 50% which can be explained by the presence of cellular free arachidonate that could have protected the enzyme from higher inhibition (Figure 4.4.6B).

## Discussion

The present study was initiated by the observation that long-term exposure of RAW 264.7 macrophages to LPS resulted in Tyr-nitration of a 72kDa protein, which by immunoprecipitation with anti-3-NT antibodies yielded PGHS-2 as the main nitrated protein. Nitration of PGHS-2 had already been reported for the LPS-stimulated cell line J774.A1 (Clancy 2000) and positive staining for 3-NT was also demonstrated in LPS-stimulated RAW 264.7 macrophages however the nitrated proteins were not identified (Pfeiffer 2001a; Pfeiffer 2001b).

The observations of this work clarify several issues on the interaction of the  $\bullet$ NO-pathway with prostanoid biosynthesis. Tyr-nitrations can be a consequence of peroxynitrite formation which requires the simultaneous generation of  $\bullet$ NO and  $\bullet$ O<sub>2</sub><sup>-</sup>. Since peroxynitrite is unstable and only formed transiently, nitrite as the stable end product of  $\bullet$ NO synthesis was reported to be utilized by the myeloperoxidase/H<sub>2</sub>O<sub>2</sub> pathway to cause nitrations at Tyr-residues (Baldus 2002; Sampson 1998; Gaut 2002). This appears as an unspecific oxidation mechanism during phagocytosis or autodestructive processes in tissues while regulatory functions have not yet been found associated with this pathway. As the main novel finding, we herein provide evidence that PGHS-2 is autocatalytically inactivated in the presence of nitrite by a heme-catalyzed formation of the  $\bullet$ NO<sub>2</sub> radical which subsequently nitrates and inactivates the

enzyme. In accordance with literature (Kulmacz 1995), the peroxide requirements for PGHS-2 were detected very low since no additions of peroxides were necessary for the peroxidatic activation of  $\text{NO}_2^-$  and catalase was inhibitory. In the absence of arachidonate, nitrite levels of around  $10\mu\text{M}$  were sufficient for the nitration of PGHS-2. However, in the presence of arachidonate the inhibition was effectively suppressed thus allowing to conclude a rather simple mechanism of nitrite activation as depicted in Figure 4.4.7. Since peroxides generate a



**Figure 4.4.7. PGHS nitration by nitrite.** Resting ferric PGHS-2 is first activated by peroxides to form  $\text{Tyr}$ ,  $\text{Fe}^{4+}=\text{O}$  at the active site of the enzyme. On the left, the normal catalytic cycle of arachidonate oxygenation and reformation of the active state by  $\text{PGG}_2$  is depicted. In the presence of  $\text{NO}_2^-$ ,  $\text{Fe}^{4+}=\text{O}$  can activate  $\text{NO}_2^-$  to form the  $\text{NO}_2$  radical which subsequently recombines with a  $\text{Tyr}$  radical yielding the nitrated and inactivated enzyme.

Tyr-radical at the active site-located Tyr<sup>371</sup> it is very likely, although still not proven, that this residue becomes nitrated by combination with the  $\bullet\text{NO}_2$  radical (Hsi 1994; Tsai 1998). From this mechanism a new interpretation of related data in literature could be derived. First, the reported nitration of this Tyr<sup>371</sup>-residue by high concentrations of  $\bullet\text{NO}$  (1-2mM) (Gunther 1997; Goodwin 1998; Tsai 1994) under aerobic conditions could follow the suggested pathway since nitrite readily forms from  $\bullet\text{NO}$  and dioxygen. This would also apply for the detection of nitrated PGHS-2 in LPS/IFN- $\gamma$ / $\bullet\text{NO}$  -treated J774.A1 macrophages (Clancy 2000). Second, the observed increase of 3-NT in LPS-stimulated RAW 264.7 macrophages could be assumed to originate from a self-catalyzed nitration of PGHS-2 without involvement of MPO. There is no doubt that PGHS-2 can also be nitrated by peroxynitrite, but from earlier data indicating peroxynitrite levels of more than 100 $\mu\text{M}$  as necessary for an inhibition (Zou 1997) as well as from the time course of NOS-2 induction and PGHS-2 inhibition found in this work, nitrite as the precursor of the Tyr-nitrating process appears as a rather convincing alternative explanation.

A physiological function of nitrite as a regulator of prostanoid formation however still remains a matter of speculation. A strong activation of macrophages for massive induction of NOS-2 is certainly required to generate sufficient amounts of  $\bullet\text{NO}$ . This can be converted to nitrite by several pathways and will accumulate with time until it reaches concentrations that allow the self-catalyzed nitration of PGHS-2. Since this process was found to be competitive with arachidonate-oxygenation, the levels of nitrite have to rise well above the threshold of 10 $\mu\text{M}$  as detected for isolated PGHS-2. Indeed, levels as high as 50-80 $\mu\text{M}$  have been measured in inflamed tissue and more than 70 $\mu\text{M}$  can accumulate in the supernatants of LPS-stimulated macrophages. If at the resolution of the inflammatory phase a decline of phospholipase A<sub>2</sub> activity would lower the levels of free arachidonate, a synergistic effect would arise for a rapid inhibition of prostanoid formation. The requirement for a high output

of  $\bullet\text{NO}$  may limit the significance of PGHS-nitration to the PGHS-2 isoform which usually is associated with NOS-2 induction. PGHS-1 can become nitrated by peroxynitrite (Deeb 2002; Boulos 2000) and also by nitrite (unpublished), but under physiological conditions the levels of  $\bullet\text{NO}$  from NOS-1 or NOS-3 activity could be regarded as too low for an effective inhibition.

In summary, our data support an autocatalytic inhibition of PGHS-2 mediated by the peroxidatic generation of a  $\bullet\text{NO}_2$  radical which subsequently nitrates an essential Tyr residue at the active site of the enzyme.

## **4.5. Nitration of PGHS-2 Inhibits Prostanoid Release in Rat Alveolar Macrophages**

### **Introduction**

Alveolar macrophages (AM) represent the first line of defence against various inhaled particles adhering to the alveolar epithelial surface (Laskin 1995; Lombry 2004). As part of the innate immune system AM are involved in the initiation and maintenance of the bronchial inflammatory cascade releasing various proinflammatory mediators like reactive oxygen species ( $\bullet\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$ ,  $\bullet\text{OH}$ ), reactive nitrogen species ( $\bullet\text{NO}$ ,  $\text{ONOO}^-$ ,  $\bullet\text{NO}_2$ ) and eicosanoids like thromboxane  $\text{A}_2$  ( $\text{TxA}_2$ ),  $\text{PGE}_2$ ,  $\text{PGD}_2$  or leukotrienes (Wizemann 1994; Pendino 1993; Nathan 1987; Suzuki 1993; Steudel 1997). Formation of such mediators is usually terminated after removal of the pathogen, but when AM are excessively or continuously activated, pathophysiological situations like pneumonia, asthma, COPD or pulmonary hypertension can be provoked (Uhlir 1996; Steinberg 1994; Maddox 2002; Barnes 2003). Endotoxin (lipopolysaccharide, LPS) serves as a model for such severe challenges and triggers a plethora of responses which include the release of bronchioconstrictive  $\text{TxA}_2$  by AM (Uhlir 1996; Bannerman 1999).  $\text{PGE}_2$  is also formed and can either counteract or synergize this action depending on the local distribution of  $\text{PGE}_2$ -receptor subtypes (Breyer 2001). Hence macrophage-derived prostanoids contribute essentially to the physiological and pathophysiological response of the lung.

Prostanoids originate from the prostaglandin endoperoxide  $\text{H}_2$  synthase (PGHS) pathway (Smith 1972; Vane 1971). The constitutively expressed PGHS-1 maintains housekeeping functions at various sites like the stomach, vascular endothelial cells or platelets whereas the

inducible PGHS-2 is mainly expressed under pathophysiological conditions like atherosclerosis, ischemia-reperfusion or endotoxemia (Hirata 1997; Langenbach 1995; Liou 2000; Schildknecht 2004; Schonbeck 1999; Domoki 1999). Lung AM constitute a basal PGHS-1 activity but can rapidly upregulate PGHS-2 in response to inflammatory mediators or mechanical stimulation (O'Sullivan 1992; Hempel 1994; Ermert 2000). Both isoenzymes of PGHS possess two interconnected catalytic sites, a cyclooxygenase- and a peroxidase domain which together share a single heme prosthetic group (Dietz 1988; Picot 1994; Kulmacz 1994). Regulation of PGHS-activity seems to be governed by a complex interaction between reactive nitrogen- and oxygen species, however no clear-cut picture on the mechanism has emerged so far (Goodwin 1999). A direct interaction of  $\bullet\text{NO}$  with isolated PGHS enzyme has been found inhibitory (Gunther 1997; Goodwin 1998), however the high concentrations required (1-2mM) exclude a biological significance of this reaction (Tsai 1994).  $\bullet\text{NO}$  has also been reported to enhance or inhibit PGHS-2 expression, though these interactions on the transcriptional and translational level appear highly specific for the respective cell type (Habib 1997; Tetsuka 1996). Peroxynitrite, originating from the nearly diffusion-limited reaction of  $\bullet\text{NO}$  with superoxide ( $\bullet\text{O}_2^-$ ) was alternatively suggested as a nitrating species for the two PGHS-isoenzymes (Deeb 2002). However, for nitration and inhibition of purified PGHS-2, bolus additions of 100 $\mu\text{M}$  peroxynitrite were required thus ruling out peroxynitrite as a nitrating species (Zou 1997). In contrast, endogenously formed levels even resulted in a stimulation of PGHS activity (Landino 1996). This phenomenon was explained by the requirement of peroxides for the initiation and maintenance of the PGHS enzymatic turnover and known by the term "peroxide tone". For PGHS-2 the peroxide requirements were reported as low as 2nM whereas PGHS-1 needed about 10-fold higher levels (21nM) (Kulmacz 1995). Nitration of PGHS-2 in LPS-stimulated J774.A1 macrophages has been demonstrated (Clancy 2000) and could be explained by our recent observation of a nitrite-

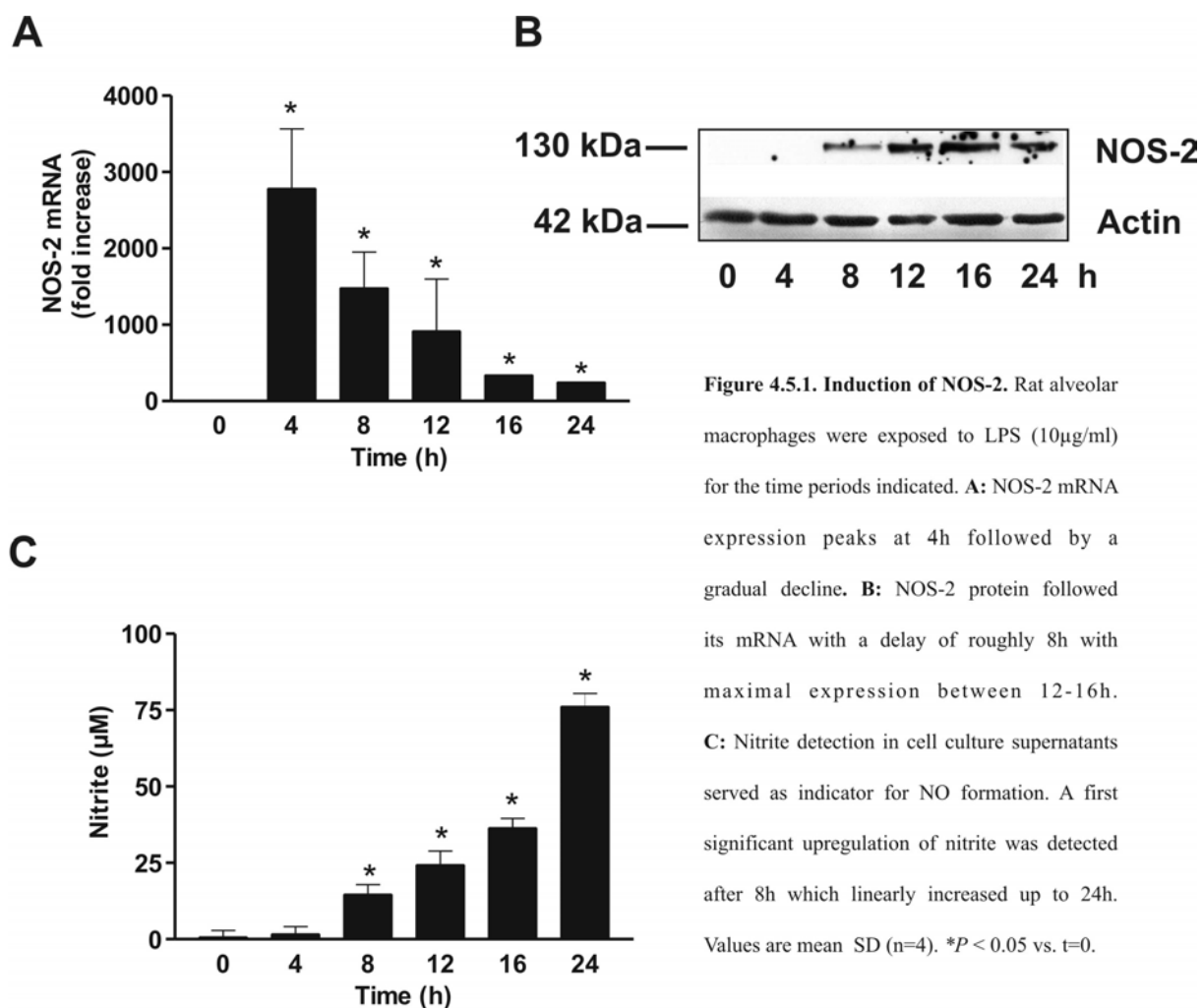
dependent Tyr-nitration and inhibition of PGHS-2 arising from an autocatalytic activation of nitrite by the intrinsic peroxidase activity of PGHS-2 (see chapter 4.4).

This new mechanism on the regulation of PGHS-2 was tested in our present investigations with emphasis on three questions: i) can PGHS-2-nitration be observed in LPS-treated rat AM, ii) is this reaction mediated by peroxynitrite or by nitrite, iii) could PGHS-2-nitration represent a regulatory mechanism for the release of  $\text{TxA}_2$  and  $\text{PGE}_2$  in LPS-treated AM. For all three questions positive answers could be obtained by the experimental data presented in this work.

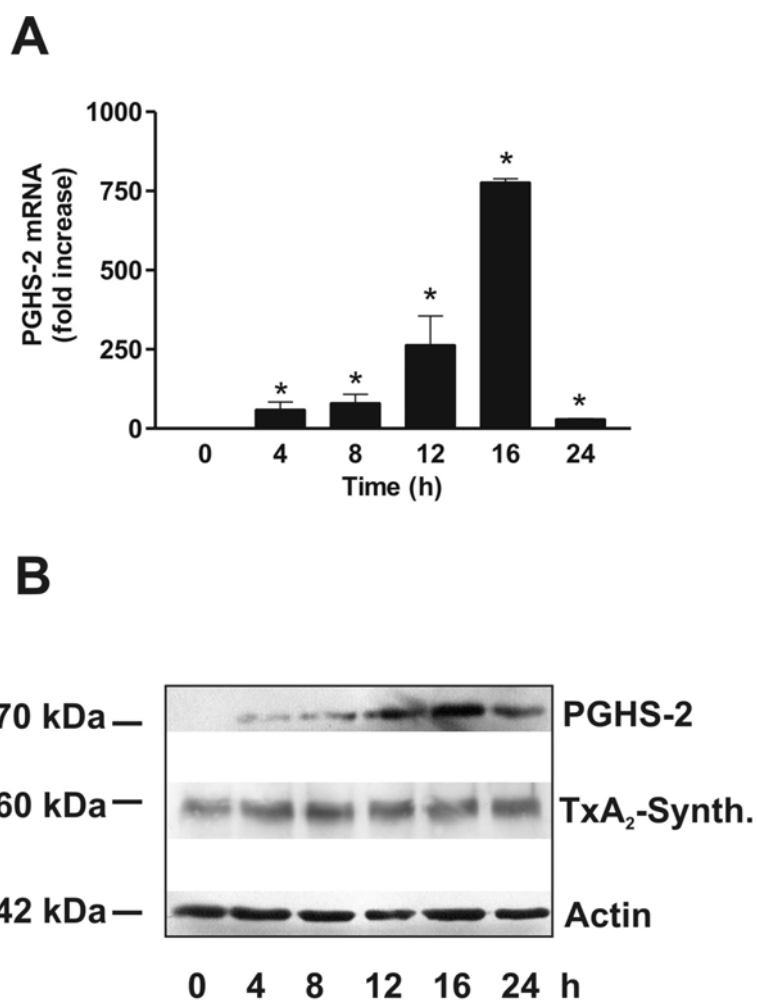
## Results

### *Induction of NOS-2 and PGHS-2 in rat alveolar macrophages*

Exposure of resting rat AM to LPS (10 $\mu$ g/ml) resulted in the appearance of NOS-2 mRNA after 4h followed by an exponential decline until 24h (Fig. 4.5.1A). NOS-2 protein followed its corresponding mRNA but with a considerable delay of 4-8h exhibiting an expression plateau between 12-16h (Fig. 4.5.1B). Nitrite as a stable product of  $\bullet$ NO accumulated with time and its levels closely matched with the increase of NOS-2 protein expression (Fig. 4.5.1C).



For PGHS-2-induction the time course for mRNA transcription with a late maximum at 16h appears rather unusual for the expression of an early immediate gene and was followed by a rapid decrease to almost zero levels at 24h (Fig. 4.5.2A). PGHS-2 protein closely followed its mRNA and demonstrated maximal expression between 12-16h (Fig. 4.5.2B). TxA<sub>2</sub>-synthase protein expression was unaltered over the investigated time period. To test a possible inhibition of TxA<sub>2</sub>-synthase, homogenates of cells incubated with LPS for 0h and 16h were treated with 1mM aspirin to inhibit endogenous formation of PGH<sub>2</sub> and then were incubated with exogenous PGH<sub>2</sub> for 3min. TxB<sub>2</sub> formation was measured by EIA resulting in no alteration of TxB<sub>2</sub> formation (not shown).



**Fig. 4.5.2. Induction of PGHS-2.** Rat alveolar macrophages were exposed to LPS (10 $\mu$ g/ml) for the time periods indicated.

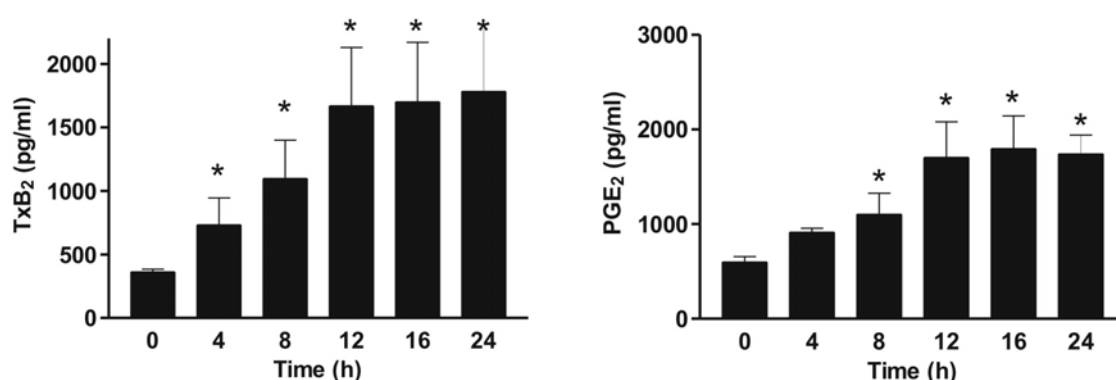
**A:** PGHS-2 mRNA exhibited a gradual increase with a peak at 16h followed by a rapid decline to levels of unstimulated cells.

**B:** PGHS-2 protein closely followed its corresponding mRNA and demonstrated maximal expression between 12-16h.

Values are mean  $\pm$  SD (n=3). \* $P$  < 0.05 vs. t=0.

*Self- inhibition of prostanoid formation by nitration of PGHS-2*

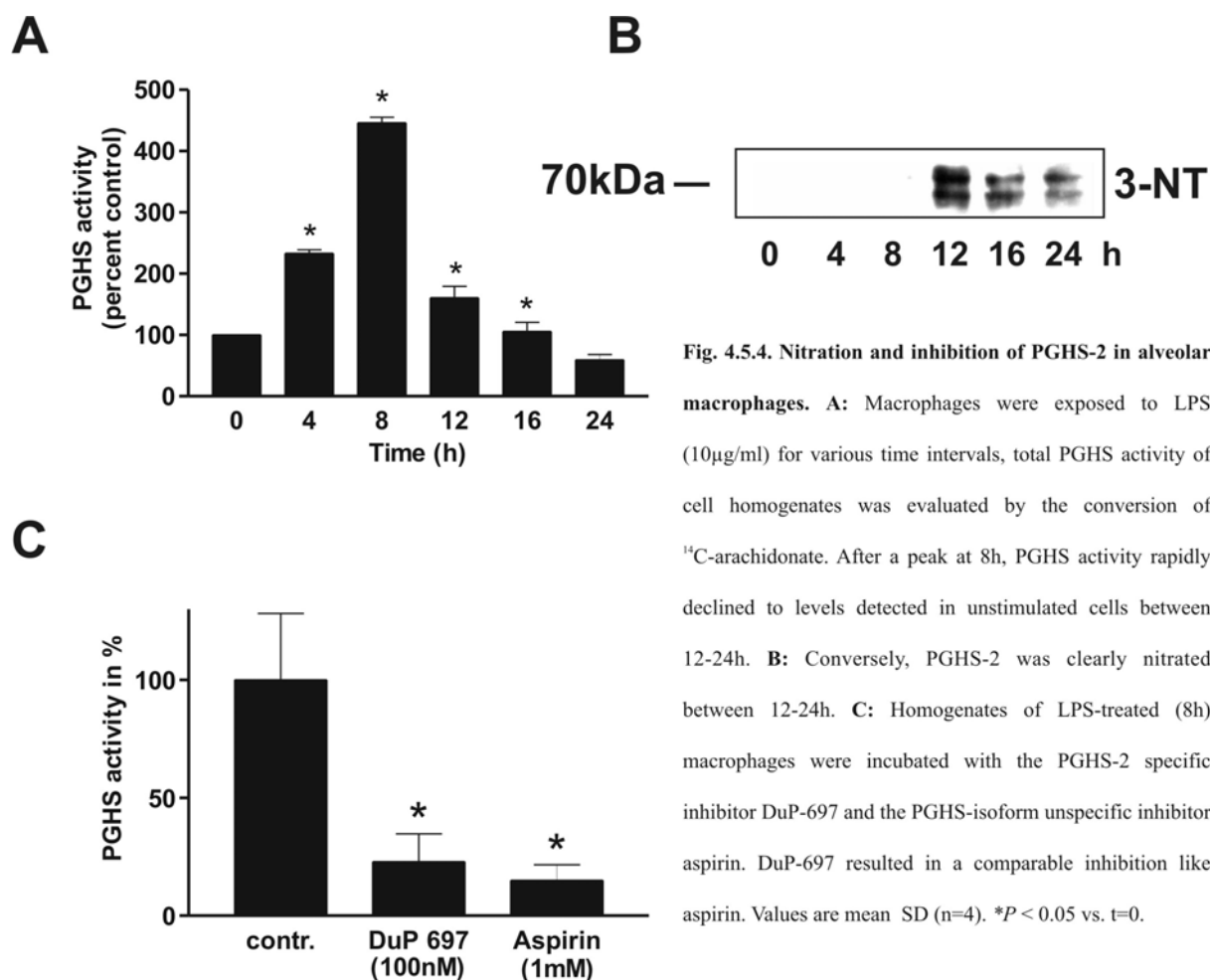
When rat AM were exposed to LPS (10 $\mu$ g/ml) for various time intervals, a time-dependent accumulation of both TxB<sub>2</sub> and PGE<sub>2</sub> was observed in the supernatants (Fig. 4.5.3). Between 12-24h no significant additional formation of either TxB<sub>2</sub> or PGE<sub>2</sub> occurred although PGHS-2 protein was still highly expressed (see Fig. 4.5.2B). Hence it could be assumed that PGHS-2 after 12h of LPS exposure had become inactive.



**Figure 4.5.3. Prostanoid formation by alveolar macrophages.** Prostanoids were detected after incubation with LPS for the various time intervals respectively. The stable hydration product TxB<sub>2</sub> serves as marker for TxA<sub>2</sub> production. TxB<sub>2</sub> originates from the non-enzymatic hydration of unstable TxA<sub>2</sub>. Constant formation of both TxB<sub>2</sub> and PGE<sub>2</sub> was observed between 0-12h while no additional release could be detected between 12-24h. Values are mean SD (n=4). \*P 0.05 vs. t=0.

To directly assess PGHS activity in AM exposed to LPS for various time periods, cell lysates were incubated with <sup>14</sup>C-arachidonate in order to follow the total prostanoid pattern. PGHS activity demonstrated a peak after 8h of LPS incubation with a subsequent decline between 12-24h to levels of unstimulated cells (Fig. 4.5.4A). This regression coincided with the lack of additional TxB<sub>2</sub> and PGE<sub>2</sub> synthesis between 12-24h as shown in Fig. 4.5.3, but was in contrast with the observation of maximal PGHS-2-expression after 16h (Fig. 4.5.2B) indicating a posttranscriptional inhibition of PGHS-2.

When 3-nitrotyrosine (3-NT) was probed with a commercially available anti-3-nitrotyrosine (3-NT) antibody at the protein level, a major nitrated double-band previously identified as PGHS-2 appeared, indicating a markedly elevated Tyr-nitration of PGHS-2 between 12-24h (Fig. 4.5.4B). Hence nitration paralleled the inhibition of PGHS-2 activity (Fig. 4.5.4A) and the inhibited release of  $\text{TxB}_2$  and  $\text{PGE}_2$  (Fig. 4.5.3). To establish PGHS-2 over PGHS-1 as the dominating contributor to the entire PGHS activity detected in cell homogenates, the PGHS-2 specific inhibitor DuP-697 and its effect in relation to the PGHS-isoform unspecific inhibitor aspirin was verified (Fig. 4.5.4C).

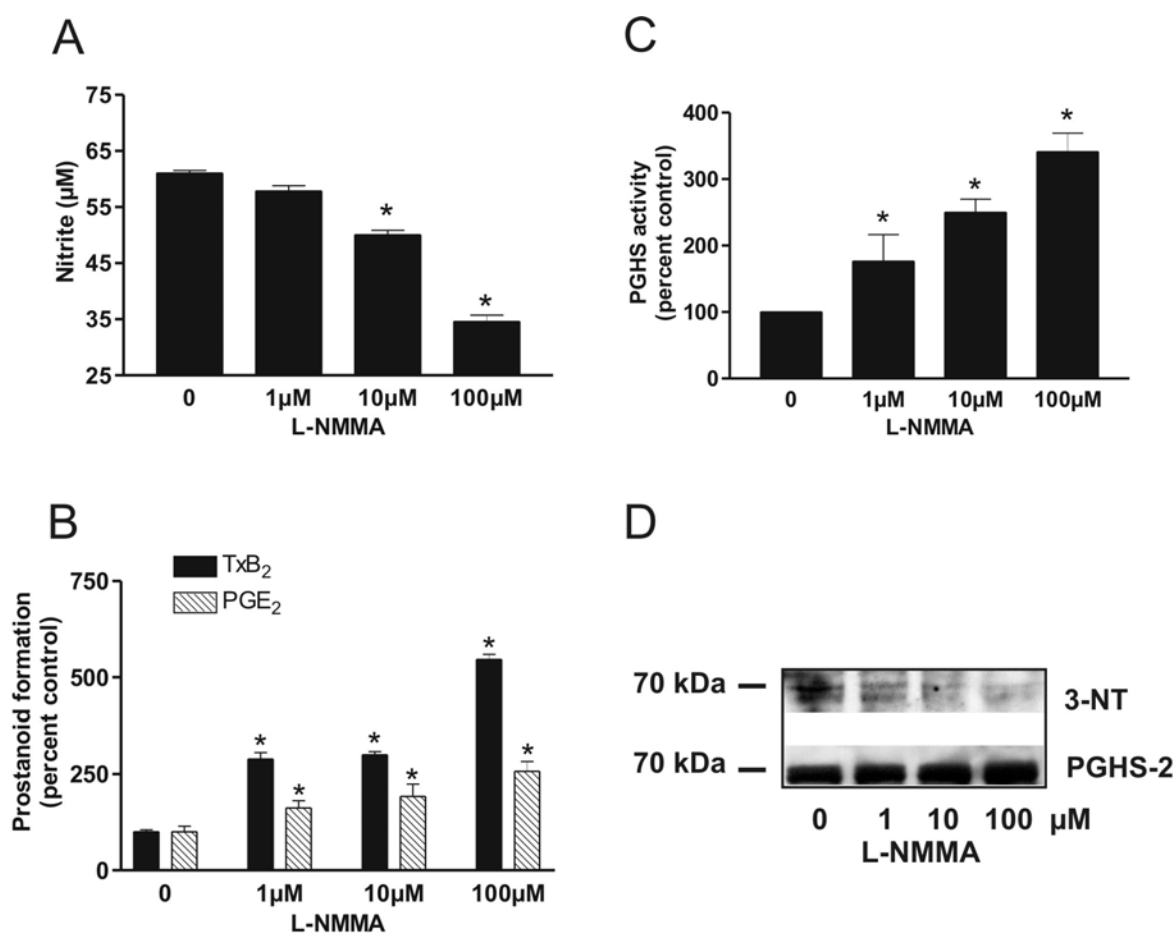


In order to exclude a reported impact of the PGHS inhibitors on PGHS-2 expression, cells were treated with LPS for 8h to allow induction of PGHS-2 and then the homogenates were

incubated with the inhibitors. Aspirin at 1mM resulted in the expected inhibition of more than 80%, a similar extent was also reached with 100nM of the PGHS-2-selective inhibitor DuP-697 (Fig.4.5.4C).

#### *Inhibition of $\bullet$ NO synthesis promotes prostanoid formation*

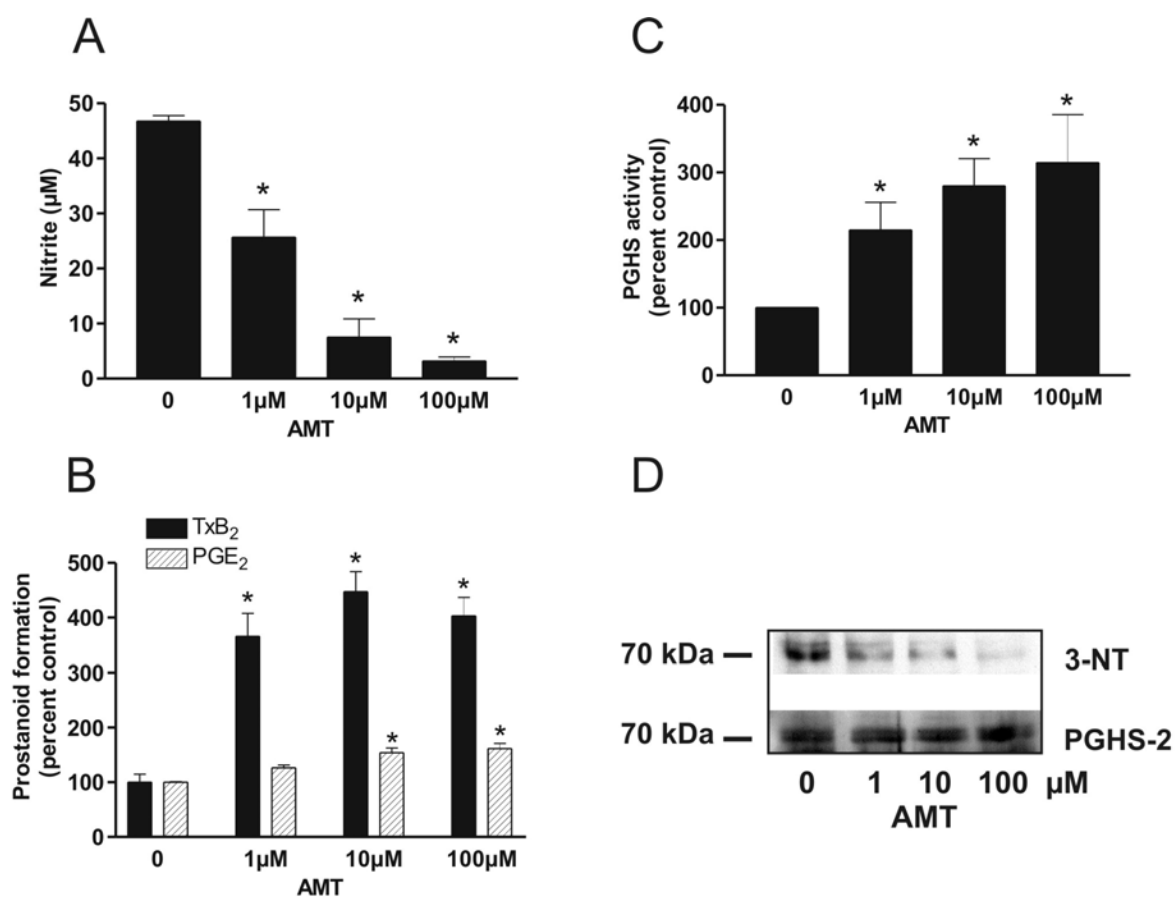
To prove the hypothesis that Tyr-nitration of PGHS-2 is the reason for an inhibition of the enzyme, AM were incubated with LPS and different concentrations (0-100 $\mu$ M) of the NOS-



**Figure 4.5.5. Impact of the NOS-inhibitor L-NMMA on PGHS activity.** Cells were incubated with LPS (10 $\mu$ g/ml) and various concentrations of the NOS-inhibitor L-NMMA for 20h. **A:** With an IC<sub>50</sub> of 6 $\mu$ M (murine NOS-2) 100 $\mu$ M L-NMMA suppressed nitrite formation by nearly 50%. Release of both TxB<sub>2</sub> and PGE<sub>2</sub> (**B**) was elevated with increased inhibition of NO synthesis by L-NMMA. The effect was found more pronounced for TxB<sub>2</sub> compared with PGE<sub>2</sub>. PGHS activity of cell homogenates dose-dependently increased with elevated inhibition of NO synthesis (**C**). Conversely, when western-blot were stained with an anti-3-nitrotyrosine antibody, inhibition of NO-synthesis prevented PGHS-2 from nitration (**D**). Levels of nitrite, TxB<sub>2</sub> and PGE<sub>2</sub> at L-NMMA = 0 were defined as 100%.

inhibitor L-NMMA for 20h. Release of nitrite as the stable end product of  $\bullet$ NO-formation was concentration-dependently inhibited (Fig. 4.5.5A). In contrast, release of  $\text{TxB}_2$  was elevated 5-fold and  $\text{PGE}_2$  release increased 2.5-fold (Fig. 4.5.5B).

To test whether this increased prostanoid release accounts for a reduced inhibition of PGHS-2, total PGHS activity in AM lysates was evaluated and showed an increase of PGHS activity with increasing inhibition of  $\bullet$ NO-synthesis (Fig. 4.5.5C). Reciprocal to the rise in PGHS activity,



**Figure 4.5.6. Impact of the NOS-inhibitor AMT on PGHS activity.** Alveolar macrophages were incubated with LPS (10µg/ml) and various concentrations of AMT for 20h. (A) Due to the lower  $\text{IC}_{50}$  of AMT (4.2nM for murine NOS-2) 1µM AMT demonstrated a comparable inhibition of nitrite formation than 100µM L-NMMA (Fig. 5). (B) Maximal elevation of  $\text{TxB}_2$  release by AMT was reached at 1µM AMT and was not further stimulated by higher concentrations. (C) PGHS activity of cell homogenates dose-dependently increased with elevated inhibition of NO-synthesis. whereas PGHS-2 was prevented from nitration. Values are mean  $\pm$  SD (n=3). \* $P < 0.05$  vs. AMT =0.

PGHS-2-nitration gradually declined indicating that Tyr-nitration of PGHS-2 is accompanied by an inhibition of the enzyme. Under the conditions employed, PGHS-2 protein expression was not affected by the NOS inhibitor (Fig. 4.5.5D). Since L-NMMA has a relatively high  $IC_{50}$  of  $6\mu\text{M}$  (murine NOS-2), the NOS-2-selective inhibitor AMT ( $0\text{--}100\mu\text{M}$ ) with an  $IC_{50}$  of  $4.2\text{nM}$  (murine macrophages) was added together with LPS for 20h. As expected, nitrite formation was inhibited more effectively in agreement with NOS-2 as the major source of  $\bullet\text{NO}$  in LPS-exposed AM (Fig. 4.5.6).  $\text{TxB}_2$  release was elevated 4-fold at already  $1\mu\text{M}$  AMT and was only slightly enhanced by a further increase of the inhibitor. It was puzzling to observe that for  $\text{PGE}_2$  the increase was much lower for which several reasons may exist like different kinetics of the corresponding synthases or different localizations of the interacting enzymes. However, blocking of NOS-2 reverted the inhibition of PGHS-2 which was definitely proven by the correlated decrease of nitration.

## Discussion

In the present study we examined the previously described Tyr-nitration and auto-inhibition of PGHS-2 by nitrite as a regulatory mechanism terminating the release of  $\text{TxA}_2$  and  $\text{PGE}_2$  in LPS-stimulated rat AM. Optimized conditions for PGHS-2 induction in these cells were selected in order to obtain prostanoid release mainly through PGHS-2. In literature some reports indicate the involvement of PGHS-1 in AM stimulated with LPS immediately after isolation (Wilborn 1995). By allowing to rest overnight, we observed a distinct preference of PGHS-2 activity over PGHS-1 in providing substrate for prostanoid synthesis. Thus, as clearly indicated by the high extent of inhibition by DuP-697, most of the prostanoid formation under the conditions employed in this work depends on the presence of newly

synthesized PGHS-2. It should be noted that the results from Fig. 4.5.3, 4.5.5 and 4.5.6 indicate quantitative differences for TxA<sub>2</sub> and PGE<sub>2</sub> in terms of induction by LPS and inhibition by •NO-products. This could be explained by a different coupling between the two PGHS-isoforms and the corresponding isomerases. Such differences are known but were not in the focus of the present investigation. For rat AM the results presented here clearly demonstrate an inhibition of TxA<sub>2</sub> and PGE<sub>2</sub> release which coincided with a sudden onset of Tyr-nitration and inhibition of PGHS-2. Blocking •NO-synthesis released the inhibition without affecting PGHS-2 protein expression under the conditions employed. This strongly points to a negative posttranslational regulation of PGHS-2-dependent TxA<sub>2</sub> and PGE<sub>2</sub> formation and raises the question which •NO-derived species mainly accounts for the nitration and inhibition observed. A direct interaction of •NO with PGHS-2 was reported in vitro (Gunther 1997; Goodwin 1998), however the unphysiological high levels (1-2mM) nearly exclude a biological significance of this reaction (Tsai 1994). Due to its Tyr-radical and heme-catalyzed reaction mechanism, another likely candidate for the nitration of PGHS-2 could be peroxynitrite. In fact, isolated PGHS-2 in the absence of arachidonate can easily be nitrated by peroxynitrite. However, the presence of arachidonate, which reflects the situation in activated macrophages, potently prevents from such an inhibition (Thomas 2002) and bolus additions of at least 100µM peroxynitrite were reported necessary for the inhibition of PGHS-2 (Zou 1997). Furthermore, in the presence of arachidonate, intracellular peroxynitrite levels not only failed to inhibit, but even stimulated PGHS-2 activity. Inhibition of intracellular peroxynitrite-formation in LPS-stimulated AM by an antioxidant or a spin-trap was demonstrated to inhibit the release of PGE<sub>2</sub> (Hempel 1994). This effect can be explained by the necessity of peroxides for the initiation and maintenance of the PGHS catalytic cycle (Buckley 1991). Interestingly, the peroxide tone of PGHS-1 (21nM) was discovered 10-fold higher than that of PGHS-2 (2nM) (Kulmacz 1995). As a consequence, the high intracellular reductive potential together with the higher peroxide tone of PGHS-1 would prevent from an

uncontrolled prostanoid formation by the constitutively expressed PGHS-1. Following stimulation of a cell, peroxynitrite was found a potent intracellular activator of the newly synthesized PGHS-2 (Landino 1996) which would be favored against PGHS-1 due to its lower requirement for peroxides. All these observations together would exclude peroxynitrite as the major nitrating species and leave nitrite as the likely candidate for the nitration of PGHS-2.

In a previously published work in RAW 264.7 macrophages (Bachschmid), the intrinsic peroxidase activity of PGHS-2 was found sufficient for a one-electron oxidation of nitrite to yield the highly reactive  $\bullet\text{NO}_2$  radical. It was hypothesized that  $\bullet\text{NO}_2$  would subsequently interact with the  $\bullet\text{Tyr}$  radical formed during PGHS catalysis (Tyr<sup>385</sup> in PGHS-1; Tyr<sup>371</sup> in PGHS-2) to form nitrotyrosine. This agrees with reports on Tyr-nitration in LPS-stimulated RAW 264.7 macrophages in which nitrite was regarded to support nitration of not further identified proteins (Pfeiffer 2001a; Pfeiffer 2001b). Due to the discovery of PGHS-2 as the major nitrated protein in the same cellular system, we assume the intrinsic peroxidase activity of PGHS-2 and not the catalytic action of myeloperoxidase (Sampson 1998) as mainly responsible for the peroxidatic activation of nitrite and the subsequent nitration and inhibition of PGHS-2.

Data obtained with isolated PGHS-2 indicated nitrite levels of at least 10 $\mu\text{M}$  as necessary for the observed inhibition (Bachschmid). Since the interaction of nitrite with PGHS-2 occurs in competition with arachidonate, the inhibitory levels of intracellular nitrite will vary and can be expected lower when phospholipase A<sub>2</sub> activity also declines as has been shown to occur between 12 and 24h in stimulated peritoneal macrophages (Naraba 1998). While exogenous nitrite concentration-dependently inhibited PGHS activity when added to cell homogenates, it was ineffective when added to intact cells (unpublished). This indicates an effective export of nitrite and therefore implies a direct control of PGHS-2 nitration by the intracellular NOS-2 activity. The nearly parallel expression of NOS-2 and PGHS-2-nitration as shown in Fig.

4.5.1B and 4.5.4B further support this hypothesis. The physiological significance of this regulation by NOS-2 activity becomes obvious when the extreme delay of NOS-2 protein expression compared with that of PGHS-2 is taken into account (Sunil 2002). While the time course of PGHS-2 mRNA and protein expression develop almost in parallel, there is an unusual wide time lag of about 8-12h between the maxima of NOS-2-mRNA and -protein expression. This delay opens a time window of about 8h in which prostanoid synthesis can proceed. The mechanism causing this delay is not known but a concomitant expression of NOS-2 and PGHS-2 would have abolished prostanoid formation already at a much earlier time point.

In conclusion, our data suggest a nitrite-dependent autocatalytic inhibition of PGHS-2-derived TxA<sub>2</sub> formation in LPS-exposed rat AM. It has to be noted that this mechanism is certainly limited to cell types expressing high amounts of NOS-2. For instance, LPS-exposed vascular smooth muscle cells that exert only little generation of •NO demonstrated no significant inhibition of PGHS-2-dependent prostacyclin synthesis (Schildknecht 2004). For the constitutive PGHS-1 a similar mechanism seems to exist (unpublished) but should be without physiological importance since the required nitrite levels are not reached under non-inflammatory conditions in the absence of NOS-2. Since TxA<sub>2</sub> is a potent bronchioconstrictor, a self-inhibition of its synthesis by AM appears as a beneficial process and makes nitrite an endogenous PGHS-2 inhibitor for terminating the inflammatory response in the lung. The question remains whether this endogenous inhibition coincides with phospholipase A<sub>2</sub> inactivation or whether the unmetabolized arachidonate is channeled into other pathways like the formation of leukotrienes, lipoxins or 15-HPETE.

## 5. General Discussion

The following chapters correspond to the five publications in which experimental details and results can be found. In addition to the respective discussion sections in each of these chapters, the following general discussion emphasises on the general relevance of these findings for biology and medicine and provide a molecular explanation for events in the vasculature associated with massive inflammation. The two novel concepts of peroxynitrite as cellular activator of PGHS and the autocatalytic nitration and inhibition of PGHS by nitrite allow new insights into the regulation of the PGHS catalytic cycle and ask for a new interpretation of literature data with respect to the interaction of the NOS and the PGHS pathways.

### 5.1. Prostacyclin Synthesis by Vascular Smooth Muscle Cells.

In recent years the elaborate work of Fitzgerald (McAdam 1999, FitzGerald 2004) has uncovered the vascular endothelium as a major source of systemic prostacyclin ( $\text{PGI}_2$ ) formation under normal conditions. Prostacyclin exerts vasodilatory, anti-aggregatory and anti-adhesive properties and therefore becomes a central regulator of vascular homeostasis (Noll 1998, Furchgott 1980, Moncada 1978). Patients in sepsis or septic shock suffer from severe systemic hypotension. Several *in vivo* studies clearly indicated that plasma levels of 6-keto- $\text{PGF}_{1\alpha}$ , the stable hydrolysis product of  $\text{PGI}_2$ , are highly elevated in these patients (Halushka 1985). These findings would lead to the suggestion that under conditions of systemic endotoxin exposure, the endothelium would mainly account for the increased formation of  $\text{PGI}_2$ .

However, studies performed during the last decade by our group clearly indicated that endothelial PGI<sub>2</sub>-synthase becomes inactivated under several pathophysiological conditions like endotoxemia, ischemia-reperfusion, diabetes or atherosclerosis (Zou 1996, 1997, 1999). Inactivation of PGI<sub>2</sub>-synthase is mediated by peroxynitrite, originating from the diffusion-limited interaction of •NO and •O<sub>2</sub><sup>-</sup>. Peroxynitrite nitrates a tyrosine residue near the active site catalyzed by the heme prosthetic group of the P450-enzyme PGI<sub>2</sub>-synthase. This explains that already at concentrations of only 50nM of authentic peroxynitrite, nitration and inhibition can be observed (Zou 1997). These findings opened the question where the observed high levels of plasma 6-keto-PGF<sub>1α</sub> in septic individuals are originating from. A first hint was given by a work of Smith and DeWitt published in the early 1980's which demonstrates vascular smooth muscle cells as a rich source of PGI<sub>2</sub>-synthase. Surprisingly, under physiological conditions it lacks any significant prostaglandin endoperoxide H<sub>2</sub> synthase (PGHS) activity (DeWitt 1983) and a plausible answer to the question on a physiological function of this "silent pool" of PGI<sub>2</sub>-synthase could not be given yet. Based on these findings together with the discovery of an inducible isoform of PGHS in the early 1990's (PGHS-2), the work presented herein was initiated by the hypothesis that smooth muscle cells (SMC) could be involved in the systemic formation of PGI<sub>2</sub> in septic patients.

The current thesis now provides a conclusive model which introduces the LPS-exposed vascular smooth muscle as a potent source of PGI<sub>2</sub> and therefore combines the contradictory observations mentioned above. The observed high output of PGI<sub>2</sub> in response to LPS is achieved by an induction of PGHS-2 which provides the constitutively expressed PGI<sub>2</sub>-synthase with substrate (Schildknecht 2004). These findings allow to postulate vascular smooth muscle as an alternative regulator of vascular integrity under conditions of a functionally impaired endothelium.

For a better understanding of the complexity of events in the LPS-exposed vasculature, we propose two phases of vascular activation which provide a molecular explanation for the clinical observations of severe hypotension in septic patients.

**“Phase I”** which requires no *de novo* protein synthesis occurs within the first hour of LPS exposure and is limited to the endothelium. In this phase, production of endothelial  $\bullet\text{O}_2^-$  is upregulated which reacts with endothelial  $\bullet\text{NO}$  to form peroxynitrite (Bachschnid 2003). Peroxynitrite subsequently nitrates and inactivates endothelial  $\text{PGI}_2$ -synthase. Therefore, the increase of endothelial  $\bullet\text{O}_2^-$  formation not only reduces the levels of free  $\bullet\text{NO}$  which exerts similar biological properties as  $\text{PGI}_2$ , but also limits  $\text{PGI}_2$  release by triggering the peroxynitrite-mediated inhibition of its synthase. As a result of the reduced availability of  $\text{PGI}_2$  and  $\bullet\text{NO}$ , proaggregatory, proadhesive and vasoconstrictive conditions prevail. Furthermore, endothelial PGHS activity is increased by the peroxide tone while  $\text{PGI}_2$ -synthase as the major recipient of  $\text{PGH}_2$  is inhibited under these conditions resulting in the release of  $\text{PGH}_2$ . This can either bind to the  $\text{TxA}_2/\text{PGH}_2$  receptor on smooth muscle cells or platelets to cause additional vasoconstriction and platelet aggregation (Mais 1985, Ogletree 1985) and/or it can be converted to  $\text{PGE}_2$  by  $\text{PGE}_2$ -synthase localized on SMCs (Soler 2000).  $\text{PGE}_2$  is essentially involved in the host's immune response by triggering the expression of preformed P-selectin on the surface of endothelial cells thus enabling the trapping and “rolling” of leukocytes (Hailer 2000). This transmigration of activated leukocytes from the blood to the site of an infection would follow at the end of “Phase I” by a gating of the endothelial barrier. Thus, the switch which is performed during “Phase I” from conditions of vascular homeostasis to a functionally impaired endothelium must not be regarded as a dysfunctional process, but should rather be seen as one of the first stages in the complex series of events enabling leukocytes to migrate from the blood to the sites of infection. While Phase I is usually characterized by a hypertensive state, clinicians are more familiar with the opposite phenotype of severe hypotension in septic patients. These events are summarized as **“Phase**

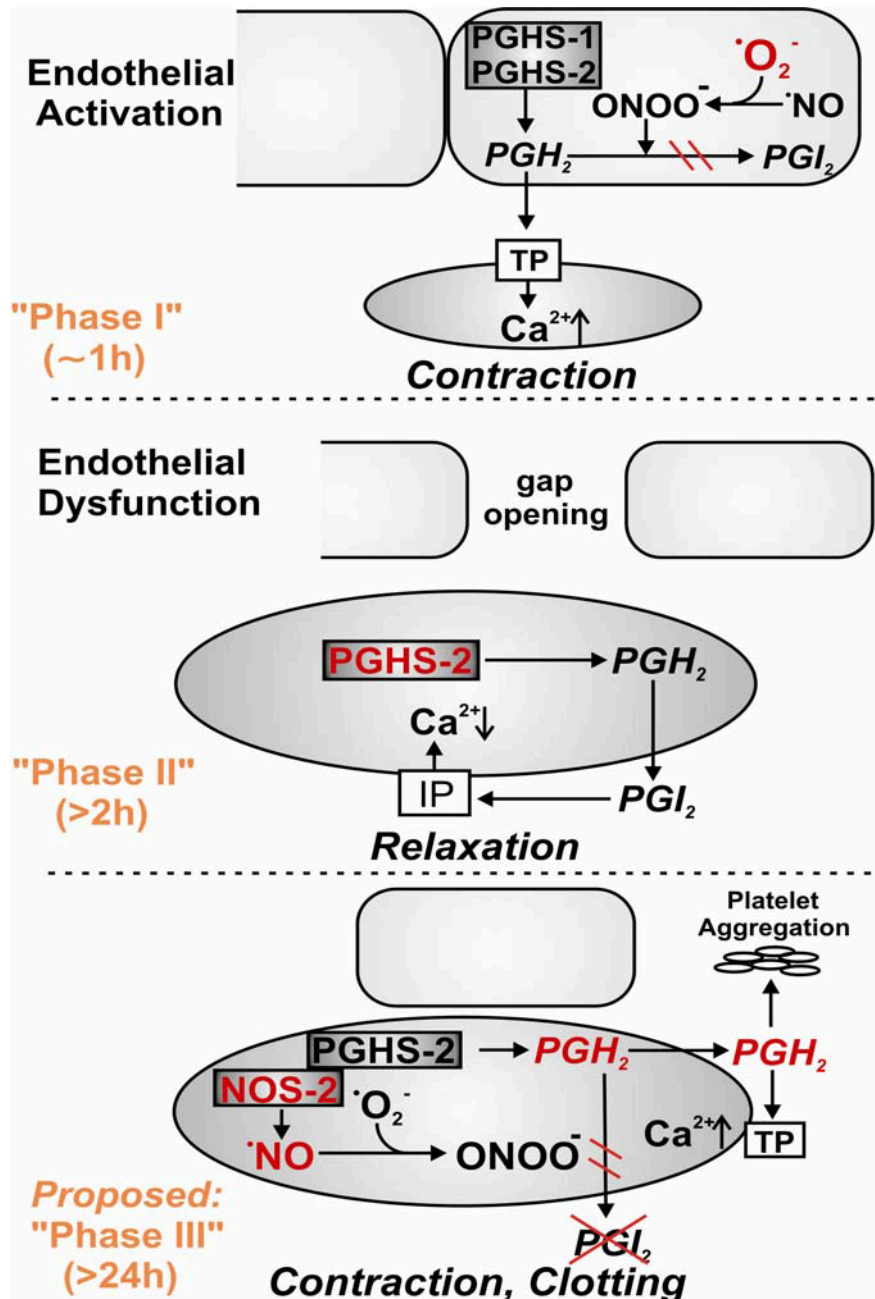
II” of vascular activation which is initiated after about 2h of endotoxin challenge and includes the expression of early immediate genes like ICAMs or VCAMs on the surface of endothelial cells as well as the induction of PGHS-2 and NOS-2. Access of circulating endotoxin to the smooth muscle layers of septic individuals would be achieved by a leaky endothelial barrier, an increased transendothelial shuttling and in some cases even by a local detachment of endothelial cells (Gaynor 1970, Maeda 1995). The high output of PGI<sub>2</sub> by SMC in “Phase II” not only compensates the lack of endothelial PGI<sub>2</sub> and •NO release but also counteracts the PGH<sub>2</sub>-mediated activation of the TxA<sub>2</sub>/PGH<sub>2</sub> receptor cascade. Since this PGI<sub>2</sub> release by SMC nearly completely depends on the induction of PGHS-2, this enzyme could represent a promising target for the treatment of severe hypotension in septic shock.

Stimulated by the assumption of a “good” PGHS-1 and a “bad” PGHS-2, several PGHS-2-specific inhibitors were developed during the past decade. By the end of 2004, the public became aware of long-term side effects of such pharmaceuticals like Vioxx which were mostly used in the treatment of rheumatoid arthritis patients. These side effects definitely came not as a surprise since several studies had clearly demonstrated that PGHS-2 is vitally involved in PGI<sub>2</sub> formation by the endothelium and other physiological processes even in healthy subjects (McAdam 1999). It could be speculated that PGHS-2 is surely not equally expressed throughout the body’s endothelium but should be found at distinct sites of the vasculature exposed to increased shear forces (Okahara 1998). Deduced from the fact that administration of PGHS-2-specific inhibitors to healthy subjects exerts no detrimental side-effects, it appears that the pharmacological inhibition of PGHS-2-dependent PGI<sub>2</sub> formation in endothelial cells under normal conditions is effectively counterregulated either by an increased involvement of PGHS-1 or by an elevated release of •NO. In contrast, two independent clinical trials (VIGOR, CLASS) (Bombardier 2000, Silverstein 2000) demonstrated that patients at higher risk like chronic vascular or coronary disorders exhibit a significantly higher incidence of myocardial infarction when receiving PGHS-2-specific

inhibitors compared with classical NSAIDs. This indicates that under such conditions, the induction of PGHS-2 in response to pathological stimuli may be involved as a counterregulatory mechanism.

Based on the current findings, the treatment of patients in acute sepsis with PGHS-2 inhibitors could represent a useful tool to correct the severe hypotension. However, since the PGHS-2-dependent release of PGI<sub>2</sub> by SMC has to be regarded as a beneficial process as elaborated in this work, a total blockade of PGHS-2 should be avoided and only a precisely monitored partial inhibition appears to be an advisable strategy for clinicians.

Another interesting question that arises from the data presented in this thesis and previously published results of our group is why PGI<sub>2</sub>-synthase becomes nitrated and inhibited in LPS-exposed endothelial cells while the same treatment causes a sustained release of high amounts of PGI<sub>2</sub> by SMC. One essential feature that prevents peroxynitrite-mediated nitration of PGI<sub>2</sub>-synthase in SMC is a lack of NOS-2 induction within the time frame investigated in human and bovine cells (Schildknecht 2004). This limits intracellular peroxynitrite formation just by a limited availability of •NO. The second important parameter that distinguishes endothelial and smooth muscle cells seems to be a higher antioxidative capacity of SMC that would keep intracellular levels of peroxynitrite very low. This potential can be maintained by the action of glutathione/glutathione peroxidase, low weight antioxidant molecules like vitamins C and E, lipoic acid, but also by peroxiredoxins which are capable to directly reduce peroxides as well as by glutaredoxins which were reported to reduce glutathionylated proteins (Nordberg 2001). The results presented in this work mainly cover the time between 2-24h of LPS-exposure. Preliminary data of timely extended LPS challenges however demonstrate a biphasic induction pattern of PGHS-2. A first peak was detected after roughly 4-6h, followed by a subsequent decline of both mRNA and protein expression and a second peak between 48-72h



**Vascular LPS model of Septic Shock.** In “Phase I” which proceeds within the first hour of LPS exposure, an increase in endothelial superoxide ( $\cdot\text{O}_2^-$ ) production causes endothelial activation.  $\cdot\text{O}_2^-$  reacts with  $\cdot\text{NO}$  to form peroxynitrite ( $\text{ONOO}^-$ ) which subsequently nitrates endothelial  $\text{PGI}_2$ -synthase.  $\text{PGH}_2$ , which is released under these conditions, binds to the  $\text{TxA}_2/\text{PGH}_2$  (TP) receptor on smooth muscle cells (SMC) and evokes vasoconstriction. In “Phase II”, prostaglandin endoperoxide  $\text{H}_2$  synthase-2 (PGHS-2) is induced in SMC and supplies the constitutively expressed  $\text{PGI}_2$ -synthase with substrate.  $\text{PGI}_2$  activates its (IP) receptor in an autocrine manner to evoke vasorelaxation. This systemic vasodilation is a characteristic feature of sepsis and septic shock. Preliminary data indicate an induction of NOS-2 in human and bovine SMC after 24h of endotoxin exposure. It can be speculated that this increased availability of  $\cdot\text{NO}$  could be a prerequisite to terminate “Phase II” and would lead to a third phase in the LPS-challenged vasculature. The following increase of  $\cdot\text{NO}$  would either lead to a recovery of endothelial function or, if  $\cdot\text{O}_2^-$  levels are still high, to elevated intracellular levels of  $\text{ONOO}^-$  which could nitrate and inactivate  $\text{PGI}_2$ -synthase (PGIS) in SMC. The subsequent rise of unmetabolized  $\text{PGH}_2$  would then lead to vasoconstriction, platelet aggregation and vessel collapse.

of LPS exposure. Interestingly, in such progressive stages of endotoxin exposure, bovine SMC also revealed an induction of NOS-2 which was suppressed within the first 24h. It could be speculated that under such conditions (LPS >48h) PGHS-2 would again be highly active to provide the PGI<sub>2</sub>-precursor PGH<sub>2</sub>. The induction of NOS-2 together with a declined antioxidative capacity of the smooth muscle cell could then favor nitration and inactivation of PGI<sub>2</sub>-synthase. Since such oxidative conditions would support PGHS-2 activity by an increase of the peroxide tone, the levels of unmetabolized PGH<sub>2</sub> would rise and could activate the TxA<sub>2</sub>/PGH<sub>2</sub>-receptor (Saussy 1986) on the surface of SMC and platelets to evoke systemic vasoconstriction and aggregation. Such speculative events in the vascular smooth muscle could essentially contribute to the high mortality of patients undergoing severe sepsis. This scenario however represents a hypothesis at the time of writing and is part of further investigations.

## **5.2 Peroxynitrite Provides the Cellular Peroxide Tone for PGHS-2**

During the work on PGI<sub>2</sub> release by bovine SMC it turned out that within the first 24h of LPS-incubation no inhibition of PGI<sub>2</sub>-synthase could be observed. This rather came as a surprise, since the same treatment causes nitration of PGI<sub>2</sub>-synthase by peroxynitrite in endothelial cells. One explanation for the lack of endogenous generation of peroxynitrite sufficient for the inactivation of PGI<sub>2</sub>-synthase was the absence of NOS-2 induction in human and bovine SMC under the conditions employed. As proof of principle, we therefore added the peroxynitrite-generating compound SIN-1 and expected a concentration-dependent decline of PGI<sub>2</sub> formation. However, SIN-1 in concentrations up to 1mM, which corresponds to a peroxynitrite generation of approximately 100nM/sec ( $t_{1/2}=1$ sec) (Frein, unpublished), not

only failed to nitrate PGI<sub>2</sub>-synthase in SMC but even stimulated PGI<sub>2</sub> formation. This posed the following two questions:

First, how does peroxynitrite cause a concentration-dependent increase of PGI<sub>2</sub> release, and second, how do SMC prevent PGI<sub>2</sub> synthase from inhibition by exogenously added peroxynitrite ?

It has been described in literature that both isolated PGHS-1 and PGHS-2 require the presence of peroxides for the initiation of their catalytic cycle. According to the so-called “branched chain” reaction mechanism, which today is a generally accepted model to explain PGHS catalysis, the peroxide initially reacts with the resting ferric enzyme to form an oxoferryl species ( $\text{Fe}^{4+}=\text{O}$  PPIX) and a tyrosyl radical (Dietz 1988, Karthein 1988). Formation of this radical is essential since it reacts with the substrate arachidonate to initiate the cyclooxygenase reaction. The following cycles are then activated in an autocatalytic manner by newly generated PGG<sub>2</sub>. According to this model which was established with purified enzyme, a single activation of PGHS by a peroxide should be sufficient for a sustained enzymatic turnover (see Introduction Fig. 1.5). Within a cell however, a potent antioxidant system usually keeps the levels of peroxides very low.

It was demonstrated in this work that among a variety of different candidates, peroxynitrite turned out as one of the most potent cellular activators of PGHS-2. Interestingly, the peroxide tone for isolated PGHS-2 was reported in literature as 10-fold lower (2nM) than that of PGHS-1 (21nM) (Kulmacz 1995). The findings of the present work revealed that exogenously added peroxynitrite can nearly double PGI<sub>2</sub> output by SMC which indicates that the intracellular peroxide tone works at half-saturating levels of around 1nM peroxynitrite. As an essential physiological consequence, such low levels of intracellular formed peroxynitrite are insufficient to nitrate and inactivate PGI<sub>2</sub> synthase which requires concentrations of at least 50nM. Peroxynitrite formed either endogenously or by exogenous addition seems to be tightly controlled by a powerful antioxidant capacity of the cell which effectively protects PGI<sub>2</sub>-

synthase from inactivation. Therefore, vascular smooth muscle can be regarded as a cell type optimized for the sustained release of PGI<sub>2</sub> to counteract a functionally impaired endothelium under severe pathophysiological conditions.

Several reports on the interaction of the PGHS and the NOS pathway exist in literature, however no clear-cut picture has emerged so far. Most of these studies are only of limited significance since attention is limited on the impact of •NO on PGHS activity alone.

In contrast, the concept of peroxynitrite as activator of PGHS includes both •NO and •O<sub>2</sub><sup>-</sup> as regulatory elements. Formation of •NO and •O<sub>2</sub><sup>-</sup> is highly cell-type specific and with our proposed model, most of the sometimes diverging observations in literature can be satisfactorily explained. Interestingly, in vascular SMC the endogenous generation of •NO and •O<sub>2</sub><sup>-</sup> appeared to occur in a nearly 1:1 ratio. This is of particular importance since with an excess of either •NO or •O<sub>2</sub><sup>-</sup> an increased decomposition of peroxynitrite can be observed (Jourdeuil 2001). Formation of •NO and •O<sub>2</sub><sup>-</sup> by different independent enzymes in a precise 1:1 ratio however appears rather unlikely. Instead it could be speculated that •O<sub>2</sub><sup>-</sup> represents a regulator of endogenous •NO formation. In SMC, NOS-1 can be assumed as mainly responsible for the formation of the low levels of •NO observed (Brophy 2000). In contrast to NOS-2 and NOS-3, Stuehr et al. have shown that NOS-1 exerts a unique mechanistic feature that distinguishes it from the other two isoforms (Stuehr 2004). During catalysis, NOS-1 predominantly occurs in the [Fe<sup>II</sup> NO] form which arises from [Fe<sup>III</sup> NO] as part of a futile cycle. One could speculate that under cellular conditions •O<sub>2</sub><sup>-</sup> would directly combine with •NO of the [Fe<sup>III</sup> NO] complex to form peroxynitrite. This interaction would not only stimulate •NO synthesis by reverting the enzyme back to the free [Fe<sup>III</sup>] state ready for initiating a new catalytic cycle, but could also explain the observed endogenous 1:1 ratio of •NO and •O<sub>2</sub><sup>-</sup>.

Formation of  $\bullet\text{O}_2^-$  for generating intracellular peroxynitrite would therefore become a key element in the regulation of prostanoid biosynthesis in SMC.

This finely tuned redox environment, which is required for the sustained release of  $\text{PGI}_2$  by SMC, is most likely not provided within the entire cytoplasm but only in distinct cellular compartments. In recent years, attention has been focused on the so-called caveolae, small plasma invaginations involved in a variety of cellular signal transduction pathways. One might speculate that the finely tuned conditions for PGHS-2-dependent  $\text{PGI}_2$  release are limited to such cellular compartments which could harbor both the enzymes involved in prostanoid biosynthesis as well as  $\bullet\text{NO}$  and  $\bullet\text{O}_2^-$  generating systems. This assumption is supported by literature data indicating a colocalization of PGHS-2 and caveolin-1 (Liou 2001) as well as  $\text{PGI}_2$ -synthase and caveolin-1 (Spisni 2001) in human fibroblasts and endothelial cells respectively. Interestingly, NOS-1 was also reported to be closely localized to the caveolae and exists in an inactive state when associated with the caveolin-1 multi-complex (Liu 1996). Following stimulation of a cell, it regains enzymatic activity by dissociating from caveolin-1 but remains in close vicinity to the vesicle. Taken together, the assumption that  $\text{PGI}_2$  biosynthesis in SMC occurs mainly in caveolae would not only support the precisely defined conditions necessary for the formation of  $\text{PGI}_2$ , but would also favor its release into the extracellular space.

Ongoing work of our laboratory emphasises on a possible involvement of the concept of peroxide tone in the PGHS-1-dependent formation of thromboxane  $\text{A}_2$  ( $\text{TxA}_2$ ) in human platelets. Deduced from preliminary studies, peroxynitrite also appears to play an essential role in the regulation of PGHS-1 activity in this system. As already mentioned, the peroxide requirement of PGHS-1 is about 10-fold higher than that of PGHS-2. This implies that intracellular levels of peroxynitrite can also be expected higher when compared with SMC. In contrast to  $\text{PGI}_2$ -synthase,  $\text{TxA}_2$ -synthase demonstrates a higher resistance against oxidative

inactivation and should not be inhibited by the higher concentrations of peroxynitrite necessary for PGHS-1 activation in platelets.

### **5.3. Mutual Formation of either PGI<sub>2</sub> or •NO by Vascular Smooth Muscle Cells from Different Species**

The studies mentioned in the previous chapters were performed with primary cultures of bovine aortic SMC. It was elaborated that one factor that prevents nitration of PGI<sub>2</sub>-synthase in these cells is the absence of NOS-2 induction in the time frame investigated. In view of several reports in literature performed with rat SMC which clearly indicated an upregulation of NOS-2, SMC from rat, cattle and man were compared with respect to their induction pattern of PGHS-2 and NOS-2 in response to endotoxin. In human and bovine SMC, no significant induction of NOS-2 was observed within 24h of LPS exposure. Instead, PGI<sub>2</sub> was released in a PGHS-2 dependent manner. In contrast, rat SMC significantly upregulated NOS-2 while only little induction of PGHS-2 and PGI<sub>2</sub> formation was detected. In all three species, the response of the vascular smooth muscle to LPS represents an alternative mechanism to counterregulate a functional impaired endothelium. PGI<sub>2</sub> and •NO evoke similar physiological properties like vasorelaxation or platelet disaggregation. Previous work of our group indicated a high sensitivity of PGI<sub>2</sub>-synthase to oxidative inactivation (Zou 1996, 1997). It was demonstrated in this work that in bovine SMC such an inhibition of PGI<sub>2</sub>-synthase is effectively prevented by different mechanisms at least within the first 24h of LPS incubation. Within this time frame, the physiological consequence of a severe systemic hypotension is nearly the same in man, cattle and rat. The interesting question however is what happens in later stages of endotoxin exposure. As already mentioned, in such progressive stages NOS-2

induction was also observed in bovine SMC. The subsequent elevation of  $\bullet$ NO formation represents a prerequisite for increased intracellular peroxynitrite levels. Nitration of PGI<sub>2</sub>-synthase occurs at peroxynitrite concentrations of already 50nM (Zou 1997) whereas inhibition of NOS activity by peroxynitrite requires concentrations between 10-20 $\mu$ M and occurs mainly due to oxidation of the NOS cofactor BH<sub>4</sub> (Pasquet 1996; Patel 2002). Therefore, it could be speculated that the well-known higher resistance of rats to endotoxin could at least in part be explained by a main focus on the NOS pathway and its higher resistance against oxidative inactivation. Since the rat is a widely used model for studying the sepsis syndrome, the observed species-dependent differences have to be considered when such data are extrapolated to the situation in man.

#### **5.4. Nitration of PGHS-2 by Nitrite**

During studies on PGI<sub>2</sub>-synthase nitration in RAW 264.7 macrophages a protein band of around 70kDa was positively stained by anti-3-nitrotyrosine antibodies and later identified as PGHS-2. Since peroxynitrite was elaborated as an activator and not inhibitor of PGHS-2, an alternative mechanism of nitration came into the center of our research. In literature the peroxidatic activation of inorganic nitrite was proposed as an alternative process leading to biological tyrosine nitrations (Baldus 2002, Sampson 1998). In vivo this reaction is mainly performed by activated granulocytes which secrete high myeloperoxidase activities.

As a novel finding of this work, both cellular and isolated PGHS-2 were detected to be nitrated and inhibited by nitrite in an autocatalytic manner under involvement of the heme-prosthetic group. The proposed mechanism is likely to arise only in cell systems which concomitantly express PGHS-2 and NOS-2 like in LPS-stimulated RAW 264.7 macrophages. However, since preliminary data on long-term LPS-challenges of SMC (>48h) indicated an

upregulation of NOS-2, one could speculate on a possible involvement of this mechanism also in SMC in progressive stages of endotoxic shock. It has to be considered that an induction of NOS-2 in SMC would first lead to intracellular peroxynitrite levels sufficient for the inhibition of PGI<sub>2</sub>-synthase. The subsequent release of PGH<sub>2</sub> would lead to vasoconstriction and platelet aggregation. Therefore, a possible nitration of PGHS-2 by nitrite could represent a mechanism to terminate the inflammatory response, however it remains highly elusive whether nitrite levels in the high micromolar range can actually be formed in the vasculature.

Another important aspect in the interaction of the PGHS-2 enzyme with nitrite is the availability of its substrate arachidonate. While in the presence of arachidonate nitrite levels of around 10 $\mu$ M were established as sufficient for the autocatalytic nitration of isolated PGHS-2, preincubation with arachidonate effectively prevented from such a nitration. This implies a competitive behavior of arachidonate and nitrite for the one-electron oxidation occurring during the PGHS catalytic cycle.

This competitive behavior could exert fundamental consequences for PGHS-2 activity in whole cells. Since nitrite can easily accumulate within a cell, activity of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) would become a central regulator of PGHS-2 nitration. A reduction in PLA<sub>2</sub> activity would therefore not only limit prostanoid biosynthesis by a reduced supply with the substrate arachidonate, but could also prone PGHS for a rapid autocatalytic nitration and inactivation. Cell systems like macrophages potently induce both PGHS-2 and NOS-2 in response to endotoxin challenge. Our proposed mechanism would allow a time window of around 8h in which PGHS-2-dependent prostanoid biosynthesis could proceed without significant interference with the NO-synthase pathway. After this time, highly elevated intracellular nitrite levels could rapidly lead to the inactivation of PGHS-2 as soon as PLA<sub>2</sub> activity is reduced. A rapid breakdown of prostanoid release by the nitration of PGHS-2 could hereby represent a new general endogenous feedback mechanism to terminate the inflammatory

response. This has to be considered when a pharmacological intervention by NOS-2 specific inhibitors in the treatment of septic shock is discussed.

Preliminary data obtained with isolated PGHS-1 indicated that the proposed mechanism applies for both PGHS isoforms. However, nitrite levels in the micromolar range are usually only achieved by the induction of NOS-2 in response to a pathological stimulus. Since under these conditions PGHS-2 dominates, nitration of PGHS-1 as a regulatory mechanism seems of minor physiological importance.

Besides the presented posttranslational modifications of the enzymes involved in prostanoid biosynthesis,  $\bullet$ NO and its derivatives are also likely to affect gene- and protein expression of PGHS-2. During the work with RAW 264.7 macrophages it turned out that inhibition of endogenous NO-synthesis markedly reduced PGHS-2 protein expression (unpublished). According to literature, Tyr-nitration of I $\kappa$ -B by peroxynitrite and the subsequent translocation of NF- $\kappa$ B into the nucleus could account for the observed change in PGHS-2 expression (Matata 2002). In addition, exogenously added  $\bullet$ NO was shown to result in a superinduction of PGHS-2 in a AP-1 converged manner (von Knethen 2000). At least for LPS-stimulated RAW 264.7 macrophages, peroxynitrite could therefore be regarded as also involved in the regulation of gene expression by interfering with the NF- $\kappa$ B pathway. Since these effects were not observed in rat alveolar macrophages and literature also reveals no clear-cut answer, the interaction of  $\bullet$ NO-derived species with PGHS-2 mRNA and protein expression appears as a highly cell-type specific interaction.

### 5.5. Nitration of PGHS-2 in Alveolar Macrophages.

The autocatalytic nitration and inhibition of PGHS-2 by nitrite represents a novel posttranslational modification with fundamental impact on prostanoid biosynthesis in cell types which distinctly induce NOS-2. Since the lung is one of the first organs affected in sepsis, we tested whether the novel concept of nitrite-dependent nitration of PGHS-2 also applies in LPS-challenged rat alveolar macrophages. The potent bronchioconstrictor thromboxane A<sub>2</sub> (TxA<sub>2</sub>) was identified as the major prostanoid formed in a PGHS-2-dependent manner while the observed accumulation of nitrite nearly exclusively depended on the induction of NOS-2. Inhibition of •NO-formation in *ex-vivo* alveolar macrophages released the observed nitration and inhibition of PGHS-2-dependent TxA<sub>2</sub> generation. With respect to the situation in alveolar macrophages, the use of NOS-2 specific inhibitors could therefore indeed be useful to inhibit an exaggerated release of •NO, though it has to be considered that at the same time an endogenous regulatory mechanism terminating the release of bronchioconstrictive TxA<sub>2</sub> is also inhibited. In progressive stages of LPS-exposure to lung macrophages the ongoing release of TxA<sub>2</sub> may therefore outcompete the beneficial effects of an inhibited •NO synthesis. It has to be reminded that also in the lung species dependent variations seem to exist. As observed in vascular SMC, alveolar macrophages from rats explicitly induce NOS-2 in response to LPS whereas unpublished observations indicate a far less pronounced formation of •NO in cells obtained from humans (personal communication L. Wollin, F. Gantner).

## 5.6. Conclusions

The present work has highlighted two novel mechanisms involved in the regulation of prostanoid biosynthesis by the NO-synthase pathway. The key element affected by these interactions is prostaglandin endoperoxide H<sub>2</sub> synthase-2 (PGHS-2, COX-2) which is modulated by derivatives of nitric oxide ( $\bullet$ NO) in different ways.

- 1) **Peroxynitrite**, originating from the interaction of  $\bullet$ NO and  $\bullet$ O<sub>2</sub><sup>-</sup> was demonstrated to **activate PGHS-2** turnover in SMC by serving as a potent provider of the intracellular peroxide tone. Concentrations in the low nanomolar range were elaborated as sufficient for an activation of PGHS-2.
- 2) **Nitrite**, as the end product of  $\bullet$ NO, was demonstrated to **inhibit PGHS-2** by a self-catalyzed nitration of the enzyme in the presence of low peroxide levels. Nitrite concentrations above 10 micromolar were necessary to observe this inhibition which was competitive with arachidonate.

Since the two mechanisms are highly dependent on the intracellular concentrations of  $\bullet$ NO and  $\bullet$ O<sub>2</sub><sup>-</sup>, both interactions could in principle be observed within the same cell, however in a strictly defined sequence of events.

Human and bovine SMC upon exposure to LPS demonstrated only little formation of  $\bullet$ NO. These cells appear optimized for a sustained release of prostacyclin (PGI<sub>2</sub>) by allowing intracellular peroxynitrite levels in the low nanomolar range sufficient for the activation of PGHS-2, but inadequate for the nitration and inhibition of PGI<sub>2</sub>-synthase.

This is in contrast to the situation in LPS-exposed macrophages which release high amounts of  $\bullet$ NO by the induction of NOS-2. The peroxynitrite-mediated peroxide tone is therefore not

limiting PGHS-2 activity in these cells. However, nitrite levels in the range of 20-60 $\mu$ M can easily be reached which was found sufficient for an autocatalytic inhibition of PGHS-2.

Based on these new concepts, we not only can explain the events associated with LPS action on the vasculature, but also several diverging observations in literature on the interaction between the NOS and the PGHS pathways.

## 6. Summary

According to the aims of this thesis, the following results were obtained and published in five separate papers:

### 1. Induction of PGHS-2

- It was elaborated that vascular smooth muscle cells upon exposure to LPS induce PGHS-2 which supplies the constitutively expressed PGI<sub>2</sub>-synthase with substrate. The corresponding PGI<sub>2</sub> release compensates the loss of endothelial PGI<sub>2</sub>, caused by nitration in the first phase of LPS action, and the vasospasm elicited by endothelial PGH<sub>2</sub>.
- PGI<sub>2</sub>-synthase demonstrates no nitration and inhibition in smooth muscle cells which could be explained by the lack of NOS-2 induction and a high peroxidase activity.

### 2. Involvement of the “Peroxide Tone”

- Addition of the peroxynitrite-releasing compound SIN-1 resulted in a concentration-dependent upregulation of PGI<sub>2</sub> release.
- Peroxynitrite levels formed endogenously by smooth muscle cells were found sufficient for providing the peroxide tone for PGHS-2 but inadequate to nitrate and inhibit PGI<sub>2</sub>-synthase.
- Endogenous formation of  $\bullet\text{NO}$  or  $\bullet\text{O}_2^-$ , which together form peroxynitrite, was detected to occur in a nearly 1:1 ratio. Inhibition of either one radical resulted in a declined release of PGI<sub>2</sub>.

### 3. Species-dependent Mutual Induction of PGHS-2 or NOS-2

- Human and bovine vascular smooth muscle cells mainly induce PGHS-2 in response to LPS thus enabling the release of PGI<sub>2</sub>.
- In rat smooth muscle cells only a minimal induction of PGHS-2 was observed while NOS-2 was significantly upregulated to release •NO. Thus, rat vascular tissue is not an inflammatory model for human sepsis.

### 4. Nitration of PGHS-2 by Nitrite.

- LPS-challenged macrophages demonstrated induction of both PGHS-2 and NOS-2. Accumulated nitrite was found to be activated competitively to arachidonate by the heme catalytic site in presence of nanomolar peroxide levels.
- Nitration of PGHS-2 was catalyzed by the intrinsic peroxidase activity of PGHS-2.

### 5. Physiological Relevance of PGHS-2 Nitration

- Rat alveolar macrophages mainly release thromboxane A<sub>2</sub> in response to LPS which is nearly exclusively dependent on the induction of PGHS-2.
- Elevated levels of nitrite resulted in an autocatalytic inhibition of PGHS-2 and therefore terminate thromboxane A<sub>2</sub> formation.

## 7. Zusammenfassung

Neuere Arbeiten von Fitzgerald et al. haben gezeigt, daß das Gefäßendothel maßgeblich an der Freisetzung von Prostacyclin ( $\text{PGI}_2$ ) in gesunden Probanden beteiligt ist. Klinische Befunde deuten darauf hin, daß die Plasmaspiegel an 6-keto- $\text{PGF}_{1\alpha}$ , dem stabilen Hydrolyseprodukt von  $\text{PGI}_2$ , in septischen Patienten stark erhöht sind. Dies würde zunächst auf eine gesteigerte Freisetzung von  $\text{PGI}_2$  durch das Gefäßendothel hindeuten, jedoch haben frühere Arbeiten unserer Gruppe eine deutliche Nitrierung und Hemmung der endothelialen  $\text{PGI}_2$ -Synthase unter diesen Bedingungen dargelegt.

Frühere Beobachtungen durch Smith und DeWitt haben darauf hingewiesen, daß der vaskuläre Glattmuskel eine starke Expression der  $\text{PGI}_2$ -Synthase aufzeigt, jedoch im ruhenden Zustand nur eine marginale Cyclooxygenaseaktivität besitzt.

Als Ausgangspunkt für die vorliegende Arbeit ergab sich hieraus die Hypothese wonach der vaskuläre Glattmuskel durch die Induktion der Prostaglandin Endoperoxide  $\text{H}_2$  Synthase (PGHS-2) zur beobachteten Produktion von  $\text{PGI}_2$  beiträgt.

Die Ergebnisse dieser Arbeit können unter folgenden Teilaspekten diskutiert werden:

1. Induktion der PGHS-2 und  $\text{PGI}_2$ -Freisetzung durch Glattmuskelzellen.
2. Die Rolle des „Peroxid Tonus“ für die PGHS-2-abhängige  $\text{PGI}_2$  Produktion.
3. Spezies-Unterschiede in der Induktion von PGHS-2 und NO-Synthase-2 (NOS-2).
4. Nitrierung und Inhibition der PGHS-2 durch Nitrit in RAW 264.7 Makrophagen.
5. Physiologische Bedeutung der PGHS-2-Nitrierung in Alveolarmakrophagen.

Die wichtigsten Ergebnisse sind wie folgt zusammengefasst:

### 1. Induktion der PGHS-2

- Die konstitutiv exprimierte PGI<sub>2</sub>-Synthase wird durch die Endotoxin-stimulierte Induktion der PGHS-2 in einen funktionell aktiven Zustand überführt. Der vaskuläre Glattmuskel kann somit durch die Freisetzung von PGI<sub>2</sub> die Regulation der Gefäßhomeostase unter Bedingungen eines in seiner Funktion gestörten Endothels übernehmen.
- Aufgrund der nur sehr geringen Expression der NOS-2 und eines hohen zellulären Redoxpotentials findet im Glattmuskel keine Nitrierung der PGI<sub>2</sub>-Synthase statt.

### 2. Die Rolle des „Peroxid Tonus“

- Die Zugabe von Peroxynitrit-freisetzenden Substanzen wie SIN-1 führt in Glattmuskelzellen zu einer gesteigerten Produktion von PGI<sub>2</sub>.
- Peroxynitrit konnte als einer der potentesten endogenen Aktivatoren der PGHS-2 ausgemacht werden.
- Die intrazellulär gebildeten Spiegel von Peroxynitrit erlauben eine Aktivierung der PGHS-2, sind jedoch für eine Nitrierung der PGI<sub>2</sub>-Synthase nicht ausreichend.
- Die endogene Freisetzung von  $\cdot\text{NO}$  und  $\cdot\text{O}_2^-$ , welche zur Bildung von Peroxynitrit führt, verläuft nahezu im Verhältnis 1:1, wobei die Hemmung von  $\cdot\text{NO}$  oder  $\cdot\text{O}_2^-$  in einer reduzierten Freisetzung von PGI<sub>2</sub> resultiert.

### 3. Speziesabhängige Unterschiede in der Induktion von PGHS-2 oder NOS-2

- Vaskuläre Glattmuskelzellen des Menschen und des Rindes induzieren nach Behandlung mit LPS nahezu ausschließlich PGHS-2 und setzen dadurch PGI<sub>2</sub> frei.
- Im Gegensatz dazu kann bei der Ratte nur eine marginale Expression der PGHS-2 beobachtet werden, wohingegen hier eine signifikante Induktion der NOS-2 und eine gesteigerte Freisetzung von •NO zu beobachten ist.

### 4. Nitrierung der PGHS-2 durch Nitrit

- In LPS-behandelten RAW 264.7 Makrophagen kann sowohl eine Induktion der PGHS-2 als auch der NOS-2 gezeigt werden.
- Die endogene Peroxidaseaktivität der PGHS-2 kann in einem autokatalytischen Mechanismus zur Aktivierung von Nitrit und einer sich daran anschließenden Nitrierung und Hemmung der PGHS-2 führen.

### 5. Physiologische Bedeutung der PGHS-2-Nitrierung

- Alveolarmakrophagen der Ratte setzen nach Stimulation mit LPS Thromboxane A<sub>2</sub> (TxA<sub>2</sub>) frei, dessen Produktion nahezu ausschließlich an die Induktion der PGHS-2 gekoppelt ist
- Ein Anstieg der Nitritkonzentration führt zur autokatalytischen Nitrierung der PGHS-2 und somit zur Hemmung der TxA<sub>2</sub>-Freisetzung.

## 8. References

Abraham E., Wunderink R., Silverman H., Perl T.M., Nasraway S., Levy H., Bone R., Wenzel R.P., Balk R., Allred R. Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome: a randomized, controlled, double-blind, multicenter clinical trial. *JAMA* 1995; **273**: 934-941.

Abu-Soud H.M., Ichimori K., Presta A., and Stuehr D.J. Electron transfer, oxygen binding, and nitric oxide feedback inhibition in endothelial nitric-oxide synthase. *J. Biol. Chem.* 2000; **275**: 17349-17357.

Akarasereenont P., Mitchell J.A., Bakhle Y.S., Thiermann C., Vane J.R. Comparison of the induction of cyclooxygenase and nitric oxide synthase by endotoxin in endothelial cells and macrophages. *Eur. J. Pharmacol.* 1995; **273**: 121-128.

Akarasereenont P., Bakhle Y.S., Thiermann C., Vane J.R. Cytokine-mediated induction of cyclooxygenase-2 by activation of tyrosine kinase in bovine endothelial cells stimulated by bacterial lipopolysaccharide. *Brit. J. Pharmacol.* 1995; **115**: 401-408.

Alayash A., Ryan B.A., Cashion R.E. Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins. *Arch. Biochem. Biophys.* 1998; **349**: 65-73

Angus D.C., Linde-Zwirble W.T., Lidicker J., Clermont G., Carcillo J., Pinsky M.R. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* 2001; **29**: 1303-1310.

Arditi M., Zhou J., Dorio R., Rong G.W., Goyert S.M., and Kim K.S. Endotoxin-mediated endothelial cell injury and activation: role of soluble CD14. *Infect. Immun.* 1993; **61**: 3149-3156.

Babior B.M. NADPH Oxidase: An update. *Blood* 1999; **93**: 1464-1376.

Bachschnid M., Thureau S., Zou M.H., and Ullrich V. Endothelial cell activation by endotoxin involves superoxide/NO-mediated nitration of prostacyclin synthase and thromboxane receptor stimulation. *FASEB J.* 2003; **17(8)**: 914-916.

Bachschnid, M., Schildknecht, S., Heinz, K., Hamacher, J., Kavakli, C., Nusing, R., Daiber, A., and V. Ullrich. Autocatalytic tyrosine nitration of prostaglandin endoperoxide synthase-2 in LPS-stimulated RAW 264.7 macrophages.

Bachschnid M., Ullrich V. Redox signalling in endothelial cells. *BIF Futura* 2003; **18**: 223-230.

- Bagby R. Organization of contractile/ cytoskeletal elements. In "Biochemistry of Smooth Muscle " N.L. Stephens, ed. Vol. 1, pp1-84. 1983 CRC Press, Boca Raton, FL.
- Bakovic M., and Dunford H.B. Intimate relation between cyclooxygenase and peroxidase activities of prostaglandin H synthase. Peroxide reaction of ferulic acid and its influence on the reaction of arachidonuc acid. *Biochemistry* 1994; **33**: 6475-6482.
- Baldus S., Eiserich J.P., Brennan M.L., Jackson R.M., Alexander C.B., Freeman B.A. Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic. Biol. Med.* 2002; **33**: 1010
- Balsinde J., and Dennis E.A. Distinct roles in signal transduction for each of the phospholipase A2 enzymes present in P388D1 macrophages. *J. Biol. Chem.* 1996; **271**: 6758-6765.
- Balsinde J., Barbour S.E., Bianco I.D., and Dennis E.A. Arachidonic acid mobilization in P388D1 macrophages is controlled by two distinct Ca<sup>2+</sup>-dependent phospholipase A2 enzymes. *Proc. Natl. Acad. Sci. USA.* 1994; **91**: 11060-11064.
- Bannerman, D.D., and S.E. Goldblum. Direct effects of endotoxin on the endothelium: barrier function and injury. *Lab. Invest.* 1999; **79**: 1181-1199.
- Barnes P.J. New concepts in chronic obstructive pulmonary disease. *Annu. Rev. Med.* 2003; **54**: 113-129.
- Bates T.E., Loesch A., Burnstock G., and Clark J.B. Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. *Biochem. Biophys. Res. Commun.* 1995; **213**: 896-900.
- Beckam J.S., and Koppenol W.H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol.* 1996; **271**: C1424-C1437.
- Beckman J.S., Ye Y.Z., Anderson P.G., Chen J., Accavitti M.A., Tarpey M.M., and White C.R. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem.* 1994; **375**: 81-88.
- Berman R.S., Frew J.D., and Martin W. Endotoxin-induced arterial endothelial barrier dysfunction assessed by an in vitro model. *Br. J. Pharm.* 1993; **110**: 1282-1284.
- Beyer W., Imlay J., and Fridovich I. Superoxide dismutases. *Prog. Nucleic Acid Res. Mol. Biol.* 1991; **40**: 221-253.

- Bhagat K., Moss R., Collier J., and Vallance P. Endothelial “stunning” following a brief exposure to endotoxin: a mechanism to link infection and infarction? *Cardiovasc. Res.* 1996; **32**: 822-829.
- Bian K., Davis K., Kuret J., Binder L., Murad F. Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry. *Am. J. Physiol.* 1999; **277**: F33-F40.
- Bian K., Gao Z., Weisbrodt N., Murad F. The nature of heme/iron-induced protein tyrosine nitration. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 5712-5717.
- Bing R.J., and Lomnicka M. Why do cyclo-oxygenase-2 inhibitors cause cardiovascular events? *J. Am. Coll. Cardiol.* 2002; **39**: 521-522.
- Bishop-Bailey D., Hla T., and Mitchell J.A. Cyclo-oxygenase-2 in vascular smooth muscle. *Int. J. Mol. Med.* 1999; **3**: 41-48.
- Bishop-Bailey D., Larkin S.W., Warner T.D., Chen G., Mitchell J.A. Characterization of the induction of nitric oxide synthase and cyclo-oxygenase in rat aorta in organ culture, *Brit. J. Pharmacol.* 1997; **121**: 125-133.
- Bolotina V.M., Najibi S., Palacino J.J., Pagano P.J., and Cohen R.A. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 1994; **368**: 850-853.
- Bombardier C., Laine L., Reicin A., Shapiro D., Burgos-Vargas R., Davis B., Day R., Ferraz M.B., Hawkey C.J., Hochberg M.C., Kvien T.K., and Schnitzer T.J. Comparison of upper gastrointestinal toxicity of rofecoxib and naxoprofen in patients with rheumatoid arthritis. VIGOR Study Group. *N. Engl. J. Med.* 2000; **343**: 1520-1528.
- Bone R.C. The pathogenesis of sepsis. *Ann. Intern. Med.* 1991; **115**: 457-469.
- Bone R.C., Balk R.A., Cerra F.B., Dellinger R.P., Fein A.M., Knaus W.A., Schein R.M., Sibbald W.J. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest* 1992; **101**: 1644-1655.
- Bone R.C., Fisher C.J., Clemmer T.P., Slotman G.J., Metz C.A., Balk R.A. A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock. *N. Engl. J. Med.* 1987; **317**: 653-658.
- Boulanger C.M., Heymes C., Benessiano J., Geske R.S., Levy B.I., and Vanhoutte P.M. Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by Angiotensin II in hypertension. *Circ. Res.* 1998; **83**: 1271-1278

- Boulos, C., Jiang, H., and M. Balazy. Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues. *J. Pharmacol. Exp. Ther.* 2000; **293**: 222-229.
- Bourassa J.L., Ives E.P., Marqueling A.L., Shimanovich R, Groves J.T. Myoglobin catalyzes its own nitration. *J. Am. Chem. Soc.* 2001; **123**: 5142-5143
- Bredt, D.S., and Snyder, S.H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* 1990; **87**: 682-685.
- Breyer, R.M., Bagdassarian, C.K., Myers, S.A., and M.D. Breyer. Prostanoid receptors: subtypes and signaling. *Annu. Rev. Pharmacol. Toxicol.* 2001; **41**: 661-690.
- Brigham K.L., and Meyrick B. Endotoxin and lung injury. *Am. Rev. Respir. Dis.* 1986; **133**: 913-927.
- Brock T.G., McNish R.W., and Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E<sub>2</sub>. *J. Biol. Chem.* 1999; **274**: 11660-11666.
- Brophy C.M., Knoepp L., Xin J., and Pollock J.S. Functional expression of NOS-1 in vascular smooth muscle. *Am. J. Physiol.* 2000; **278**: H991-H997
- Brown G.C., and Cooper C.E. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* 1994; **356**: 295-298.
- Buckley B.J., Kent R.S., and Whorton R. Regulation of endothelial cell prostaglandin synthesis by glutathione. *J. Biol. Chem.* 1991; **266**: 16659-16666.
- Bult H., Beetens U., and Herman A.G. Blood levels of 6-oxo-prostaglandin F<sub>1α</sub> during endotoxin-induced hypotension in rabbits. *Eur. J. Pharmacol.* 1980; **63**: 47-56.
- Buttery L.D., Springall D.R., Chester A.H., Evans T.J., Stanfield E.N., Parums D.V., Yacoub M.H., and Polak J.M. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab. Invest.* 1996; **75**: 77-85.
- Camper S.A., Yao Y.A., and Rottman F.M. Hormonal regulation of the bovine prolactin promoter in rat pituitary tumor cells. *J. Biol. Chem.* 1985; **260**: 12246-12251.
- Capdevila J.H., Morrow J.D., Belosludtsev Y.Y., Beauchamp D.R., DuBois R.N., and Falck J.R. The catalytic outcomes of the constitutive and the mitogen inducible isoforms of prostaglandin H<sub>2</sub> synthase are markedly affected by glutathione and glutathione peroxidase(s). *Biochemistry* 1995; **34**, 3325-3337

- Cassina A.M., Hodara R., Souza J.M., Thomson L., Castro L., Ischiropoulos H., Freeman B.A., Radi R. Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* 2000; **275**: 21409-21415
- Catella-Lawson F., Reilly M.P., Kapoor S.C., Cucchiara A.J., DeMarco S., Tournier B., Vyas S.N., and Fitzgerald G.A. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *N. Engl. J. of Med.* 2001; **345**: 1809-1817.
- Caughey G.E., Cleland L.G., Penglis P.S., Galmbly J.R., James M.J. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. *J. Immunol.* 2001; **167**: 2831-2838.
- Chandrasekharan N.V., Dai H., Roos K.L., Evanson N.K., Tomsik J., Elton T.S., and Simmons D.L. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/ antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci.* 2002; **99**: 13926-13931.
- Chauhan S.D., Seggara G., Vo P.A., Macallister R.J., Hobbs A.J., Ahluwalia A. Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. *FASEB J.* 2003; **17**: 773-775.
- Chen W., Pawelek T.R., and Kulmacz R.J. Hydroperoxide dependence and cooperative cyclooxygenase kinetics in prostaglandin H synthase-1 and -2. *J. Biol. Chem.* 1999; **274**: 20301-20306
- Chenevard R., Hürlimann D., Bechir M., Enseleit F., Spieker L., Hermann M., Riesen W., Gay S., Gay R., Neidhart M., Michel B., Lüscher T., Noll T., Ruscitzka F. Selective COX-2 inhibition improves endothelial function in coronary artery disease. *Circulation* 2003; **107**: 405-409.
- Cheng Y., Austin S.C., Rocca B., Koller B.H., Coffman T.M., Grosser T., Lawson J.A., FitzGerald G.A. Role of prostacyclin in the cardiovascular response to thromboxane A<sub>2</sub>. *Science* 2002; **296**: 539-541.
- Chilton F.H., Hadley J.S., and Murphy R.C. Incorporation of arachidonic acid into 1-acyl-2-lyso-sn-glycero-3-phosphocholine of the human neutrophil. *Biochim. Biophys. Acta.* 1987; **917**: 48-56.
- Cines D.B., Pollak E.S., Buck C.A., Loscalzo J., Zimmerman G.A., McEver R.P., Pober J.S., Wick T.M., Konkle B.A., Schwartz B.S., et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 1998; **91**: 3527-3561.
- Clancy, R., Varenika, B., Huang, W., Ballou, L., Attur, M., Amin, A.R., and S.B. Abramson. Nitric oxide synthase/ COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. *J. Immunol.* 2000; **165**: 1582-1587.

- Clark J.D., Schievella A.R., Nalefski E.A., and Lin L.L. Cytosolic phospholipase A2. *J. Lipid Mediat. Cell. Signal.* 1995; **12**: 83-117.
- Cleland L.G. Oxy radicals, "peroxide tone" and inflammation. *J. Rheumatol.* 1984; **11**: 725-726.
- Coker S.J., Hughes B., Parratt J.R., Rodger I.W., and Zeitlin I.J. The release of prostanoids during the acute pulmonary response to E. coli endotoxin in anaesthetized cats. *Br. J. Pharmacol.* 1983; **78**: 561-570.
- Connolly E., Bouchier-Hayes D.J., Kaye E., Leaky A., Fitzgerald D., and Belton O. Cyclooxygenase isozyme expression and intimal hyperplasia in a rat model of balloon angioplasty. *J. Pharmacol. Exp. Ther.* 2002; **300**: 393-398.
- Conti M.A., and Adelstein R.S. The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5'cAMP-dependent protein kinase. *J. Biol. Chem.* 1981; **256**: 3178-3181.
- Cosentino F., Hishikawa K., Katusic Z.S. and Lüscher T.F. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 1997; **96**: 25-28.
- Crane B.R., Arvai A.S., Gachhui R., Wu C., Ghosh D.K., Getzoff E.D., Stuehr D.J., Trainer J.A. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* 1997; **278**: 425-431.
- Cudd A. and Fridovich I. Electrostatic interactions in the reaction mechanisms of bovine erythrocyte superoxide dismutase. *J. Biol. Chem.* 1982; **257**: 11443-11447.
- Cupillard L., Koumanov K., Mattei M.G., Lazdunski M., and Lambeau G. Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A2. *J. Biol. Chem.* 1997; **272**: 15745-15752.
- Curzen N.P., Griffiths M.J.D., and Evans T.W. Role of the endothelium in modulating the vascular response to sepsis. *Clin. Sci.* 1994; **86**: 359-374.
- D'Angelo D.D., Eubank J.J., Davis M.G., and Dorn G.W. Mutagenic analysis of platelet thromboxane receptor cysteines. *J. Biol. Chem.* 1996; **271**: 6233-6240.
- Daiber A., Frein D., Namgaladze D., and Ullrich V. Oxidation and nitrosation in the nitrogen monoxide/superoxide system. *J. Biol. Chem.* 2002; **277**: 11882-11888
- Daiber A., Herold S., Schoneich C., Namgaladze D., Peterson J.A., Ullrich V. Nitration and inactivation of cytochrome P450BM-3 by peroxynitrite. Stopped-flow measurements prove ferryl intermediates. *Eur. J. Biochem.* 2000; **267**: 6729-6739

- Daniel E.E., Jury J., Wang Y.F. nNOS in canine lower esophageal sphincter: colocalized with Cav-1 and Ca<sup>2+</sup>-handling proteins ? *Am. J. Physiol.* 2001; **281**: G1101-1114.
- Davenpeck K.L., Gauthier T.W., Albertine K.H., and Lefer A.M. Role of P-selectin in microvascular leukocyte-endothelial interaction in splanchnic ischemia-reperfusion. *Am. J. Physiol.* 1994; **267**: H622-H630.
- Davidge S.T., Baker P.N., Laughlin M.K., and Roberts J.M. Nitric oxide produced by endothelial cells increases production of eicosanoid through activation of prostaglandin H synthase. *Circ. Res.* 1995; **77**: 274-283
- Deeb, R.S., Resnick, M.J., Mittar, D., McCaffrey, T., Hajjar, D.P., and R.K. Upmacis. Tyrosine nitration in prostaglandin H(2) synthase. *J. Lipid Res.* 2002; **43**: 1718-1726.
- Deitch E.A. Animal models of sepsis and shock: a review and lessons learned. *Shock* 1998; **9**: 1-11.
- Dennis E.A. The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends. Biochem Sci.* 1997; **22**: 1-2.
- Dennis E.A., Diversity of group types, regulation, and function of phospholipase A2. *J. Biol. Chem.* 1994; **269**: 13057-13060.
- DeWitt D.L., and Smith W.L. Purification of prostacyclin synthase from bovine aorta by immunoaffinity chromatography. Evidence that the enzyme is a hemoprotein. *J. Biol. Chem.* 1983; **258**: 3285-3293.
- DeWitt D.L., Day J.S., Sonnenburg W.K., and Smith W.L. Concentrations of prostaglandin endoperoxide synthase and prostaglandin I<sub>2</sub> synthase in the endothelium and smooth muscle of bovine aorta. *J. Clin. Invest.* 1983; **72**: 1882-1886 .
- Dietz, R., Nastainczyk, W., and H.H. Ruf. Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G2. *Eur. J. Biochem.* 1988; **171**: 321-328.
- Domoki, F., Veltkamp, R., Thrikawala, N., Robins, G., Bari, F., Louis, T.M., and D.W. Busija. Ischemia-reperfusion rapidly increases COX-2 expression in piglet cerebral arteries. *Am. J. Physiol.* 1999; **277**: H1207-H1214.
- Doni M.G., Whittle B.J., Palmer R.M., Moncada S. Actions of nitric oxide on the release of prostacyclin from bovine endothelial cells in culture. *Eur. J. Pharmacol.* 1988; **151**: 19-25
- Dorn G.W. Cyclic oxidation-reduction reactions regulate thromboxane A2/prostaglandin H2 receptor number and affinity in human platelet membranes. *J. Biol. Chem.* 1990; **265**: 4240-4246.

- Doyle M.P. and Hoekstra J.W. Oxidation of nitrogen oxides by bound dioxygen in heme proteins. *J. Inorg. Biochem.* 1981; **14**: 351-358.
- Egan R.W., Paxton J., Kuehl F.A. Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J. Biol. Chem.* 1976; **251**: 7329-7335
- Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 1998; **391**: 393-397.
- Eldor A., Falcone D.J., Hajjar D.P., Minick C.R., and Weksler B.B. Recovery of prostacyclin production by de-endothelialized rabbit aorta: critical role of the neointimal smooth muscle cells. *J. Clin. Invest.* 1981; **67**: 735-74.
- Ermert, L., Ermert, M., Merkle, M., Goppelt-Struebe, M., Duncker, H.R., Grimminger, F., and W. Seeger. Rat pulmonary cyclooxygenase-2 expression in response to endotoxin challenge: differential regulation in the various types of cells in the lung. *Am. J. Pathol.* 2000; **156**: 1275-1287.
- Ersoy Y., Özerol E., Baysal Ö., Temel I., MacWalter R.S., Meral Ü., and Altay Z.A. Serum nitrate and nitrite levels in patients with rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis. *Ann. Rheum. Dis.* 2002; **61**: 76-78.
- Espey M.G., Xavier S., Thomas D.D., Miranda K.M., Wink D.A. Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proc Natl. Acad. Sci. USA* 2002; **99**: 3481-3486
- Euroth C., Eger B.R., Ohamoto K., Nishimo T., Nishimo T, and Pai E.F. Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. *Proc. Natl. Acad. Sci. U.S.A.* 2001; **97**: 10723-10728.
- Fierro I.M., and Serhan C.N. Mechanisms in anti-inflammation and resolution: the role of lipoxins and aspirin-triggered lipoxins. *Brazilian J. Med. Biol. Res.* 2001; **34**: 555-566.
- Fisher C.J., Agosti J.M., Opal S.M., Lowry S.F., Balk R.A., Sadoff J.C., Abraham E., Schein R.M., Benjamin E. Treatment of septic shock with the tumor necrosis factor receptor: Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. *N. Engl. J. Med.* 1996; **334**: 1697-1702.

- Fisher C.J., Slotman G.J., Opal S.M., Pribble J.P., Bone R.C., Emmanuel G., Ng D., Bloedow D.C., Catalano M.A. Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: a randomized, open-label, placebo-controlled multicenter trial. *Crit. Care Med.* 1994; **22**: 12-21.
- Fisslthaler B., Popp R., Kiss L., Potente M., Harder D.R., Fleming I., Busse R. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999; **401**: 493-497.
- FitzGerald G.A. Mechanisms of platelet activation: thromboxane A<sub>2</sub> as an amplifying signal for other agonists. *Am. J. Cardiol.* 1991; **68**: 11B-15B.
- FitzGerald G.A. Coxibs and cardiovascular disease. *N. Engl. J. Med.* 2004; **351**: 1709-1711.
- Fitzgerald G.A., Cheng Y., Austin S. COX-2 inhibitors and the cardiovascular system. *Clin. Exp. Rheumatol.* 2001; **19**: S31-S36.
- Fitzgerald G.A., Patrono C. The coxibs, selective inhibitors of cyclooxygenase-2. *N. Engl. J. Med.* 2001; **345**: 433-442.
- Fletcher J.R., and Ramwell P.W. Lidocaine or indomethacin improves survival in baboon endotoxic shock. *J. Surg. Res.* 1978; **24**: 154-160.
- Fletcher J.R., and Ramwell P.W. Modification by aspirin and indomethacin, of the hemodynamic and prostaglandin releasing effects of E. coli endotoxin in the dog. *Br. J. Pharmacol.* 1977; **61**: 175-181.
- Ford P.C., Wink D.A., and Stanbury D.M. Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* 1993; **326**: 1-3.
- Francis S.H., Noblett B.D., Todd B.W., Wells J.N., and Corbin J.D. Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol. Pharmacol.* 1988; **34**: 506-517.
- Freedland R.A., Crozier G.L., Hicks B.L., and Meijer A.J. Arginine uptake by isolated rat liver mitochondria. *Biochim. Biophys. Acta.* 1984; **802**: 407-412.
- Frey E.A., Miller D.S., Jahr T.G., Sundan A., Bazil V., Espevik T., Finlay B.B., Wright S.D. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med.* 1992; **176**: 1665-1671.
- Fridovich I. Mitochondria: are they the seat of senescence ? *Aging Cell* 2004; **3**: 13-16.

- Fries D.M., Paxinou E., Themistocleous M., Swanberg E., Griending K.K., Salvemini D., Slot J.W., Heijnen H.F., Hazen S.L., Ischiropoulos H. Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species. *J. Biol. Chem.* 2003; **278**: 22901-22907.
- Fu J.Y., Masferrer J.L., Seibert K., Raz A., and Needleman P. J. The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *Biol. Chem.* 1990; **265**: 16737-16740.
- Furchgott R.F. Role of endothelium in responses of vascular smooth muscle. *Circ. Res.* 1983; **53**: 557-573.
- Furchgott R.F., and Zawadzki J.V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; **288**: 373-376.
- Furchgott R.F., Cherry P.D., Zawadzki J.V., and Jothianandan D. Endothelial cells as mediators of vasodilation of arteries. *J. Cardiovasc. Pharmacol.* 1984; **6**: S336-S343.
- Galinanes M., Matata B.M. Protein nitration is predominantly mediated by a peroxynitrite-dependent pathway in cultured human leucocytes. *Biochem. J.* 2002; **367**: 467-473
- Garavito R.M. and DeWitt D.L. The cyclooxygenase isoforms: structural insights into the conversion of arachidonic acid to prostaglandins. *Biochim. Biophys. Acta* 1999; **1441**: 278-287.
- Garavito R.M., Mulichak A.M. The structure of mammalian cyclooxygenases. *Annu. Rev. Biophys. Biomol. Struct.* 2003; **32**: 183-206.
- Garcia-Cardena G., Fan R., Stern D.F., Liu J., Sessa W.C. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* 1996; **271**: 27237-27240.
- Garthwaite J., Charles S.L., and Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 1988; **336**: 385-388.
- Gaut J.P., Byun J., Tran H.D., Lauber W.M., Carroll J.A., Hotchkiss R.S., Belaaouaj A., Heinecke J.W. Myeloperoxidase produces nitrating oxidants in vivo. *J. Clin. Invest.* 2002; **109**: 1311-1319.
- Gaynor E., Bouvier C., and Spaet T.H. Vascular lesions: possible pathogenetic basis of the generalized Schwartzman reaction. *Science* 1970; **170**: 986-988.
- Ghafourifar P., and Richter C. Nitric oxide synthase activity in mitochondria. *FEBS Lett.* 1997; **418**: 291-296.

- Giulivi C., Poderoso J.J., and Boveris A. Production of nitric oxide by mitochondria. *J. Biol. Chem.* 1998; **273**: 11038-11043.
- Goldblum S.E., Ding X., Brann T.W., and Campbell-Washington J. Bacterial lipopolysaccharide induces actin reorganization, intercellular gap formation, and endothelial barrier dysfunction in pulmonary vascular endothelial cells: concurrent F-actin depolymerization and new actin synthesis. *J. Cell Physiol.* 1993; **157**: 13-23.
- Goldstein S., Czapski G., Lind J., and Merenyi G. Tyrosine nitration by simultaneous generation of NO and O<sub>2</sub><sup>-</sup> under physiological conditions. HOW THE RADICALS DO THE JOB. *J. Biol. Chem.* 2000; **275**: 3031-3036
- Goodwin, D.C., Gunther, M.R., Hsi, L.C., Crews, B.C., Eling, T.E., Mason, R.P., and L.J. Marnett. Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. *J. Biol. Chem.* 1998; **273**: 8903-8909.
- Goodwin, D.C., Landino, L.M., and L.J. Marnett. Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. *FASEB J.* 1999; **13**: 1121-1136.
- Gorman R.R., Hamilton R.D., and Hopkins N.K. Stimulation of human foreskin fibroblasts adenosine 3', 5'-cyclic monophosphate levels by prostacyclin (prostaglandin I<sub>2</sub>). *J. Biol. Chem.* 1979; **254**: 1671-1676.
- Green L.C., Tannenbaum S.R., and Goldman P. Nitrate synthesis in the germfree and conventional rat. *Science* 1981; **212**: 56-58.
- Greenman R.L., Schein R.M., Martin M.A., et al. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. The XOMA Sepsis Study Group. *JAMA* 1991; **266**: 1097-1102.
- Griendlin K.K., Sorescu D., Lassegue B., Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler. Thromb. Vasc. Biol.* 2000; **20**: 2175-2183.
- Griendling K.K., and Ushio-Fukai M. NADH/NADPH oxidase and vascular function. *Trends Cardiovasc. Med.* 1997; **7**: 301-307.
- Griendling K.K., and Ushio-Fukai M. Redox control of vascular smooth muscle proliferation. *J. Lab. Clin. Med.* 1998; **132**: 9-15.
- Griendling K.K., Minieri C.A., Ollerenshaw J.D., and Alexander R.W. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.* 1994; **74**: 1114-1148.

- Griendling K.K., Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* 2000; **86**: 494-501.
- Griffith O.W., and Stuehr D.J. Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* 1995; **57**: 707-736.
- Griffiths M.J., Liu S., Curzen N.P., Messent M., and Evans T.M. In vivo treatment with endotoxin induces nitric oxide synthase in rat main pulmonary artery. *Am. J. Physiol.* 1995; **268**: L509-L518.
- Grisham M.B., Jourdain D., Wink D.A. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am. J. Physiol.* 1999; **276**: G315-G321.
- Gruetter C.A., Barry B.K., McNamara D.B., Gruetter D.Y., Kadowitz P.J., Ignarro L. Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosamine. *J. Cyclic Nucleotide Res.* 1979; **5**: 224.
- Gumina R.J., Newman P.J., Kenny D., Warltier D.C., Gross G.J. The leukocyte cell adhesion cascade and its role in myocardial ischemia-reperfusion injury. *Basic Res. Cardiol.* 1997; **92**: 201-213.
- Gunther M.R., Hsi L.C., Curtis J.F., Gierse J.K., Marnett L.J., Eling T.E., and Mason R.P. Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *J. Biol. Chem.* 1997; **272**: 17086-17090
- Habib A. Bernard C., Leuret M., Creminon C., Esposito B., Tedgui A., Maclouf J. Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J. Immunol.* 1997; **158**: 3845-3851.
- Hailer N.P., Oppermann E., Leckel K., Cinatl J., Markus B.H., and Blatheta R.A. Prostaglandin E2 induces expression of P-selection (CD62P) on cultured human umbilical vein endothelial cells and enhances endothelial binding of CD4-T-cells. *Transplantation* 2000; **70**: 236-240.
- Halushka P.V., Reines H.D., Barrow S.E., Blair I.A., Dollery C.T., Rambo W., Cook J.A., and Wise W.C. Elevated plasma 6-keto-prostaglandin F 1 alpha in patients in septic shock. *Crit. Care Med.* 1985; **13**: 451-453.
- Hamaguchi M., Ishibashi T., and Imai S. Involvement of charybdotoxin-sensitive K<sup>+</sup> channel in the relaxation of bovine tracheal smooth muscle by glyceryl trinitrate and sodium nitroprusside. *J. Pharmacol. Exp. Ther.* 1992; **262**: 263-270.

- Hamberg M. and Samuelsson B. Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 1973; **70**: 899-903.
- Hamberg M., Svensson J., Wakabayashi T. and Samuelsson B. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 1974; **71**: 345-349.
- Harris R.C., McKauna J.A., Akai Y., Jacobson H.R., Dubois R.N., and Breyer M.D. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J. Clin. Invest.* 1994; **94**: 2504-2510.
- Hatare O., Tokuda M., Itano T., Matsui H., and Doi A. Purification and characterization of calmodulin from rat liver mitochondria. *Biochem. Biophys. Res. Commun.* 1982; **104**: 673-679.
- Hemler M., and Lands W.E.M. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J. Biol. Chem.* 1976; **251**: 5575-5579
- Hempel, S.L., Monick, M.M., and G.W. Hunninghake. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.* 1994; **93**: 391-396.
- Hempel, S.L., Monick, M.M., He, B., Yano, T., and G.W. Hunninghake. Synthesis of prostaglandin H synthase-2 by human alveolar macrophages in response to lipopolysaccharide is inhibited by decreased cell oxidant tone. *J. Biol. Chem.* 1994; **269**: 32979-32984.
- Herold S., Rehmann F. Kinetic and mechanistic studies of the reactions of nitrogen monoxide and nitrite with ferryl myoglobin. *J. Biol. Inorg. Chem.* 2001; **6**: 543-555
- Hevel J.M., White K.A., and Marletta M.A. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* 1991; **266**: 22789-22791.
- Hille R., and Nishino T. Xanthine oxidase and xanthine dehydrogenase. *FASEB J.* 1995; **9**: 995-1003.
- Hink U., Li H., Mollman H., Oelze M. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ. Res.* 2001; **88**: F14-F22.
- Hirata, T., Ukawa, H., Yamakuni, H., Kato, S., and K. Takeuchi. Cyclo-oxygenase isozymes in mucosal ulcerogenic and functional responses following barrier disruption in rat stomachs. *Br. J. Pharmacol.* 1997; **122**: 447-454.

Hotchkiss R.S., Swanson P.E., Freeman B.D., Tinsley K.W., Cobb J.P., Matuschak G.M., Buchman T.G., Karl I.E. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit. Care Med.* 1999; **27**: 1230-1251.

Hsi L.C., Hoganson C.W., Babcock G.T., Smith W.L. Characterization of a tyrosyl radical in prostaglandin endoperoxide synthase-2. *Biochem. Biophys. Res. Commun.* 1994; **202**: 1592-1598.

Huie R.E. and Padmaja S. The reaction rate of nitric oxide with superoxide. *Free Radical Res. Commun.* 1993; **18**: 195-199.

Husain S., Andrews N., Mulcahy D., Panza J.A., Quyyumi A.A. Aspirin improves endothelial dysfunction in atherosclerosis. *Circulation* 1998; **97**: 716-720.

Ichida K., Amaya Y., Noda K., Minoshima S., Hosoya T., Sakai O., Shimizu N. and Nishino T. Cloning of the cDNA encoding human xanthine dehydrogenase (oxidase): structural analysis of the protein and chromosomal location of the gene. *Gene* 1993; **133**: 279-284.

Ignarro L.J. Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. *Blood Vessels* 1991; **28**: 67-73.

Ignarro L.J., Buga G.M., Wood K.S., Byrns R.E., and Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* 1987; **84**: 9265-9269.

Imlay J.A., and Fridovich I. Assay of superoxide production in Escherichia coli. *J. Biol. Chem.* 1991; **266**: 6957-6965.

Inoue H., Taba Y., Miwa Y., Yokota C., Miyagi M., Sasaguri T. Transcriptional and posttranscriptional regulation of cyclooxygenase-2 expression by fluid shear stress in vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 2002; **22**: 1415-1420.

Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* 1998; **356**: 1-11.

Ishii Y., Shuyi W., and Kitamura S. Soluble CD14 in serum mediates LPS-induced increase in permeability of bovine pulmonary arterial endothelial cell monolayers in vitro. *Life Sci.* 1995; **56**: 2263-2272.

Jeffcoat R. The biosynthesis of unsaturated fatty acids and its control in mammalian liver. *Essays Biochem.* 1979; **15**: 1-36.

- Johnson R.M., and Lincoln T.M. Effects of nitroprusside, glyceryl trinitrate and 8-bromo cyclic GMP on phosphorylase a formation and myosin light chain phosphorylation in rat aorta. *Mol. Pharmacol.* 1985; **27**: 333-342.
- Jones O.T. The regulation of superoxide production by the NADPH oxidase of neutrophils and other mammalian cells. *Bioessays.* 1994; **16**: 919-923.
- Jourd'heuil D., Jourdeuil F.L., Kutchukian P.S., Musah R.A., Wink D.A., and Grisham M.B. Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo. *J. Biol. Chem.* 2001; **276**: 28799-28805
- Kalgutkar A.S., Crews B.C., and Rowlinson S.W. Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science* 1998; **280**: 1268-1270.
- Kamisaki Y., Wada K., Bian K., Balabanli B., Davis K., Martin E., Behod F., Lee Y.C., Murad F. An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 11584-11589.
- Kanai A., Peara L.L., Clemens P.R., Binder L.A., VanBibber M.M., Choi S.Y., de Groat W.C., and Peterson J. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. *Proc. Natl. Acad. Sci. U.S.A.* 2001; **98**: 14126-14131.
- Karthein R., Dietz R., Nastainczyk W. and Ruf H.H. Higher oxidation states of prostaglandin H synthase. EPR study of a transient tyrosyl radical in the enzyme during the peroxidase reaction. *Eur. J. Biochem.* 1988; **171**: 313-320.
- Katsuki S., Arnold W.P., and Murad F. Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J. Cyclic Nucleotide Res.* 1977; **3**: 239-247.
- Kilinc K., Kilinc A., Wolf R.E., Grisham M.B. Myoglobin-catalyzed tyrosine nitration: no need for peroxynitrite. *Biochem. Biophys. Res. Comm.* 2001; **285**: 273-276
- Knowles R.G., and Moncada S. Nitric oxide synthases in mammals. *Biochem. J.* 1994; **298**: 249-258.
- Koppenol W.H., Moreno J.J., Pryor W.A., Ischiropoulos H., and Beckman J.S. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* 1992; **5**: 834-842.
- Krippeit-Drews P., Morel N., Godfraind T. J. Effect of nitric oxide on membrane potential and contraction of rat aorta. *Cardiovasc. Pharmacol.* 1992; **20**: S72-S75.

- Kujubu D.A., Fletcher B.S., Varnum B.C., Lim R.W., and Herschman H.R. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* 1991; **266**: 12866-12872.
- Kulmacz, R.J. (1998) Cellular regulation of prostaglandin H synthase catalysis. *FEBS Lett.* **430**, 154-157
- Kulmacz, R.J., and L.H. Wang. Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2. *J. Biol. Chem.* 1995; **270**: 24019-24023.
- Kulmacz, R.J., Pendleton, R.B., and W.E. Lands. Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics. *J. Biol. Chem.* 1994; **269**: 5527-5536.
- Kurumbail R.G., Stevens A.M., Gierse J.K., McDonald J.J., Stegeman R.A., Pak J.Y., Gildehaus D., Miyashiro J.M., Penning T.D., Seibert K., Isakson P.C., Stallings W.C. Structural basis for selective inhibitors of cyclooxygenase-2 by anti-inflammatory agents. *Nature* 1996; **384**: 644-648.
- Kuwata H., Nakatani Y., Murakami M., and Kudo I. Cytosolic phospholipase A2 is required for cytokine-induced expression of type IIA secretory phospholipase A2 that mediates optimal cyclooxygenase-2-dependent delayed prostaglandin E2 generation in rat 3Y1 fibroblasts. *J. Biol. Chem.* 1998; **273**: 1733-1740
- Kuzkaya N., Weissmann N., Harrison D.G., Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols. *J. Biol. Chem.* 2003; **278**: 22546-22554.
- Kwon N.S., Stuehr D.H., and Nathan C.F. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* 1991; **174**: 761-767.
- Lambeir A.M., Markey C.M., Dunford H.B., and Marnett L.J. Spectral properties of the higher oxidation states of prostaglandin H synthase. *J. Biol. Chem.* 1985; **260**: 14894-14896.
- Landino L.M., Crews B.C., Gierse J.K., Hauser S.D., and Marnett L.J. Mutational analysis of the role of the distal histidine and glutamine residues of prostaglandin-endoperoxide synthase-2 in peroxidase catalysis, hydroperoxide reduction, and cyclooxygenase activation. *J. Biol. Chem.* 1997; **272**: 21565-21574
- Landino L.M., Crews B.C., Timmons M.D., Morrow J.D., and Marnett L.J. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 1996; **93**: 15069-15074
- Langenbach, R., Morham, S.G., Tian, H.F., Loftin, C.D., Ghanayem, B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D. et al. Prostaglandin synthase 1 gene disruption in mice reduces

arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* 1995; **83**: 483-492.

Laskin D.L., Pendino K.J. Macrophages and inflammatory mediators in tissue injury. *Annu. Rev. Pharmacol. Toxicol.* 1995; **35**: 655-677.

Lecomte M., Laneuville O., Ji C., DeWitt D.L., and Smith W.L. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.* 1994; **269**: 13207-13215.

Lederer J.A., Rodrick M.L., and Mannick J.A. The effects of injury on the adaptive immune response. *Shock* 1999; **11**: 153-159.

Leslie C.C. Properties and regulation of cytosolic phospholipase A2. *J. Biol. Chem.* 1997; **272**: 16709-16712.

Lin C.C., Hsiao L.D., Chien C.S., Lee C.W., Hsieh J.T., Yang C.M. Tumor necrosis factor-alpha-induced cyclooxygenase-2 expression in human tracheal smooth muscle cells: involvement of p42/p44 and p38 mitogen-activated protein kinases and nuclear factor-kappaB. *Cell Signal.* 2004; **16**: 597-607.

Linares E., Giorgio S., Mortara R.A., Santos C.X., Yamada A.T., and Augusto O. Role of peroxynitrite in macrophage microbicidal mechanisms in vivo revealed by protein nitration and hydroxylation. *Free Rad. Biol. Med.* 2001; **30**: 1234-1242.

Lincoln T.M. Effects of nitroprusside and 8-bromo-cyclic GMP on the contractile activity of the rat aorta. *J. Pharmacol. Exp. Ther.* 1983; **224**: 100-107.

Lincoln T.M., Cornwell T.L., and Taylor A.E. cGMP-dependent protein kinase mediates the reduction of  $Ca^{2+}$  by cAMP in vascular smooth muscle cells. *Am. J. Physiol.* 1990; **258**: C399-C407.

Liou J.Y., Deng W.G., Gilroy D.W., Shyue S.K., Wu K.K. Colocalization and interaction of cyclooxygenase-2 with caveolin-1 in human fibroblasts. *J. Biol. Chem.* 2001; **276**:34975-34982

Liou, J.Y., Shyue, S.K., Tsai, M.J., Chung, C.L., Chu, K.Y., and K.K. Wu. Colocalization of prostacyclin synthase with prostaglandin H synthase-1 (PGHS-1) but not phorbol ester-induced PGHS-2 in cultured endothelial cells. *J. Biol. Chem.* 2000; **275**: 15314-15320.

Liu J., Garcia-Cardena G., Sessa W.C. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry* 1996; **35**: 13277-13281.

- Lombry, C., Edwards, D.A., Preat, V., and R. Vanbever. Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules. *Am. J. Physiol.* 2004; **286**: L1002-1008.
- Loschen G., Azzi A., and Flohe L. Mitochondrial H<sub>2</sub>O<sub>2</sub> formation: relationship with energy conservation. *FEBS Lett.* 1973; **33**: 84-88.
- Loschen G., Azzi A., Richter C., and Flohe L. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Lett.* 1974; **42**: 68-72.
- Lu G., Tsai A.L., Van Wart H.E., and Kulmacz R.J. Comparison of the peroxidase reaction kinetics of prostaglandin H synthase -1 and -2. *J. Biol. Chem.* 1999; **274**: 16162-16167.
- Luong C., Miller A., Barnett J., Chow J., Ramesha C., Browner M.F. Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat. Struct. Biol.* 1996; **3**: 927-933.
- MacMillan-Crow L.A., Thompson J.A. Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (Y34F) by peroxynitrite. *Arch. Biochem. Biophys.* 1999; **366**: 82-88.
- Maddox, L., and D.A. Schwartz. The pathophysiology of asthma. *Annu. Rev. Med.* 2002; **53**: 477-498.
- Maeda K., Abello P.A., Abraham M.R., Wetzel R.C., Robotham J.L., and Buchman T.G. Endotoxin induces organ-specific endothelial cell injury. *Shock* 1995; **3**: 46-50.
- Mais D.E., Saussy D.L., Chaihouni A., Kochel P.J., Knapp D.R., Hamanaka N., and Halushka P.V. Pharmacological characterization of human and canine thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub> receptors in platelets and blood vessels: evidence for different receptors. *J. Pharmacol. Exp. Ther.* 1985; **233**: 418-424.
- Malinski T., Bailey F., Zhang Z.G., and Chopp M. Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion: *J. Cereb. Blood Flow Metab.* 1993; **13**: 355-358.
- Manku M.S., Horrobin D.F., Huang Y.S., and Morse N. Fatty acids in plasma and red cell membranes in normal humans. *Lipids* 1983; **18**: 906-908.
- Marcus A.J., Weksler B.B., Jaffe E.A., Broekman M.J. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. *J. Clin. Invest.* 1980; **66**: 979-986.
- Margalit A., Hauser S.D., Zweifel B.S., Anderson M.A., and Isakson P.C. Regulation of prostaglandin biosynthesis in vivo by glutathione. *Am J Physiol.* 1998; **274**: R294-R302

- Markey C.M., Alward A., Weller P.E., and Marnett L.J. Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. Reducing substrate specificity and the relationship of peroxidase and cyclooxygenase activities. *J. Biol. Chem.* 1987; **262**: 6266-6279
- Marletta M.A. Nitric oxide synthase: function and mechanism. *Adv. Exp. Med. Biol.* 1993; **338**: 281-284.
- Marnett L.J. Cyclooxygenase mechanisms. *Curr. Opin. Chem. Biol.* 2000; **4**: 545-552.
- Marnett L.J., Rowlinson S.W., Goodwin D.C., Kalgutkar A.S., and Lanzo C.A. Arachidonic acid and oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J. Biol. Chem.* 1999; **274**: 22903-22906.
- Marotta P., Sautebin L., and DiRosa M. Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774. *Br. J. Pharm.* 1992; **107**: 640-641.
- Marshall P.J., Kulmacz R.J., and Lands W.E.M. Constraints on prostaglandin biosynthesis in tissues. *J. Biol. Chem.* 1987; **262**: 3510-3517
- Martin M.A. Epidemiology and clinical impact of gram-negative sepsis. *Infect. Dis. Clin. North Am.* 1991; **5**: 739-752.
- Masferrer J.L., Zweifel B.S., Manning P.T., Hauser S.D., Leaky K.M., Smith W.G., Isakson P.C., and Seibert K. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci.* 1994; **91**: 3228-3232.
- Matata B.M., Galinanes M. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor- $\kappa$ B DNA binding activity. *J. Biol. Chem.* 2002; **277**: 2330-2335.
- Matoba T., Shimokawa H., Morikawa K., Kubota H., Kunihiro I., Urakami-Harasawa L., et al. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler. Thromb. Vasc. Biol.* 2003; **23**: 1224-1230.
- Matthews J.S., McWilliams P.J., Key B.J., and Keen M. Inhibition of prostacyclin release from cultured endothelial cells by nitrovasodilator drugs. *Biochim. Biophys. Acta.* 1995; **1269**: 237-242
- McAdam B.F., Catella-Lawson F., Mardini I.A., Kapoor S., Lawson J.A., FitzGerald G.A. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. *Proc. Natl. Acad. Sci. USA* 1999; **96**: 272-277.

- McCord J.M. and Fridovich I. Superoxide dismutase: An enzyme function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* 1969; **244**: 6049-6055.
- McCord J.M. Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* 1985; **312**: 159-163.
- McCord J.M., and Fridovich I. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* 1968; **243**: 5753-5760.
- McCormack J.G., and Browne H.M. Studies on mitochondrial Ca<sup>2+</sup> transport and matrix Ca<sup>2+</sup> using fura-2-loaded rat heart mitochondria. *Biochim. Biophys. Acta.* 1989; **973**: 420-427.
- Meneshian A., and Bulkley G.B. The physiology of endothelial xanthine oxidase: from urate catabolism to reperfusion injury to inflammatory signal transduction. *Microcirculation* 2002; **9**: 161-175.
- Meyrick B.O., Ryan U.S., and Brigham K.L. Direct effects of E. coli endotoxin on structure and permeability of pulmonary endothelial monolayers and the endothelial layer of intimal explants. *Am. J. Pathol.* 1986; **122**: 140-151.
- Mitaka C., Hirata Y., Yokoyama K., Wakimoto H., Hirokawa M., Nosaka T., Imai T. Relationships of circulatory nitrite/nitrate levels to severity and multiple organ dysfunction syndrome in systemic inflammatory response syndrome. *Shock* 2003; **19**: 305-309.
- Miyamoto T., Ogino N., Yamamoto S., and Hayaishi O. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* 1976; **251**: 2629-2636
- Moncada S., and Vane J.R. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A<sub>2</sub> and prostacyclin. *Pharmacol. Rev.* 1978; **30**: 293-331.
- Moncada S., Gryglewski R.J., Bunting S., and Vane J.R. A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance (prostaglandin X) which prevents platelet aggregation. *Prostaglandins* 1976; **12(5)**: 715-737
- Moncada S., Vane J.R. Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. *N. Engl. J. Med.* 1979; **300**: 1142-1147.
- Montesano R., Orci L., and Vassalli P. Human endothelial cell cultures: phenotypic modulation by leukocyte interleukins. *J. Cell Physiol.* 1985; **122**: 424-434.
- Morita I. Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid. Mediat.* 2002; **68**: 165-175.

- Mukherjee D. Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. *Biochem. Pharmacol.* 2002; **63**: 817-821.
- Mukherjee D., Nissen S.E., and Topol E.J. Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA* 2001; **286**: 954-959.
- Murray R., Shipp L., FitzGerald G.A. Prostaglandin endoperoxide/thromboxane A<sub>2</sub> receptor desensitization. Cross-talk with adenylate cyclase in human platelets. *J. Biol. Chem.* 1990; **265**: 21670-21675.
- Muscara M.N., Vergnolle N., Lovren F., et al. Selective cyclo-oxygenase-2 inhibition with celecoxib elevates blood pressure and promotes leukocyte adherence. *Br. J. Pharmacol.* 2000; **129**: 1423-1430.
- Nakagawa O., Tanaka I., Usui T., Harada M., Sasaki Y., Itoh H., Yoshimasa T., Namba T., Narumiya S., and Nakao K. Molecular cloning of human prostacyclin receptor cDNA and its gene expression in the cardiovascular system. *Circulation* 1994; **90**: 1643-1647.
- Naraba H., Murakami M., Matsumoto H., Shimbara S., Ueno A., Kudo I., Oh-ishi S. Segregated coupling of phospholipases A<sub>2</sub>, cyclooxygenases, and terminal prostanoid synthases in different phases of prostanoid biosynthesis in rat peritoneal macrophages. *J. Immunol.* 1998; **160**: 2974-2982.
- Nathan C.F. Secretory products of macrophages. *J. Clin. Invest.* 1987; **79**: 319-326.
- Neubert K., Haberland A., Kruse I., Wirth M., and Schimke I. The ratio of formation of prostacyclin/thromboxane A<sub>2</sub> in HUVEC decreased in each subsequent passage. *Prostaglandins* 1997; **54**: 447-462.
- Noll G., Luscher T.F. The endothelium in acute coronary syndromes. *Eur. Heart J.* 1998; **19**: C30-C38.
- Nordberg J., and Arner E.S. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Rad. Biol. Med.* 2001; **31**: 1287-1312
- O'Bannon M.K., Sadowski H.B., Winn V. and Young D.A. A serum -and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J. Biol. Chem.* 1991; **266**: 23261-23267.
- O'Sullivan, M.G., Chilton, F.H., Huggins, E.M., and C.E. McCall. Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J. Biol. Chem.* 1992; **267**: 14547-14550.
- Oberholzer A., Oberholzer C., and Moldawer L.L. Sepsis syndromes: understanding the role of innate and acquired immunity. *Shock* 2001; **16**: 83-96.

- Ogino N., Ohki S., Yamamoto S., Hayaishi O. Prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. Inactivation and activation by heme and other metalloporphyrins. *J. Biol. Chem.* 1978; **253**: 5061-5068.
- Ogletree M.L., Harris D.N., Greenberg R., Haslanger M.F., and Nakane M. Pharmacological actions of SQ 29,548, a novel selective thromboxane antagonist. *J. Pharmacol. Exp. Ther.* 1985; **234**: 435-441.
- Ohara Y., Peterson T.E., and Harrison D.G. Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* 1993; **91**: 2546-2551.
- Okahara K., Sun B., Kambayashi J. Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 1998; **18**: 1922-1926.
- Otto J.C. and Smith W.L. Photolabeling of prostaglandin endoperoxide H synthase-1 with 3-trifluoro-<sup>3</sup> (m[125I] iodophenyl) diazirine as a probe of membrane association and the cyclooxygenase active sites. *J. Biol. Chem.* 1996; **271**: 9906-9910.
- Otto J.C., and Smith W.L. Prostaglandin endoperoxide synthases -1 and -2. *J. Lipid Mediat. Cell Signal.* 1995; **12**: 139-156.
- Oury T.D., Day B.J., and Crapo J.D. Extracellular superoxide dismutase in vessels and airways of humans and baboons. *Free Rad. Biol. Med.* 1996; **20(7)**: 957-965.
- Pagano P.J., Chanock S.J., Siwik D.A., Colucci W.S., and Clark J.K. Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. *Hypertension* 1998; **32**: 331-337.
- Pagano P.J., Clark J.K., Cifuentes-Pagano M.E., Clark S.M., Callis G.M., and Quinn M.T. Localization of a constitutively active phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc. Natl. Acad. Sci. U.S.A.* 1997; **94**: 14438-14488.
- Pagano P.J., Tornheim K., and Cohen R.A. Superoxide anion production by rabbit thoracic aorta: effect of endothelium-derived nitric oxide. *Am. J. Physiol.* 1993; **265**: H707-H712.
- Palmer R.M., Ferrige A.G., and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; **327**: 524-526.
- Parente L., and Perretti M. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem. Pharmacol.* 2003; **65**: 153-159.

- Parks D.A., and Granger D.N. Xanthine oxidase: biochemistry, distribution and physiology. *Acta Physiol. Scand.* 1986; Suppl. **548**: 87-99.
- Parrillo J.E. Pathogenic mechanisms of septic shock. *N. Engl. J. Med.* 1993; **328**: 1471-1477.
- Pasquet J.P., Zou M.H., Ullrich V. Peroxynitrite inhibition of nitric oxide synthases. *Biochimie* 1996; **78**: 785-791.
- Patel K.B., Stratford M.R., Wardman P., Everett S.A. Oxidation of tetrahydrobiopterin by biological radicals and scavenging of the trihydrobiopterin radical by ascorbate. *Free Rad. Biol. Med.* 2002; **32**: 203-211.
- Patterson C., Ruef J., Madamanchi N.R., Barry-Lane P., Hu Z., Horaist C., Ballinger C.A., Brasier A.R., Bode C., Runge M.S. Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin: evidence that p47(phox) may participate in forming this oxidase in vitro and in vivo. *J. Biol. Chem.* 1999; **274**: 19814-19822.
- Pendino, K.J., Laskin, J.D., Shuler, R.L., Punjabi, C.J., and D.L. Laskin. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. *J. Immunol.* 1993; **151**: 7196-7205.
- Perkins D.J., Kniss D.A. Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. *J. Leukoc. Biol.* 1999; **65**: 792-799.
- Pfeiffer S., Lass A., Schmidt K., Mayer B. Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J. Biol. Chem.* 2001; **276**: 34051-34058
- Pfeiffer S., Lass A., Schmidt K., Mayer B. Protein tyrosine nitration in mouse peritoneal macrophages activated in vitro and in vivo: evidence against an essential role of peroxynitrite. *FASEB J.* 2001; **15**: 2355-2364
- Pfeiffer S., Mayer B. Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J. Biol. Chem.* 1998; **273**: 27280-27285
- Pfister S.L., Spitzbarth N., Nithipathikon K., Edgmond W.S., Falck J.R., Campbell W.B. Identification of the 11,14,15 -and 11,12,15-trihydroxy-eicosatrienoic acids as endothelium-derived releasing factors of rabbit aorta. *J. Biol. Chem.* 1998; **273**: 30879-30887.
- Picot D., Loll P.J., and Garavito R.M. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 1994; **367**: 243-249.

- Pittman K.M., MacMillan-Crow L.A., Peters B.P., Allen J.B. Nitration of manganese superoxide dismutase during ocular inflammation. *Exp. Eye Res.* 2002; **74**: 463-471.
- Pober J.S., Warner-Lambert/ Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. *Am. J. Pathol.* 1988; **133**: 426-433.
- Radomski M.W., Palmer R.M., and Moncada S. An L-arginine/ nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 1990; **87**: 5193-5197.
- Raetz C. Biochemistry of endotoxins. *Annu. Rev. Biochem.* 1990; **59**: 129-170.
- Raetz C., and Whitfield C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 2002; **71**: 635-700.
- Raeymaekers L., Hofmann F., Casteels R. Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. *Biochem. J.* 1988; **252**: 269-273.
- Rangel-Frausto M.S., Pittet D., Costigan M., Hwang T., Davis C.S., and Wenzel R.P. The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. *JAMA* 1995; **273**: 117-123.
- Reddy S.T., and Herschman H.R. Prostaglandin synthase-1 and prostaglandin synthase-2 are coupled to distinct phospholipases for the generation of prostaglandin D2 in activated mast cells. *J. Biol. Chem.* 1997; **272**: 3231-3237
- Rees D.D., Celtek S., Palmer R.M., Moncada S. Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.* 1990; **173**: 541-547.
- Reidy M.A., and Schwartz S.M. Endothelial injury and regeneration. Endotoxin: a nondenuding injury to aortic endothelium. *Lab. Invest.* 1983; **48**: 25-34.
- Rietschel E.T., and Brade H. Bacterial endotoxins. *Sci. Am.* 1992; **267**: 54-61.
- Rietschel E.T., Kirikae T., Schade F.U., Mamat U., Schmidt G., Loppnow H., Ulmer A.J., Zahringer U., Seydel U., Di Padova F., Schreier M., and Brade H. Bacterial endotoxin: molecular relationships of structure to activity and function: *FASEB J.* 1994; **8**: 217-225.
- Rietschel E.T., Kirikae T., Schade F.U., Ulmer A.J., Holst O., Brade H., Schmidt G., Mamat U., Grimmecke H.D., Kusumoto S., and Hringler U. The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiology* 1993; **187**: 169-190.

- Rigo A., and Viglino P. Effect of ionic strength on the activity of bovine superoxide dismutase. *FEBS Lett.* 1975; **50**: 86-88.
- Rimarachin J.A., Jacobson J.A., Szabo P., Maclouf J., Creminon C., and Weksler B.B. Regulation of cyclooxygenase-2 expression in aortic smooth muscle cells. *Arterioscler Thromb.* 1994; **14**: 1021-1031.
- Ristimäki A., Garfinkel S., Wessendorf J., Maciag T., and Hla T. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *J. Biol. Chem.* 1994; **269**: 11769-11775.
- Rosen G.M., Pou S., Ramos C.L., Cohen M.S., Britigan B.E. Free radicals and phagocytic cells. *FASEB J.* 1995; **9**: 200-209.
- Roy L., Knapp H., Robertson R.M., FitzGerald G.A. Endogenous biosynthesis of prostacyclin during cardiac catheterization and angiography in man. *Circulation* 1985; **71**: 434-440.
- Salmon J.A., Smith D.R., Flower R.J., Moncada S. and Vane J.R. Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes. *Biochim. Biophys. Acta.* 1978; **523**: 250-262.
- Salvemini D., Misko T.P., Masferrer J.L., Seibert K., Currie M.G., and Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* 1993; **90**: 7240-7244
- Salvemini D., Settle S.L., Masferrer J.L., Seibert K., Currie M.G., and Needleman P. Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Brit. J. Pharmacol.* 1995; **114**: 1171-1178.
- Sampson, J.B., Ye, Y., Rosen, H., and J.S. Beckman. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys.* 1998; **356**: 207-213.
- Sarcevic B., Brookes V., Martin T.J., Kemp B.E., and Robinson P.J. Atrial natriuretic peptide-dependent phosphorylation of smooth muscle cell particulate fraction proteins is mediated by cGMP-dependent protein kinase. *J. Biol. Chem.* 1989; **264**: 20648-20654.
- Sarkela T.M., Berthiaume J., Elfering S., Gybina A.A., and Giulivi C. The modulation of oxygen radical production by nitric oxide in mitochondria. *J. Biol. Chem.* 2001; **276**: 6945-6949.
- Sato Y., Sagami I., and Shimizu T. Identification of caveolin-1-interacting sites in neuronal nitric-oxide synthase. Molecular mechanisms for inhibition of NO formation. *J. Biol. Chem.* 2004; **279**: 8827-8836

Saussy D.L., Mais D.E., Burck R.M., Halushka P.V. Identification of a putative thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub> receptor in human platelet membranes. *J. Biol. Chem.* 1986; **261**: 3025-3029.

Schildknecht S., Bachschmid M., Baumann A., and Ullrich V. COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. *FASEB J.* 2004; **18(6)**: 757-759

Schildknecht S., Bachschmid M., Weber K., Maass D., Ullrich V. Endotoxin elicits nitric oxide release in rat but prostacyclin synthesis in human and bovine vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 2005; **327**: 43-48.

Schmidt, H.H., Pollock, J.S., Nakane M., Gorsky L.D., Förstermann, U., and Murad, F. Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. *Proc. Natl. Acad. Sci. USA* 1991; **88**: 365-369.

Schmidt P., Youhnovski N., Daiber A., Balan A., Arsic M., Bachschmid M., Przybylski M., Ullrich V. Specific nitration at tyrosine 430 revealed by high resolution mass spectrometry as basis for redox regulation of bovine prostacyclin synthase. *J. Biol. Chem.* 2003; **278**: 12813-12819.

Schonbeck U., Sukhova G.K., Graber P., Coulter S., and Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. *Am. J. Pathol.* 1999; **155**: 1281-1291.

Schultz M.J., and van der Poll T. Animal and human models for sepsis. *Ann. Med.* 2002; **34**: 573-581.

Schumann R.R., Lamping N., Kirschning C., Knopf H.P., Hoess A., and Herrmann F. Lipopolysaccharide binding protein: its role and therapeutical potential in inflammation and sepsis. *Biochem. Soc. Trans.* 1994; **22**: 80-82.

Schumann R.R., Leong S.R., Flaggs G.W., Gray P.W., Wright S.D., Mathison J.C., Tobias P.S., and Ulevitch R.J. Structure and function of lipopolysaccharide binding protein. *Science* 1990; **249**: 1429-1431.

Schwarz P.M., Kleinert H., and Förstermann U. Potential functional significance of brain-type and muscle-type nitric oxide synthase I expressed in adventitia and media of rat aorta. *Arterioscler. Thromb. Vasc. Biol.* 1999; **19**: 2584-2590

Sengupta S. Cyclooxygenase-2: A new therapeutic target. *Indian J. of Pharm.* 1999; **31**: 322-332.

Serhan C.N. Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): A jungle of cell-cell interactions or a therapeutic opportunity? *Prostaglandins.* 1997; **53**: 107-137.

Shimokawa T., Kulmacz R.J., DeWitt D.L., and Smith W.L. Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J. Biol. Chem.* 1990; **265**: 20073-20076.

- Siddhanta U., Presta A., Fau B., Wolan D., Rousseau D.L., and Stuehr D.J. Domain swapping in inducible nitric oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer. *J. Biol. Chem.* 1998; **273**: 18950-18958.
- Sies H., Sharov V.S., Klotz L.O., and Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J. Biol. Chem.* 1997; **272**: 27812-27817
- Silverstein F.E., Faich G., Goldstein J.L., Simon L.S., Pincus T., Whelton A., Makuch R., Eisen G., Agrawal N.M., Stenson W.F., Burr A.M., Zhao W.W., Kent J.D., Lefkowitz J.B., Verburg K.M., and Geis G.S. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* 2000; 284: 1247-1255.
- Simmons D.L., Xie W., Evett G., Merrill J., Robertson D.L., and Bradshaw W.S. Drug inhibition and cellular regulation of prostaglandin G/H synthase isozyme 2. *J. Lipid Mediat.* 1993; **6**: 113-117.
- Sirsjö A., Söderkvist P., Sundqvist T., Carlsson M., Ost M., and Gidlof A. Different induction mechanisms of mRNA for inducible nitric oxide synthase in rat smooth muscle cells in culture and in aortic strips. *FEBS Lett.* 1994; **338**: 191-198.
- Small J.V. and Sobieszek A. Studies on the function and composition of the 10nm (100A) filaments of vertebrate smooth muscle. *J. Cell Sci.* 1977; **23**: 243-268.
- Smith C.J., Zhang Y, and Koboldt C.M. Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl. Acad. Sci.* 1998; **95**: 13313-13318.
- Smith W. Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* 1992; **263**: F181-F191.
- Smith W.L and Lands W.E. Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. *Biochemistry* 1972; **11**: 3276-3285.
- Smith W.L., and DeWitt D.L. Prostaglandin endoperoxide H synthases -1 and -2. *Adv. Immunol.* 1996; **62**: 167-215.
- Smith W.L., DeWitt D.L., Garavito R.M. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 2000; **69**: 145-182.
- Smith W.L., Garavito R.M., and DeWitt D.L. Prostaglandin endoperoxide H synthases (cyclooxygenases) -1 and -2. *J. Biol. Chem.* 1996; **271**: 33157-33160.

- Soejima K., Ishizaka A., Urano T., Sayama K., Sakamaki F., Nakumura H., Terashima T., Waki Y., Tasaka S., Fujishima S., Kawata T., Christ W.J., and Kanazawa M. Protective effect of B 464, a lipid A analog, on endotoxin-induced cellular responses and acute lung injury. *Am. J. Respir. Crit. Care Med.* 1996; **154**: 900-906.
- Soler M., Camacho M., Escudero J.R., Iniguez M.A., and Vila L. Human vascular smooth muscle cells but not endothelial cells express prostaglandin E synthase. *Circ. Res* 2000; **87**: 504-507.
- Song I., Ball T.M., and Smith W.L. Different suicide inactivation processes for the peroxidase and cyclooxygenase activities of prostaglandin endoperoxide H synthase-1. *Biochem. Biophys. Res. Commun.* 2001; **289**: 869-875
- Spencer A.G., Thuresson E., Otto J.C., Song I., Smith T., DeWitt D.L., Garavito R.M., Smith W.L. The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2. Peptide mapping and mutational analysis. *J. Biol. Chem.* 1999; **274**: 32936-32942.
- Spisni E., Griffoni C., Santi S., Riccio M., Marulli R., Bartolini G., Toni M., Ullrich V., and Tomasi V. Colocalization prostacyclin (PGI<sub>2</sub>) synthase-- caveolin-1 in endothelial cells and new roles for PGI<sub>2</sub> in angiogenesis. *Exp. Cell Res.* 2001; **266**: 31-43
- Stadler J., Harbrecht B.G., Di Silvio M., Curran R.C., Jordan M.L., Simmons R.L., and Billiar T.R. Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J. Leukoc. Biol.* 1993; **53**: 165-172.
- Stanfield K.M., Khan N.K., and Gralinski M.R. Localization of cyclooxygenase isozymes in cardiovascular tissues of dogs treated with naproxen. *Vet. Immunol. Immunopathol.* 2001; **80**: 309-314
- Steinberg, K.P., Milberg, J.A., Martin, T.R., Maunder, R.J., Cockrill, B.A., and L.D. Hudson. Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 1994; **150**: 113-122.
- Studel, W., Kramer, H.J., Degner, D., Rosseau, S., Schutte, H., Walmrath, D., and W. Seeger. Endotoxin priming of thromboxane-related vasoconstrictor responses in perfused rabbit lungs. *J. Appl. Physiol.* 1997; **83**: 18-24.
- Stoclet J.C., Martinez M.C., Ohlmann P., Chasserot S., Schott C., and Kleschyov A.L. Induction of nitric oxide and cyclooxygenase products in regulation of arterial contraction in human septic shock. *Circulation* 1999; **100**: 107-112.

- Stralin P., Karlsson K., Johansson B.O., Marklund S.L. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler. Thromb. Vasc. Biol.* 1995; **15**: 2032-2036.
- Stuehr D., Pou S., and Rosen G.M. Oxygen reduction by nitric-oxide synthases. *J. Biol. Chem.* 2001; **276**: 14533-14536.
- Stuehr D.J., Cho H.J., Kwon N.S., Weise M.F., and Nathan C.F. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD-and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 1991; **88**: 7773-7777.
- Stuehr D.J., Kwon N.S., Nathan C.F., Griffith O.W., Feldman P.L., and Wiseman J. N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* 1991; **266**: 6259-6263.
- Stuehr D.J., Marletta M.A. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* 1985; **82**: 7738-7742.
- Stuehr D.J., Santolini J., Wang Z.Q., Wei C.C., Adak S. Update on mechanism and catalytic regulation in the NO synthases. *J. Biol. Chem.* 2004; **279**: 36167-36170.
- Sunil, V.R., Connor, A.J., Guo, Y., Laskin, J.D., and D.L. Laskin. Activation of type II alveolar epithelial cells during acute endotoxemia. *Am. J. Physiol.* 2002; **282**: L872-L880.
- Suzuki, K., Yamamoto, T., Sato, A., Murayama, T., Amitani, R., Yamamoto, K., and F. Kuze. Lipopolysaccharide primes human alveolar macrophages for enhanced release of superoxide anion and leukotriene B<sub>4</sub>: self-limitations of the priming response with protein synthesis. *Am. J. Respir. Cell Mol. Biol.* 1993; **8**: 500-508.
- Tannenbaum S.R., Fett D., Young V.R., Land P.D., and Bruce W.R. Nitrite and nitrate are formed by endogenous synthesis in the human intestine. *Science* 1978; **200**: 1487-1489.
- Tate R.M., Morris H.G., Schroeder W.R., and Repine J.E. Oxygen metabolites stimulate thromboxane production and vasoconstriction in isolated-perfused rabbit lungs. *J. Clin. Invest.* 1984; **74**: 608-613.
- Tateson J.E., Moncada S., and Vane J.R. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins* 1977; **13**: 389-399.
- Tetsuka, T., Daphna-Iken, D., Miller, B.W., Guan, Z., Baier, L.D., and A.R. Morrison. Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. *J. Clin. Invest.* 1996; **97**: 2051-2056.

- Thomas, C.J., Kapoor, M., Sharma, S., Bausinger, H., Zyilan, U., Lipsker, D., Hanau, D., and A. Surolia. Evidence of a trimolecular complex involving LPS, LPS binding protein and soluble CD14 as an effector of LPS response. *FEBS Lett.* 2002; **531**: 184-188.
- Tischfield J.A. A reassessment of the low molecular weight phospholipase A2 gene family in mammals. *J. Biol. Chem.* 1997; **272**: 17247-17250.
- Topper J.N., Cai J.C., Falb D., Gimbrone M.A. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc. Natl. Acad. Sci USA* 1996; **93**: 10417-10422.
- Trybus K.M. Assembly of cytoplasmic and smooth muscle myosins. *Curr. Opin. Cell Biol.* 1991; **3**: 105-111.
- Tsai A., and Kulmacz R.J. Tyrosyl radicals in prostaglandin H synthase-1 and -2. *Prostaglandin Other Lipid Mediat.* 2000; **62**: 231-254
- Tsai A., Kulmacz R.J. and Palmer G. Spectroscopic evidence for reaction of prostaglandin H synthase-1 tyrosyl radical with arachidonic acid. *J. Biol. Chem.* 1995; **270**: 10503-10508.
- Tsai A., Wu G., Palmer G., Bambai B., Koehn J.A., Marshall P.J., and Kulmacz R.J. Rapid kinetics of tyrosyl radical formation and heme redox state changes in prostaglandin H synthase-1 and -2. *J. Biol. Chem.* 1999; **274**: 21695-21700
- Tsai A.L., Hsi L.C., Kulmacz R.J., Palmer G., and Smith W.L. Characterization of the tyrosyl radicals in ovine prostaglandin H synthase -1 by isotope replacement and site-directed mutagenesis. *J. Biol. Chem.* 1994; **269**: 5085-5091.
- Tsai A.L., Palmer G., Xiao G., Swinney D.C., Kulmacz R.J. Structural characterization of arachidonyl radicals formed by prostaglandin H synthase-2 and prostaglandin H synthase-1 reconstituted with manganese protoporphyrin IX. *J. Biol. Chem.* 1998; **273**: 3888-3894.
- Tsai A.L., Wei C., and Kulmacz R.J. Interaction between nitric oxide and prostaglandin H synthase. *Arch. Biochem. Biophys.* 1994; **313**: 367-372
- Ueno N., Murakami M., Tanioka T., Fujimori K., Tanabe T., Urade Y., Kudo I., Coupling between cyclooxygenase, terminal prostanoid synthase, and phospholipase A<sub>2</sub>. *J. Biol. Chem.* 2001; **276**: 34918-34927.

- Uhlig, S., Nusing, R., von Bethmann, A., Featherstone, R.L., Klein, T., Brasch, F., Muller, K.M., Ullrich, V., and A. Wendel. Cyclooxygenase-2-dependent bronchioconstriction in perfused rat lungs exposed to endotoxin. *Mol. Med.* 1996; **2**: 373-383.
- Ulevitch R.J., and Tobias P.S. Recognition of endotoxin by cells leading to transmembrane signaling. *Curr. Opin. Immunol.* 1994; **6**: 125-130.
- Ullrich V. and Bachschmid M. Superoxide as a messenger of endothelial function. *Biochem. Biophys. Res. Commun.* 2000; **278**: 1-8.
- Ullrich V., Zou M., and Bachschmid M. New physiological aspects on the thromboxane A<sub>2</sub>-prostacyclin regulatory system. *Biochim. Biophys. Acta.* 2001; **1532**: 1-14.
- Upmacis R.K., Deeb R.S., Hajjar D.P. Regulation of prostaglandin H<sub>2</sub> synthase activity by nitrogen oxides. *Biochemistry* 1999; **38**: 12505-12513
- Uracz W., Uracz D., Olszanecki R., and Gryglewski R.J. Interleukin 1 $\beta$  induces functional prostaglandin E synthase cultured human umbilical vein endothelial cells. *J. Physiol. Pharmacol.* 2002; **53**: 643-654.
- Vallance P. Vascular endothelium, its physiology and pathophysiology. In: Witherall D.J., Ledingham J.G.G., Warrell D. eds. Oxford Textbook of Medicine. Oxford. Oxford University Press, 1995: 2295-2300.
- Vallance P., Collier J., and Bhagat K. Infection, inflammation and infarction: does acute endothelial dysfunction provide a link? *Lancet* 1997; **349**: 1391-1392.
- van der Dink W.A., Tsai A.L., and Kulmacz R.J. The cyclooxygenase reaction mechanism. *Biochemistry* 2002; **41**: 15451-15458
- Vane J.R. Back to aspirin a day? *Science* 2002; **296**: 474-475.
- Vane J.R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* 1971; **231**: 232-235.
- Vane J.R., and Botting R.M. Mechanism of action of nonsteroidal anti-inflammatory drugs. *Am. J. Med.* 1998; **104**: 2S-8S.
- Vane J.R., Anggard E.E., and Botting R.M. Regulatory functions of the vascular endothelium. *N. Engl. J. Med.* 1990; **323**: 27-36.
- Vane J.R., Bakhle Y.S., and Botting R.M. Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.* 1998; **38**: 97-120.

- Vane J.R., Botting R.M. Formation by the endothelium of prostacyclin, nitric oxide and endothelin. *J. Lipid Mediat.* 1993; **6**: 395-404.
- Vane J.R., Mitchell J.A., Appleton I., Tomlinson A., Bishop-Bailey D., Croxtall J., and Willoughby D.A. Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation. *Proc. Natl. Acad. Sci.* 1994; **91**: 2046-2050.
- Verma S., Satish R.R., Shewchuk L., Mather K.J., Anderson T.J. Cyclooxygenase-2 blockade does not impair endothelial vasodilator function in healthy volunteers. *Circulation* 2001; **104**: 2879-2882.
- Viriyakosol S., and Kirkland T. Knowledge of cellular receptors for bacterial endotoxin. *Clin. Infect. Dis.* 1995; **21**: S190-S195.
- Von Knethen A., Brüne B. Superinduction of cyclooxygenase-2 by NO and agonist challenge involves transcriptional regulation mediated by AP-1 activation. *Biochemistry* 2000; **39**: 1532-1540.
- Walsh M.T., Foley J.F., Kinsella B.T. The alpha, but not the beta isoform of the human thromboxane A<sub>2</sub> receptor is a target for prostacyclin-mediated desensitization. *J. Biol. Chem.* 2000; **275**: 20412-20423.
- Warner T.D., Giuliano F., Vojnovic I., Bukasa A., Mitchell J.A., and Vane J.R. Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc. Natl. Acad. Sci. USA* 1999; **96**: 7563-7568.
- Warren S. Strategies for the treatment of sepsis. *N. Engl. J. Med.* 1997; **336**: 952-953.
- Weaver J.A., Maddox J.F., Cao Y.Z., Mullarky I.K., and Sordillo L.M. Increased 15-HPETE production decreases prostaglandin synthase activity during oxidant stress in aortic endothelial cells. *Free Rad. Biol. Med.* 2001; **30(3)**: 299-308
- Wei C.W., Kulmacz R.J., and Tsai A.L. Comparison of branched-chain and tightly coupled reaction mechanisms for prostaglandin H synthase. *Biochemistry* 1995; **34**: 8499-8512.
- Wen F.Q., Watanabe K., and Yoshida M. Nitric oxide enhances PGI<sub>2</sub> production by human pulmonary artery smooth muscle cells. *Prostagl., Leukot. and Ess. Fatty Acids* 2000; **62**: 369-378.
- Wilborn, J., DeWitt, D.L., and M. Peters-Golden. Expression and role of cyclooxygenase isoforms in alveolar and peritoneal macrophages. *Am. J. Physiol.* 1995; **268**: L294-L301.
- Wise W.C., Cook J.A., Eller T., and Halushka P.V. Ibuprofen improves survival from endotoxic shock in the rat. *J. Pharmacol. Exp. Ther.* 1980; **215**: 160-164.

- Wizemann, T.M., Gardner, C.R., Laskin, J.D., Ouinones, S., Durham, S.K., Goller, N.L., Ohnishi, S.T., and D.L. Laskin. Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukoc. Biol.* 1994; **56**: 759-768.
- Woodman R.C., Ruedi J.M., Jesaitis A.J., Okamura N., Quinn M.T., Curnutte J.Z., and Babior B.M. The respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J. Clin. Invest.* 1991; **87**: 1345-1351
- Wort S.J., Evans T.W. The role of the endothelium in modulating vascular control in sepsis and related conditions. *Br. Med. Bull.* 1999; **55**: 30-48
- Wright D.H., Abran D., Bhattacharya M., et al. Prostanoid receptors: ontogeny and implications in vascular physiology. *Am. J. Phys.* 2001; **281**: R1343-R1360.
- Wright J.M. The double-edged sword of COX-2 selective NSAIDs. *CMAJ* 2002; **167**: 1131-1136.
- Wu G., Wei C., Kulmacz R.J., Osawa Y., Tsai A.L. A mechanistic study of self-inactivation of the peroxidase activity in prostaglandin H synthase-1. *J. Biol. Chem.* 1999; **274**: 9231-9237
- Wurfel M.M., Kunitake S.T., Lichenstein H., Kane J.P., and Wright S.D. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J. Exp. Med.* 1994; **180**: 1025-1035.
- Xiao G., Tsai A.L., Palmer G., Boyar W.C., Marshall P.J., and Kulmacz R.J. Analysis of hydroperoxide-induced tyrosyl radicals and lipoxygenase activity in aspirin-treated human prostaglandin H synthase -2. *Biochemistry* 1997; **36**: 1836-1845.
- Xie Q., Kashiwabara Y., Nathan C. Role of transcription factor NF-kappaB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 1994; **269**: 4705-4708.
- Xie W.L., Chipman J.G., Robertson D.L., Erikson R.L. and Simmons D.L. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* 1991; **88**: 2692-2696.
- Zafari A.M., Ushio-Fukai M., Akers M., Yin Q., Shah A., Harrison D.G., Taylor W.R., and Griendling K.K. Novel role of NADH/NADPH oxidase-derived hydrogen peroxide in angiotensin II-induced hypertrophy of rat vascular smooth muscle cells. *Hypertension* 1998; **32**: 488-495.
- Zehetgruber M., Conforto A., and Bing R.J. Vascular smooth muscle and nitric oxide. *Life Sci.* 1993; **52**: 1397-1406

Ziegler E.J., Fisher C.J., Sprung C.L., et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin- a randomized, double-blind, placebo-controlled trial. *N. Engl. J. Med.* 1991; **324**: 429-436.

Zou M.H. and Ullrich V. Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett.* 1996; **382**: 101-104.

Zou M.H. Jendral M., and Ullrich V. Prostaglandin endoperoxide-dependent vasospasm in bovine coronary arteries after nitration of prostacyclin synthase. *Br. J. Pharmacol.* 1999; **126**: 1283-1292

Zou M.H., Bachschmid M. Hypoxia-reoxygenation triggers coronary vasospasm in isolated bovine coronary arteries via tyrosine nitration of prostacyclin synthase. *J. Exp. Med.* 1999; **190**: 135-139.

Zou M.H., Klein T., Pasquet J.P., and Ullrich V. Interleukin 1beta decreases prostacyclin synthase activity in rat mesangial cells via endogenous peroxynitrite formation. *Biochem. J.* 1998; **336**: 507-512.

Zou M.H., Leist M., and Ullrich V. Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am. J. Pathol.* 1999; **154**: 1359-1365

Zou M.H., Martin C., Ullrich V. Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol. Chem.* 1997; **378**: 707-713.

Zou M.H., Shi C., Cohen R.A. High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes* 2002; **51**: 198-203.

Zou M.H., Shi C., Cohen R.A. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J. Clin. Invest.* 2002; **109**: 817-826.