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Acetaminophen inhibits prostanoid synthesis by scavenging the PGHS-activator peroxynitrite

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ABSTRACT **The primary pharmacological target of acetaminophen is prostaglandin endoperoxide H_2 synthase (PGHS). The enzymatic catalytic mechanism is radical-based, initiated, and maintained by the persistent presence of peroxides, particularly peroxynitrite, which is termed "peroxide tone". Whereas the prevailing concept assumes a direct reduction of the active, oxidized enzyme by acetaminophen, here we show that acetaminophen is a potent scavenger of peroxynitrite (peroxynitrite-mediated phenol nitration, $IC_{50}\approx72\mu M$; Sin-1-mediated DHR123 oxidation, $IC_{50}\approx11\mu M$) and thus inhibits PGHS by eliminating the peroxide tone. Nanomolar concentrations of peroxynitrite increased the activity of isolated PGHS and prostacyclin formation by aortic endothelial cells. This elevated activity was efficiently inhibited by pharmacologically relevant concentrations of acetaminophen ($IC_{50}\approx10\mu M$ for 6-keto-$PGF_{1\alpha}$) and other free radical scavengers. However, when the peroxide tone was provided by H_2O_2 or tert-butyl-OOH, acetaminophen had only negligible inhibitory effects. Our concept could help to explain the efficacy of acetaminophen to inhibit PGHS in cell types with moderate oxidant formation. However, high levels of peroxynitrite or other peroxides such as lipid peroxides formed at inflammatory sites might overwhelm the ability of acetaminophen to decrease PGHS activation. The concept presented herein provides a molecular basis to explain the excellent analgesic and antipyretic properties of acetaminophen together with its minimal anti-inflammatory effects.—Schildknecht, S., Daiber, A., Ghisla, S., Cohen, R. A., Bachschmid, M. M. Acetaminophen inhibits prostanoid synthesis by scavenging the PGHS-activator peroxynitrite.**

Key Words: endothelium · cyclooxygenase · superoxide · nitric oxide · peroxide tone

ACETAMINOPHEN (APAP; N-*ACETYL*-*P*-*AMINOP*HENOL) ranks among the most popular over-the-counter drugs for the treatment of pain and fever worldwide. Its analgesic and antipyretic properties arise primarily from inhibition of prostanoid formation within the central nervous system (1–3). The key enzyme for prostanoid synthesis is prostaglandin endoperoxide H_2 synthase (PGHS; COX), which provides the substrate for a variety of synthases such as prostacyclin (PGI_2)-synthase, PGE_2-synthase or thromboxane A_2 (TxA_2)-synthase (**Fig. 1**). Unlike aspirin or ibuprofen, which prevent the access of arachidonic acid, APAP pharmacologically interferes with PGHS catalytic activity but has no effect on arachidonic acid binding. However, the molecular mechanisms of APAP inhibition still remain unclear.

PGHS consists of two spatially distinct but mechanistically coupled catalytic sites, the cyclooxygenase domain and the peroxidase domain (4) (Figs. 1 and 7). The coupling between both activities is best explained by the branched chain mechanism (5, 6). Peroxides initiate the catalytic cycle by oxidizing the prosthetic ferric heme [Fe^{III}] of the peroxidase domain to an unstable radical cation intermediate ([Fe^{IV}=O $PPIX^{\bullet+}$]; Compound I). Electron transfer from tyrosine 385 (analogous to ovine COX-1) reduces Compound I to Compound II (Fe^{IV}=O PPIX) and results in a tyrosyl radical at the cyclooxygenase active site. This starts the controlled radical-mediated oxidation of arachidonic acid into prostaglandin endoperoxide G_2 (PGG_2; **Fig. 7**). Converting the oxidized heme-protoporphyrin back to its ferric ground state by the reductive cellular milieu continuously interrupts catalysis. Therefore, reactivation and maintenance of PGHS activity require the permanent presence of peroxides, the so-called "peroxide tone".

Numerous investigations demonstrate that APAP causes paradoxical stimulation of purified PGHS enzyme at therapeutic doses and inhibition at toxic concentrations (7, 8). In contrast, observations *in vivo* indicate inhibition of prostanoid formation at pharmacologically relevant concentrations of APAP (9, 10). The phenolic structure of APAP suggests several molecular mechanisms of PGHS inhibition: quenching of the active site COX tyrosyl radical, competitively inhibiting the peroxidase domain (11–13), lowering the peroxide

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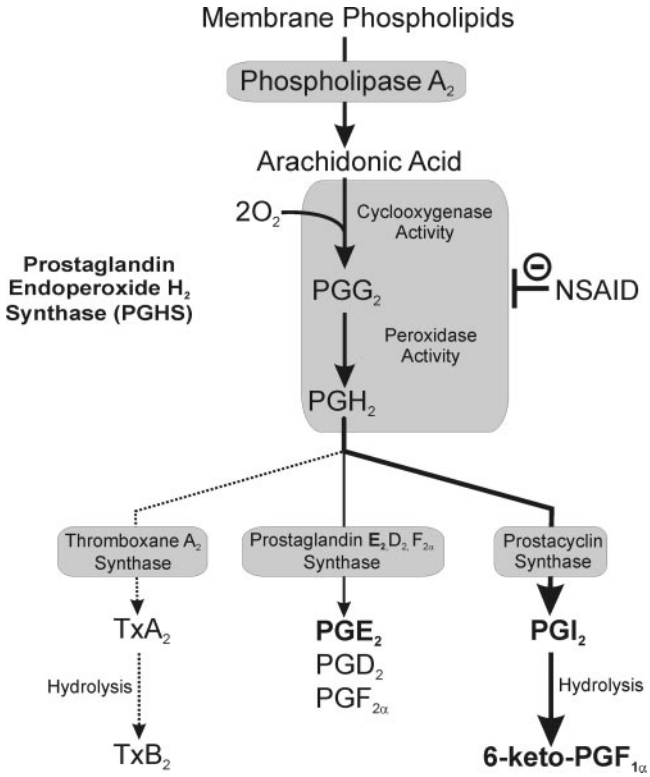

Figure 1. Schematic of prostaglandin synthesis. Arachidonic acid is released on Ca^{2+} stimulation or phosphorylation of phospholipase A_2 (PLA_2) from the membrane phospholipid pool. Prostaglandin endoperoxide H_2 synthase (PGHS) is the rate-limiting enzyme in prostaglandin synthesis that consists of two catalytic domains. The cyclooxygenase epoxygenates arachidonic acid into prostaglandin endoperoxide G_2 (PGG_2) and the peroxidase further converts PGG_2 into prostaglandin endoperoxide H_2 (PGH_2). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit PGHS and prevent prostaglandin synthesis. Predominant metabolites formed by the endothelium are prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2), which were used as measures for PGHS activity in endothelial cells. TxA_2 = Thromboxane A_2.

tone by antioxidant effects (14, 15), and altering prostanoid formation and cellular signaling by reactive APAP intermediates (16–20). It has been hypothesized that PGHS is inhibited by APAP by an enhanced reduction of the oxidized heme of PGHS to its "resting" state (Fig. 7, left side), an effect that can be overcome by an increase in peroxides (11). Thus, cells such as macrophages, which can produce high levels of peroxides, remain unaffected by APAP and may explain the absence of the drug's anti-inflammatory effects. However, no direct evidence has been demonstrated to support these notions.

We have previously reported that peroxynitrite ($ONOO^-$), originating from the reaction of nitric oxide ($^{\bullet}NO$) and superoxide ($^{\bullet}O_2^-$), is a very potent endogenous activator of PGHS (21). This activation is transient in nature and independent of covalent post-translational PGHS modifications. In this report, we provide evidence that APAP, due to its phenolic structure, is a potent scavenger of endogenously formed $ONOO^-$. APAP decreases ONOO—provided peroxide tone and the resultant diminished activation of PGHS *in vitro* and in cell culture systems. The mechanism suggested herein could, therefore, help to explain the discrepancy between the weak anti-inflammatory properties of APAP and its distinct analgesic and antipyretic effects.

MATERIALS AND METHODS

Cell culture

Primary cultures of bovine aortic endothelial cells were obtained by collagenase digestion (100 U/ml) (Type I, Worthington, NJ, USA) of aortic segments for 30 min. Endothelial cells were removed from the aortic segment with a cell scraper and grown in DMEM (Life Technologies Inc., Gaithersburg, MD, USA) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Inc.). Experiments were performed with cells between passages 3 to 5. LPS (*Escherichia coli*; Serotype 026:B26) was obtained from Sigma (St. Louis, MO, USA). Cells were quiesced by reducing the serum concentration to 1% for 24 h prior to the experiments.

6-keto-$PGF_{1\alpha}$ and PGE_2 levels

Following a 2 h pretreatment with LPS (1 μg/ml) for PGHS-2 induction, cells were washed twice with PBS and then incubated with the respective compounds in new medium for additional 30 min. The supernatant was collected for 6-keto-$PGF_{1\alpha}$ and PGE_2 detection, and cells were lysed by sonication for Western blot analysis. 6-keto-$PGF_{1\alpha}$ and PGE_2 were determined by using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's instructions.

PGHS activity

Sheep seminal vesicle microsomes were incubated at 37°C with 10 U glutathione (GSH)-peroxidase, 0.2 mM GSH, 3250 U/ml catalase in 80mM Tris-HCl, pH 8.0 containing 1μM hematin, and 500 μM phenol. 10 μM ^{12}C-arachidonic acid (^{12}C-AA) was added for 30 s, followed by Sin-1 or other reagents as indicated for 15 min. The reaction was initiated by the addition of ^{14}C-AA (30 μM) for 1 min and terminated by mixing with 4 volumes of ethyl acetate/citric acid (4 M) (30:1). After vortexing for at least 1 min, the organic phase was evaporated and spotted onto silica TLC plates (Silica 60, Merck, Darmstadt, Germany). TLC plates were subjected to chromatography with a solvent system consisting of ethyl acetate: 2,2,4-trimethylpentane: acetic acid: water (110:50:20:100). Plates were exposed to X-ray film, and the values were quantified by the detection of total PGHS metabolites using a Bio-Rad™ Imaging Densitometer (Model GS-700) and Molecular Analyst Software (Version 1.4; Bio-Rad™, Hercules, CA, USA).

Fluorescence detection

Direct interaction between APAP and peroxynitrite generated by Sin-1 was detected by dihydrorhodamine 123 (DHR 123). All reactions were carried out in 10 mM potassium phosphate buffer, pH 7.5. If necessary, pH values of the respective compounds were adjusted to pH 7.5. The reactions were performed in flat-bottom 96-well plates (BD Falcon, Franklin

Lakes, NJ, USA) using a Fluoroskan Ascent fluorescence reader (Labsystems; Thermo-Fisher, Morris Plains, NJ, USA) with excitation at 485 nm and emission at 538 nm. If not indicated otherwise, 100 μM deferoxamine was present in all preparations to prevent Fenton-chemistry reactions from occurring. APAP, aspirin, or ibuprofen was preincubated with 100 μM Sin-1 for 10 min at 37°C. Fluorescence of the oxidized product, rhodamine 123, was detected 10 min after the addition of 5 μM DHR 123 and corrected to background values obtained on the same 96-well plate without addition of Sin-1.

Western blot analysis

Proteins were separated electrophoretically by 8% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% milk PBS solution and then incubated with the primary antibodies [α-PGHS-2, (BD Bioscience, San Jose, CA, USA); α-DMPO-nitrone adduct, (Abcam, Cambridge, UK)] 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 (Amersham, Buckinghamshire, UK).

Peroxidase activity

Peroxidase activity was determined by the reduction of PPHP (5-phenyl-4-pententyl hydroperoxide) to PPA (5-phenyl-4-pentenyl alcohol) (22). Microsomal PGHS was preincubated with 1 μM hematin, 800 μM phenol, 10 μM AA in 80 mM Tris-HCl, pH 8.0, for 10 min at 4°C and with APAP or other compounds as indicated for 5 min at 37°C. The reaction was initiated by adding PPHP (10 μM final) for 10 s. The reaction was terminated by the addition of ice-cold diethyl ether/methanol/4 M citric acid (30:4:1), and vortexed thoroughly. The organic layer was evaporated to dryness and reconstituted with methanol/water (65:35) for HPLC analysis.

The HPLC system consisted of a Varian ProStar solvent delivery module and a Varian ProStar photodiode array detector. Samples were chromatographed on a 5 μm, 4.6 × 125 mm Vydac C18 reverse-phase column with a mobile phase consisting of 70% $KHPO_4$ (1 mM), pH 7.0, 30% acetonitrile, and 10 mM diethylenetriaminepentaacetic acid (DTPA) at a flow rate of 1 ml/min. PPHP and PPA were detected at 254 nm and integrated using the Varian Workstation Version 6.0. Peroxidase activity was expressed as the ratio between PPA/PPHP.

Phenol nitration

A solution of phenol (1 mM) and either APAP, phenacetin, or 4-aminophenol (25–400 μM each) in 0.1 mM potassium phosphate buffer, pH 7.4, was mixed with peroxynitrite (450 μM) (23). The samples were kept on ice, and 100 μl was subjected to HPLC analysis using a M480 HPLC pump and SP-6 UV–vis spectrophotometric detector from Gynkotek GmbH (Germering, Germany) and computer-assisted data acquisition and analysis using Data Apex Clarity software (Prague, Czech Republic). The main products, 2- and 4-nitrophenol, were isocratically eluted (0.8 ml/min flow) using a C_{18}-Nucleosil 125 × 4 100–3 reversed-phase column from Macherey-Nagel GmbH (Düren, Germany). The mobile phase contained acetonitrile (35%) in 50 mM citric acid buffer (65%), pH 2.2. The nitrophenols were detected at 287 nm and quantified using internal and external standards (2- and 4-nitrophenol). The typical retention times were 4.4 and 7.2 min, respectively. The extent of inhibition of phenol nitration based on concentrations of acetaminophen and its metabolites was used to determine IC_{50} values for these compounds.

RESULTS

Peroxynitrite stimulates PGHS activity

To demonstrate that peroxynitrite serves as a potent activator of PGHS, sheep seminal vesicle microsomes enriched in PGHS-1 were incubated with the peroxynitrite-releasing compound Sin-1, which caused a concentration-dependent increase in PGHS activity (**Fig. 2*A***). Sin-1 was used instead of authentic peroxynitrite due to the short half-life of the latter in aqueous solutions.

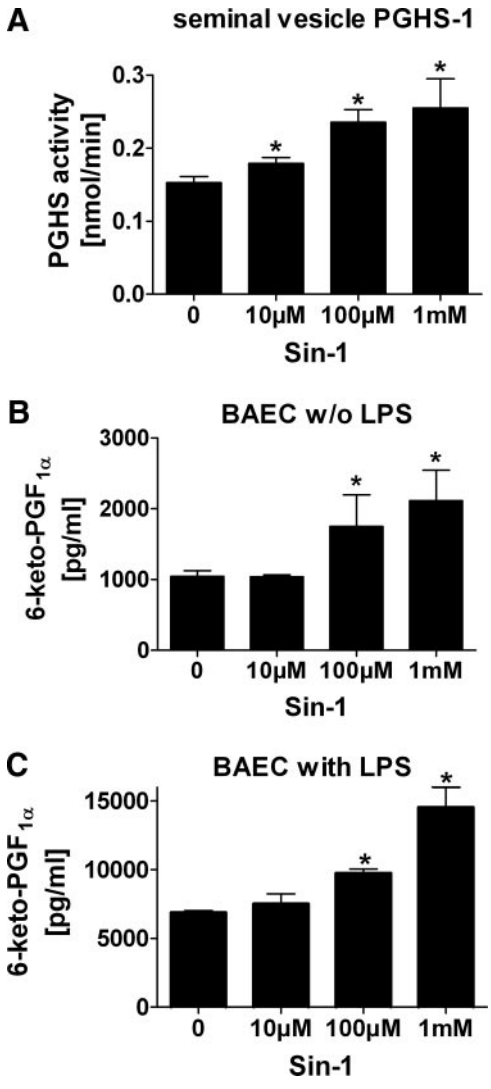

Figure 2. Peroxynitrite activates PGHS catalysis. *A*) Sheep seminal vesicle microsomes were incubated with the peroxynitrite-releasing compound Sin-1. Steady-state concentrations of peroxynitrite were approximately in the range of 1–2 × 10^{-4} of the Sin-1 concentrations indicated. Peroxynitrite in the nanomolar range caused a concentration-dependent increase in PGHS activity. Control (*B*) or LPS challenged (1 μg/ml; 2h) (*C*) primary bovine aortic endothelial cells were incubated with A23187 (1 μM) and Sin-1 at concentrations indicated for 30 min. Formation of 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2 was detected and demonstrated a concentration-dependent increase. Values are means ± SD (n=3). *$P < 0.05$ *vs.* control.

This allowed generation of steady-state fluxes of peroxynitrite by concomitant release of nitric oxide ($^{\bullet}NO$) and superoxide ($^{\bullet}O_2^-$). Under the experimental conditions, actual steady-state concentrations of peroxynitrite were $\sim 1–2 \times 10^{-4}$ of the Sin-1 concentrations applied and, therefore, in the nanomolar range representing physiological fluxes.

To test the role of peroxynitrite on PGHS in a cellular system, we measured in bovine aortic endothelial cells (BAEC) 6-keto-$PGF_{1\alpha}$, the stable breakdown product of prostacyclin (PGI_2; Fig. 2*B, C*), and PGE_2 (Supplemental Fig. S1), which represent the majority of prostaglandins formed. Cells were stimulated with the Ca^{2+}-ionophore A23187 (1 μM) and incubated with various concentrations of the peroxynitrite-releasing compound Sin-1 in order to saturate the endogenous peroxide tone. A23187 caused maximal phospholipase A_2 (PLA_2) activation and release of arachidonic acid, which makes PGHS the rate-limiting enzyme in prostaglandin synthesis. Sin-1 in the highest concentration applied slightly inhibited PLA_2 activity (Supplemental Fig. S2*A*). In addition, Sin-1 and A23187 (1 μM) following treatment with lipopolysaccharide (LPS; 1 μg/ml; 2 h), which induces PGHS-2 (Supplemental Fig. S2) and increases the endogenous formation of peroxynitrite (24), provoked a supplementary concentration-dependent increase in 6-keto-$PGF_{1\alpha}$ (Fig. 2*C*). This substantiates the concept that peroxynitrite may serve as a potent activator of prostanoid formation *in vivo* and indicates that the endogenous peroxide tone was not saturated under these conditions.

Acetaminophen, an efficient peroxynitrite scavenger

The peroxynitrite-scavenging properties of APAP were assessed by competition with the Sin-1-mediated oxidation of dihydrorhodamine 123 (DHR 123) to the fluorescent dye rhodamine 123 (**Fig. 3*A***). At pharmacologically relevant (plasma level) concentrations (0–100μM), APAP resulted in a concentration-dependent decline in DHR 123 oxidation. However, other classical PGHS inhibitors such as aspirin or ibuprofen failed to reduce Sin-1-mediated oxidation. APAP had no significant impact on the decomposition of Sin-1, which was followed by UV/VIS spectroscopy (Supplemental Fig. S3*A*). Additionally, Sin-1 was preincubated with Cu,Zn-superoxide dismutase (SOD1) to scavenge $^{\bullet}O_2^-$, rendering Sin-1 a $^{\bullet}NO$-donor. $^{\bullet}NO$ release was detected with a $^{\bullet}NO$-electrode. Addition of APAP had no significant impact on $^{\bullet}NO$ release by Sin-1 (Supplemental Fig. S3*B*), also indicating that it did not interfere with Sin-1 breakdown.

In a second assay system the concentration-dependent competition of APAP with peroxynitrite-mediated phenol nitration was assessed. 4-aminophenol followed by APAP inhibited phenol nitration at less than a 1:10 molar ratio (Fig. 3*C*, Supplemental Fig. S4*B*), whereas phenacetin showed negligible effects. Stopped-flow measurements further supported the hypothesis that

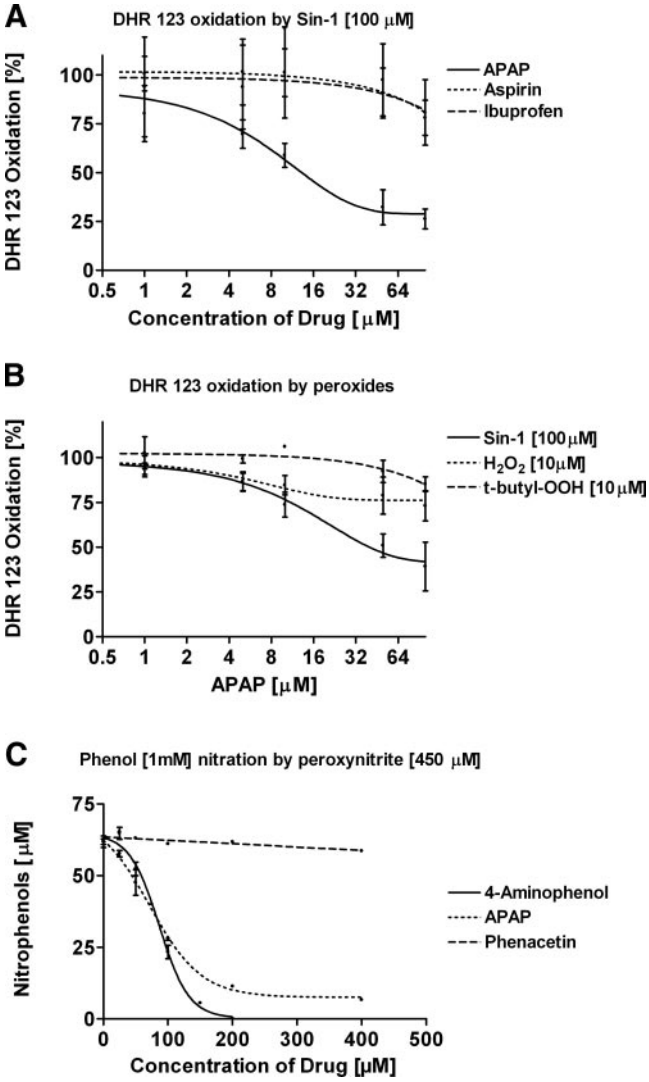

Figure 3. Acetaminophen prevents peroxynitrite-mediated DHR 123 oxidation and phenol nitration. *A*) APAP, aspirin, and ibuprofen in the concentrations indicated were preincubated with 100 μM of the peroxynitrite-generating compound Sin-1 for 10 min at 37°C. Fluorescence was detected after addition of DHR 123 (5 μM). To prevent Fenton-like reactions, 100 μM deferoxamine was present in all experiments. Separate incubations without addition of Sin-1 were performed and subtracted as background for each set of experiments, respectively. In contrast to aspirin and ibuprofen as examples of classical PGHS-inhibitors, APAP significantly lowered fluorescence, indicating direct interaction of peroxynitrite with APAP. *B*) To elucidate the potency of APAP as a specific scavenger of peroxynitrite, APAP at concentrations indicated was treated with Sin-1, t-butyl-OOH, or H_2O_2. Inhibition of the Sin-1-mediated fluorescence by APAP was significantly higher compared to t-butyl-OOH or H_2O_2, thus underlining the significance of this interaction in biological systems. *C*) APAP and 4-aminophenol efficiently blocked peroxynitrite-mediated phenol nitration at a molar ratio of 1:10, whereas phenacetin was ineffective, suggesting that APAP and 4-aminophenol react faster with peroxynitrite. This was confirmed by stopped-flow measurements. Values are means ± SD ($n \leq 8$).

APAP is a potent peroxynitrite scavenger (Supplemental Fig. S4*A*)

In contrast, when Sin-1 was replaced with other

peroxides (Fig. 3*B*) such as tert-butyl hydroperoxide (t-butyl-OOH; 10 μM), hydrogen peroxide (H_2O_2; 10 μM), or 12-hydroperoxyeicosatetraenoic acid (12-HpETE; Supplemental Fig. S5), APAP revealed poor scavenging properties toward these peroxides. Unlike the bolus addition of the peroxides, 100 μM Sin-1 generates steady-state levels of peroxynitrite that are in the physiological range between 10–20 nM. Furthermore, the scavenging efficiency of APAP and its impact on PGHS activity was compared with the peroxynitrite scavenger uric acid, the antioxidant vitamin C, or polyethylene glycol-linked (PEG) SOD and showed similar potency (**Fig. 4*A***).

Acetaminophen inhibits PGHS activity by scavenging the activator peroxynitrite

APAP in the absence of Sin-1 had only marginal inhibitory effects on microsomal PGHS activity. In contrast, Sin-1 mediated PGHS activation was efficiently suppressed by APAP (**Fig. 5*A***) in a concentration-dependent manner. Similarly, uric acid, vitamin C, and PEG-SOD also inhibited Sin-1 induced PGHS activation (Fig. 4*B*).

Endothelial cells (BAEC), which were treated (Fig. 4*C*, *D*; Supplemental Fig. S1*B*) with or without 1 μg/ml LPS (2 h) for PGHS-2 induction, were incubated with uric acid, vitamin C, the NO-synthase inhibitor L-NAME, or PEG-SOD to prevent endogenous peroxynitrite formation (no Sin-1 added). All treatments reduced A23187 stimulated 6-keto-$PGF_{1\alpha}$ or PGE_2 formation, indicating that endogenously formed peroxynitrite served as an activator of PGHS in endothelial cells, in particular in cells treated with LPS. APAP showed a similar potency of inhibition. Endothelial 6-keto-$PGF_{1\alpha}$ and PGE_2 formation was further increased by addition of Sin-1 (Fig. 5*B*, *C*; Supplemental Fig. S1*C*), suggesting that the endogenous peroxide tone was not saturated. APAP decreased Sin-1-mediated endothelial PGHS activity in a concentration-dependent manner and, at the highest concentration (100 μM), abolished the Sin-1 effect (Fig. 5*C*; Supplemental Fig. S1*C*). These data support the hypothesis that APAP has peroxynitrite scavenging properties, which can inhibit peroxynitrite-mediated peroxide tone for PGHS activity.

Interaction of acetaminophen with other peroxides

Various peroxides can provide the peroxide tone for PGHS activation, and their possible interaction with APAP alone or in conjunction with PGHS was investigated. When t-butyl-hydroperoxide was used instead of Sin-1, a concentration-dependent increase in microsomal PGHS activity was detected (Supplemental Fig. S6*A*). However, APAP demonstrated only weak inhibitory properties when t-butyl-OOH was used to stimulate PGHS (Supplemental Fig. S6*B*). Similar results were obtained in endothelial cells pretreated with uric acid to scavenge endogenously formed peroxynitrite. APAP had only moderate inhibitory properties when t-butyl-OOH was used to generate the peroxide tone (Supplemental Fig. S6*C*, *D*). Alternatively, 12-hydroperoxyeicosatetraenoic acid (12-HpETE) was applied to activate PGHS. The alkyl-hydroperoxide 12-HpETE plays a major role in activated platelets, which are known for their insensitivity toward APAP. Similar to peroxynitrite, it can also activate PGHS in the low-nanomolar range compared with the micromolar requirement of H_2O_2 or tert-butyl-OOH to evoke similar effects. 12-HpETE demonstrated no direct interactions with APAP, and microsomal PGHS was completely insensitive to APAP inhibition (Supplemental Fig. S5*B*). These results indicated that in case of t-butyl-OOH, inhibition could occur by reducing the $[Fe^{4+}{=}O\ PPIX]^{\bullet+}$ at the peroxidase domain but that scavenging of peroxynitrite might be the predominant mechanism in endothelial cells.

Direct interactions of acetaminophen with the COX active site tyrosyl radical?

5,5-dimethyl-1-pyrroline N-oxide (DMPO) can form adducts with tyrosyl radicals within proteins, which can be detected by immunostaining. Interaction of APAP

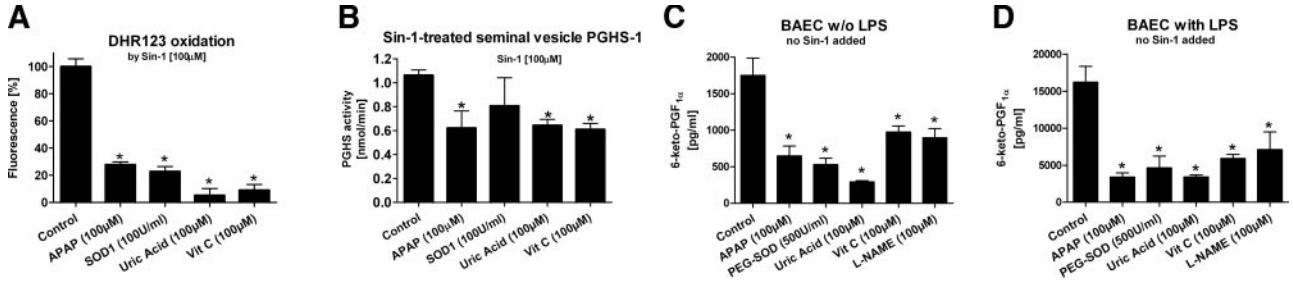

Figure 4. Effects of peroxynitrite scavengers, superoxide dismutase, and NOS inhibition on PGHS activity. *A*) Sin-1 (100 μM) and indicated compounds were incubated for 10 min following the addition of 5 μM DHR 123 in a cell-free system. APAP effectively inhibited peroxynitrite-mediated fluorescence. For comparison, the potent peroxynitrite scavenger uric acid, vitamin C as a general antioxidant, or superoxide dismutase (SOD1) were added and showed similar results. *B*) Microsomes were treated with 100 μM Sin-1, and the compounds as indicated. The stimulatory properties of peroxynitrite were reduced to a similar extent by APAP, uric acid, and vitamin C. Control cells (*C*) or LPS (1 μg/ml; 2 h) challenged cells (*D*) were incubated with the reagents indicated and 1 μM A23187 for 30 min. 6-keto-$PGF_{1\alpha}$ released within the 30 min incubation period was detected by EIA and revealed a significant reduction on endogenous peroxynitrite formation. Values are means ± SD (n=3). *$P < 0.05$ *vs.* control.

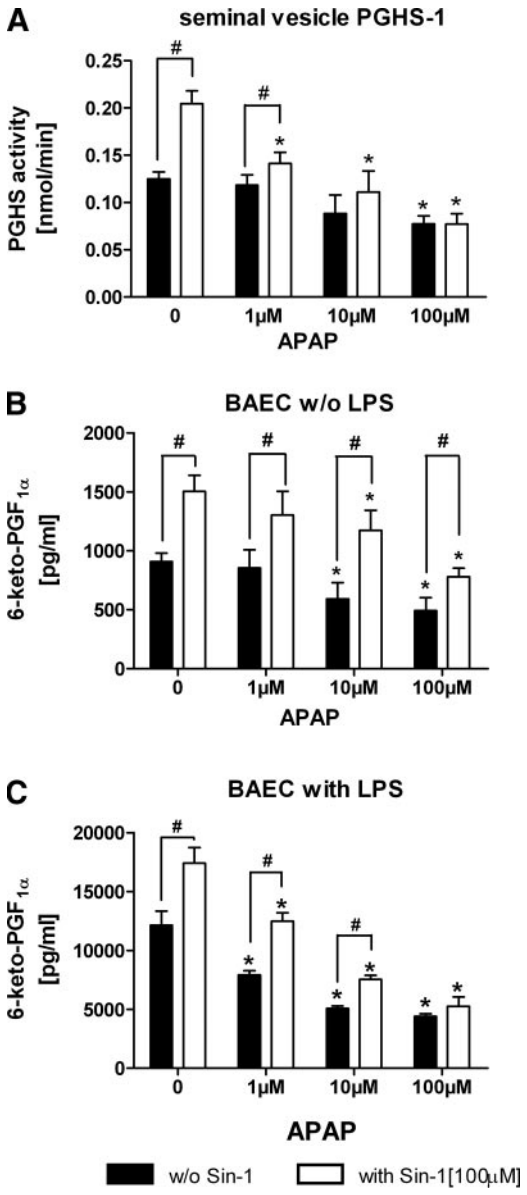

Figure 5. Acetaminophen scavenges the PGHS activator peroxynitrite. *A*) Microsomal PGHS was incubated with various concentrations of APAP in the absence or presence of Sin-1. The peroxynitrite-mediated increase in PGHS activity was concentration-dependently inhibited by APAP. Basal PGHS activity originates mainly from lipid-peroxides present in microsomal preparation and can be inhibited by high concentrations of antioxidants or peroxidases. Control (*B*) or LPS-challenged (2 h; 1 μg/ml) (*C*) bovine aortic endothelial cells were incubated with the Ca^{2+}-ionophore A23187 (1 μM) and increasing concentrations of APAP in the absence or presence of Sin-1. Values represent means ± SD (n=3). *$P <$ 0.05 *vs.* control. $^{\#}P < 0.05$ *vs.* w/o Sin-1.

with the active site tyrosyl radical should be evidenced by interference with DMPO-protein adduct. Therefore, PGHS at low concentrations (5 μM) was incubated with Sin-1, H_2O_2, or t-butyl-OOH for activation, and DMPO. Increasing concentrations of APAP demonstrated no significant impact on DMPO adduct formation (**Fig. 6*A***). PGHS total activity or PGHS-peroxidase activities were both decreased by DMPO, which was not further decreased by APAP (Fig. 6*B–D*). These observations indicate that APAP had no access to the tyrosyl radical at the cyclooxygenase domain. To test a possible competition between arachidonic acid and APAP for the tyrosyl radical of COX, the enzyme was treated with increasing APAP concentrations and two different concentrations of the substrate arachidonic acid (5 μM, 50 μM). However, no significant difference in activity was observed. This indicates that APAP does not interact directly with the active site tyrosyl radical of PGHS.

Reaction products of acetaminophen and peroxynitrite have no impact on PGHS activity

The reaction of acetaminophen with peroxynitrite resulted in the formation of a variety of products dominated by nitro-acetaminophen, acetaminophen-dimer- and -trimer (Supplemental Fig. S7). To test whether these products would have direct inhibitory properties on PGHS catalysis, nitro-acetaminophen, acetaminophen-dimer, and -trimer were purified by HPLC, identified by mass spectrometry, and added to isolated PGHS enzyme or intact cells. Unlike APAP, these products neither exhibited inhibitory effects on microsomal PGHS activity nor on 6-keto-$PGF_{1\alpha}$ formation by endothelial cells (Supplemental Fig. S8).

DISCUSSION

In the late 19th century, scientists began to search for pharmacological alternatives to natural pain and fever relieving cures. By this time, acetanilide and phenacetin were synthesized and used therapeutically. Acetaminophen, subsequently, was discovered as a degradation product in urine. Re-evaluation of the toxic effects of acetanilide in the late 1940s led to the use of APAP as the safe active metabolite. Since 1955, APAP has been sold as Tylenol® or generic medication, but the precise molecular mechanism of pharmacological action has not been fully elucidated.

Puzzling reports on acetaminophen effects: PGHS activator and inhibitor at once?

Numerous investigations, primarily conducted with purified PGHS or enriched microsomal preparations, demonstrate paradoxical stimulation of the enzyme at therapeutic plasma concentrations (30–100 μM) and inhibition at toxic concentrations (>1 mM) (7, 8). In contrast, observations *in vivo* clearly indicate inhibition of prostanoid formation in the central nervous system and the endothelium at pharmacologically relevant concentrations (9). The phenolic structure of APAP suggests several molecular mechanisms of PGHS inhibition: quenching of the active site COX tyrosyl radical, competitively inhibiting the peroxidase domain, lowering the peroxide tone by antioxidant effects (14, 15), and altering prostanoid formation and cellular signaling by reactive APAP intermediates (16–20).

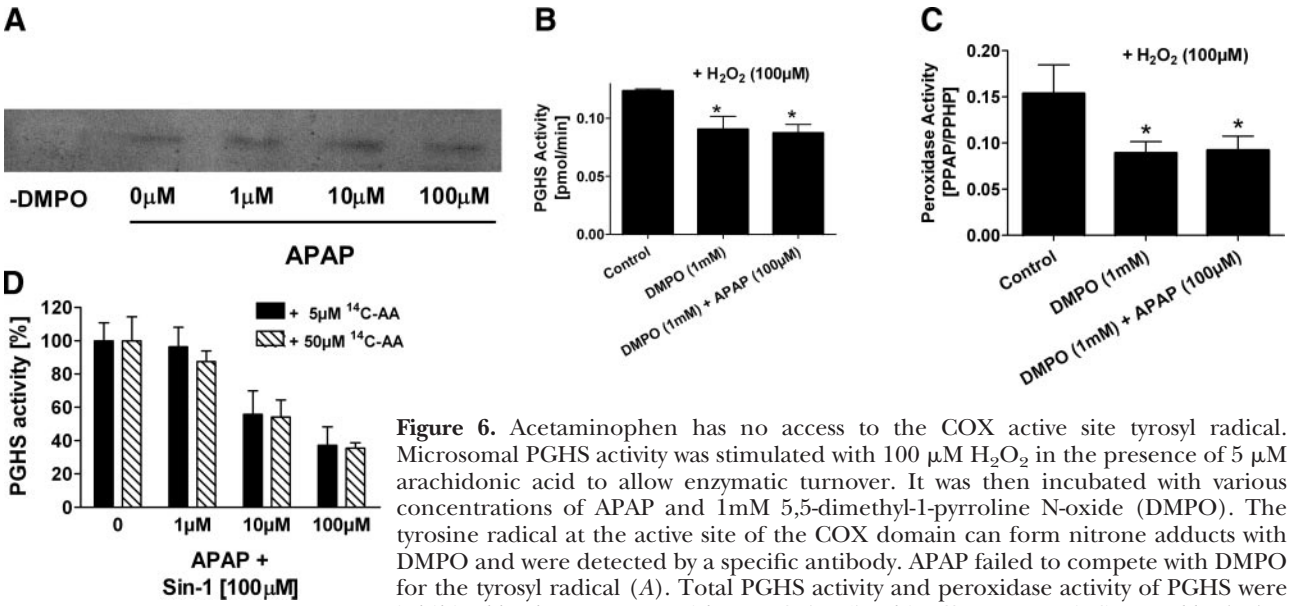

Figure 6. Acetaminophen has no access to the COX active site tyrosyl radical. Microsomal PGHS activity was stimulated with 100 μM H_2O_2 in the presence of 5 μM arachidonic acid to allow enzymatic turnover. It was then incubated with various concentrations of APAP and 1mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The tyrosine radical at the active site of the COX domain can form nitrone adducts with DMPO and were detected by a specific antibody. APAP failed to compete with DMPO for the tyrosyl radical (*A*). Total PGHS activity and peroxidase activity of PGHS were inhibited by the treatment with DMPO (*B, C*). This effect was not influenced by APAP, indicating limited access to the tyrosyl radical of COX. To test whether APAP competes with arachidonic acid for access to the cyclooxygenase active site, microsomal PGHS was incubated with increasing concentrations of APAP, 100 μM Sin-1, and either 5 μM or 50 μM ^{14}C-labeled arachidonic acid. For comparison, PGHS activity was expressed in % and demonstrated no significant differences with elevated substrate concentrations (*D*). These findings argue against a steric competition between arachidonic acid and APAP. Values are means ± SD (n=3). *$P < 0.05$ *vs.* control.

Acetaminophen effects on the cycloxygenase domain?

High and already toxic concentrations (>1mM) of APAP, analogous to other phenolic compounds such as salicylic acid, might quench the COX active site tyrosyl radical (8, 25–27). However, at pharmacologically relevant concentrations no effects were observed as demonstrated by the lack of competition of APAP with DMPO or arachidonic acid for the active site tyrosyl. Our observed lack of effect on activity is in line with other reports (27–29), and therefore this mechanism can be excluded.

Acetaminophen effects on the peroxidase domain?

Other studies have suggested that phenolic compounds can serve as peroxidase reducing-substrates that convert the activated enzyme, [Fe^{4+}=O PPIX$^{\bullet+}$] radical cation intermediate, back to the "resting" ferric state. Hence this input competes with the electron transfer from the active site tyrosine and preventing the formation of the tyrosyl radical, which is essential for arachidonic acid conversion. Thus, an elevation of cellular peroxide levels should favor the formation of the [Fe^{4+}=O PPIX$^{\bullet+}$] intermediate over the APAP-mediated reverse reaction.

Because *in vitro* assay systems lack cellular reduction systems to maintain low peroxide levels, arachidonic acid-derived peroxides saturate the peroxide tone and, instead of inhibiting the cycle, APAP may promote maximal PGHS activity by accelerating peroxidase turnover (30).

Nevertheless, whether APAP really accelerates the reduction rate of Compound I to either Compound II or the ferric ground state is debatable. However, it is clear that higher peroxide levels (PGHS-1 $EC_{50}\approx$21 nM, PGHS-2 $EC_{50}\approx$2 nM) (31) are required to activate PGHS-1 because of a slower transition from Compound I (10^2-10^3 $M^{-1}s^{-1}$) to Compound II and the tyrosyl radical (11). In contrast, PGHS-2 is only limited by the peroxidase activity (2.5×10^7 $M^{-1}s^{-1}$). Therefore, APAP should exert a PGHS-1 preference due to the longer residence of Compound I. This was observed in activity assays with isolated enzyme but not *in vivo*.

Furthermore, we demonstrated that uric acid is as potent as APAP in inhibiting Sin-1 or LPS and A23187-enhanced PGHS activity in endothelial cells (Fig. 4). Uric acid is a very efficient scavenger of peroxynitrite but not of other peroxides. However, its ability to serve as a peroxidase reducing-substrate is rather poor compared to APAP. Similar effects were observed for other antioxidants (32). These data suggest that peroxynitrite is a potent endogenous activator of PGHS. The reason for the different efficacy of peroxide species in activating PGHS may be due to the chemically higher reactivity of peroxynitrite (1.7×10^7 M^{-1} s^{-1}) (33) compared to other peroxides (*e.g.*, t-butyl hydroperoxide 5.9×10^4 M^{-1} s^{-1}) (34) and the faster formation of Compound I. In cellular systems, compartmentalization and association of PGHS with NO-synthases may prefer peroxynitrite over other peroxides to modulate PGHS activity (35).

Inhibition by scavenging the peroxide tone provider peroxynitrite?

In a recent publication, we demonstrated that peroxynitrite, originating from the interaction of nitric oxide ($^{\bullet}$NO) and superoxide ($^{\bullet}O_2^-$) is one of the most potent

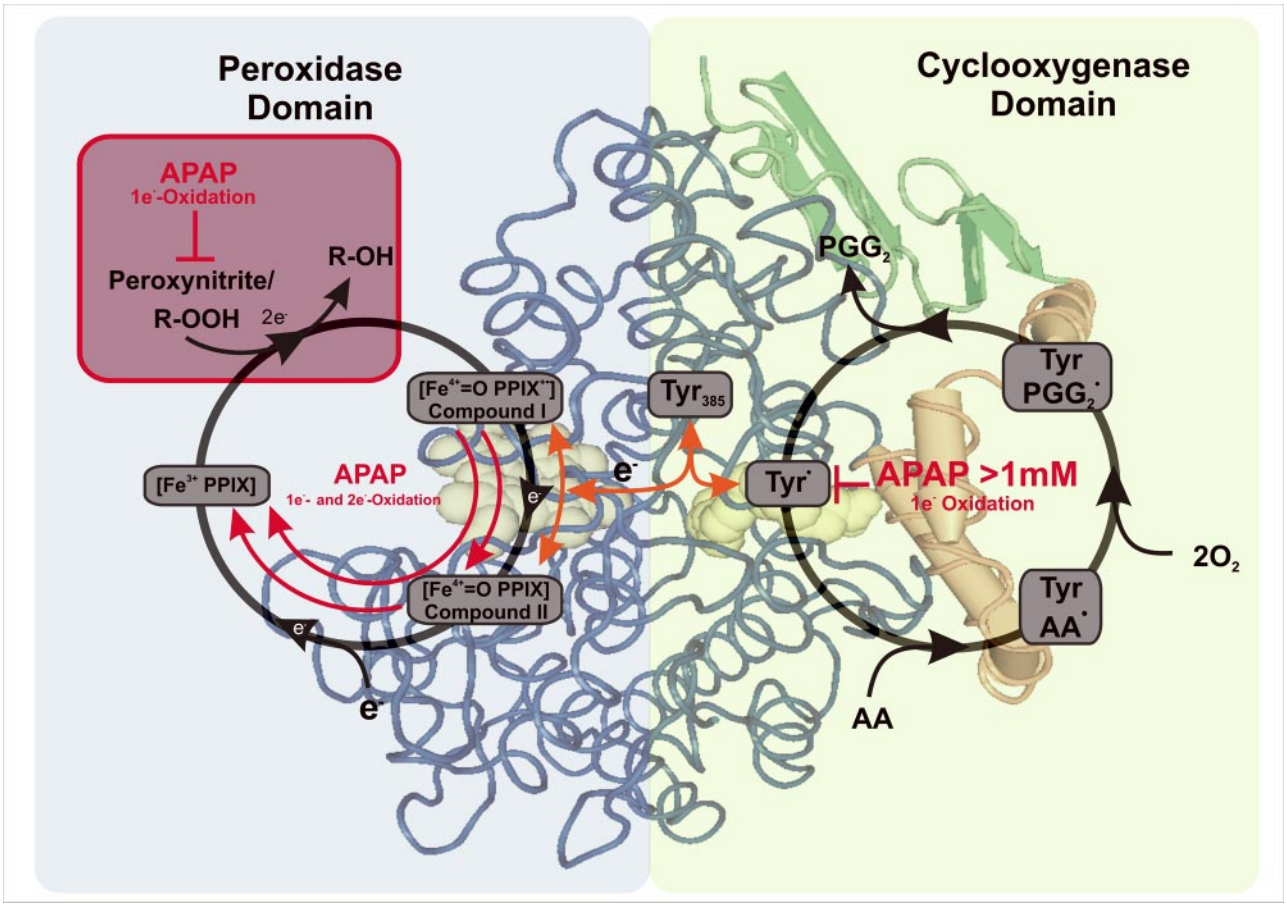

Figure 7. Acetaminophen inhibits prostanoid formation. The key enzyme in prostanoid synthesis is the homodimeric prostaglandin endoperoxide H_2 synthase (PGHS), which consists of two spatially distinct catalytic sites, the peroxidase (left panel) and the cyclooxygenase domain (right panel). Its substrate arachidonic acid (AA) is oxidized at the cyclooxygenase site by the tyrosyl radical ($Tyr^{\bullet}$) into PGG_2, which is further converted by heme (Iron Protoporphyrin IX) catalysis into PGH_2 at the peroxidase domain. The activity of PGHS is regulated by the so-called "peroxide tone", which describes the requirement of certain levels of peroxides to oxidize the heme, thus forming a tyrosyl radical at the cyclooxygenase *via* intramolecular electron transfer. Among other peroxides, peroxynitrite, originating from the interaction of nitric oxide ($^{\bullet}NO$) and superoxide ($^{\bullet}O_2^-$), emerged as one of the most potent endogenous activators of PGHS. Acetaminophen prevents PGHS activation, turnover, and prostanoid synthesis by scavenging peroxynitrite, lowering endogenous peroxynitrite-levels, or by reducing the heme back to the basal state. The background depicts the X-ray structure of the monomeric PGHS, showing (yellow) the iron protoporhyrin on the left panel and the bound arachidonic acid on the right. PP = protoporphyrin, Fe = Iron, APAP = acetaminophen, R-OOH = peroxide, R-OH = alcohol, AA = arachidonic acid, PGG_2 = prostaglandin endoperoxide G_2, PGH_2 = prostaglandin endoperoxide H_2.

intracellular activators of PGHS (21) in vascular smooth muscle cells. Unlike the high peroxide requirement for other peroxides, such as for H_2O_2, in the micromolar range, comparable PGHS activation by peroxynitrite was observed at low nanomolar concentrations in vascular smooth muscle cells (21), in platelets (Schildknecht *et al.*, unpublished), and in endothelial cells (Figs. 4 and 5). This activation process is of a transient nature and independent of post-translational covalent modifications.

In the current work, we have shown evidence that APAP scavenges the cellular PGHS activator peroxynitrite. Several methods were used to substantiate this hypothesis, including inhibition of Sin-1-mediated dihydrorhodamine 123 oxidation (Fig. 3*A*, *B*), HPLC analysis of reaction products (36), inhibition of phenol nitration (Supplemental Fig. S4*B*, Fig. 3*C*), and stopped flow measurements (Supplemental Fig. S4*A*). In addition, we provided evidence that APAP does not directly effect $^{\bullet}NO$, $^{\bullet}O_2^-$, or Sin-1 decomposition (Supplemental Fig. S3*A*, *B*). These data all suggest that APAP acts directly on peroxynitrite. Moreover, we demonstrated that extracellularly generated peroxynitrite increased PGI_2 formation in resting endothelial cells, predominantly expressing PGHS-1 (Fig. 2*B*), or in LPS-challenged cells also expressing PGHS-2 (Fig. 2*C*). This indicates that the endogenous peroxide tone was not saturated. Under these conditions, low intracellular peroxynitrite concentrations could easily be lowered by APAP and reduced below the threshold for PGHS-activation. A similar effect was observed with antioxidants (32) and NO-synthase inhibition.

Inhibition of PGHS or terminal synthases by catabolic metabolites?

PGHS and peroxidase-catalyzed 1- or 2-electron oxidations of APAP led to the formation of the reactive

intermediates N-acetyl-p-benzosemiquinone imine (NAPSQI) radical or *N-a*cetyl-*p*-benzo*q*uinone *i*mine (NAPQI), respectively (Supplemental Fig. S9). These reactive intermediates can form APAP multimers (16–18), adducts with GSH (16–18), protein-bound tyrosine or cysteine residues (16, 18, 37) and other yet unidentified cellular components. The reaction of peroxynitrite with APAP also leads to the formation of NAPQI and generates similar reaction intermediates as well as nitro-APAP (36). Thus, the reactive metabolites theoretically have the ability to interact with the enzymatic activity of proteins. However, we could show that APAP-multimers were ineffective in inhibiting PGHS activity in cell culture systems or *in vitro*. Thus, our data suggest that the metabolic end products are unlikely to PGHS inhibition.

In addition, although APAP can also undergo deacetylation by conjugating with arachidonic acid (AM404), which then can lead to potent inhibition of cyclooxygenase and activation of the endogenous canabinoid system (19, 20, 38), this occurs primarily in the brain. This might explain the very efficient antipyretic and analgesic effects of APAPs in the central nervous system but not in the periphery.

Why does acetaminophen exert weak anti-inflammatory effects?

Inflammatory sites are characterized by invading leukocytes, induction of early immediate genes such as PGHS-2, peroxidase-positive cells such as neutrophils, and formation of high amounts of oxidants, *e.g.*, peroxynitrite. Several effects might account for the lack of anti-inflammatory effects of APAP. The presence of other peroxidases (39), induction of PGHS-2, and high free radical levels might rapidly lower local APAP (peroxidase substrate) levels. A comparison between fibroblasts and macrophages has revealed that macrophages have a 16-fold higher rate of peroxidase-dependent bioactivation of phenolic compounds than fibroblasts (40). In addition, due to the high abundance and the very low requirements of PGHS-2 for activation, even low levels of peroxynitrite would still be sufficient to fully activate PGHS-2 (peroxide tone $EC_{50} \approx 2$ nM) (31). Moreover, higher levels of reactive oxygen species would overwhelm APAP's ability to maintain PGHS in the inactive basal state. These reasons could explain why a significant inhibition of prostanoid synthesis in endotoxin-challenged macrophages can only be observed at nonphysiological, *i.e.*, millimolar, concentrations of APAP. In contrast, endogenous formation of peroxynitrite in endothelial cells or smooth muscle cells (21) is relatively low compared with stimulated macrophages (41).

Summary

The data presented herein support the concept that APAP scavenges peroxynitrite, which acts as an endogenously formed cellular activator of PGHS-dependent prostanoid synthesis. Besides the established direct impact on the redox state of PGHS, the current report helps to explain the well-known differences in the effect of APAP in different cell types and could explain its beneficial role as a pain and fever-relieving compound with only marginal anti-inflammatory properties. FJ

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