Regulation of Death Receptor-Mediated Cell Demise by Glutathione

DISSERTATION

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1 Introduction

The regulation of cell death by endogenous factors as well as its control by pharmacological means is a field of great interest in biology and medicine. Physiologically occurring cell death or *apoptosis* is involved in a number of fundamental biological phenomena, and our knowledge about apoptosis signaling has dramatically increased in the past decade. The present study encompasses a number of *in vivo* studies which elaborated the impact of glutathione variations on death receptor-dependent liver injury models relying on both apoptosis (chapter 3) and necrosis (chapter 5) of hepatocytes. Additionally, the impact of glutathione alterations on a well-characterized apoptosis *in vitro* model, i.e. CD95 type I apoptosis in a leukemic cell line, was studied (chapter 4). In the first chapter, basic definitions of cell death are given, key mediators of apoptosis are introduced, and the relevance of hepatocyte apoptosis as well as the role of glutathione in cell death are discussed.

1.1 Modes of cell death: definitions and relevance

The development and maintenance of a multicellular organism requires the delicate balance of cell division and cell death. In this context, active cell death or *apoptosis* essentially serves to remove cells which are superfluous or damaged in a swift and unobtrusive manner[1,2]. Cells undergoing apoptosis display remarkable morphologic features such as shrinkage, chromatin condensation, nuclear fragmentation and wrapping up the cells' contents into sub-cellular particles termed apoptotic bodies. It must be emphasized that the word *apoptosis* was exclusively defined by these morphological features and thus describes the anatomy of cell death[3]. We learn more and more about the biochemical pathways that are responsible for these conspicuous changes, and in particular, certain proteases play a dominant role in this process[4]. Eventually, apoptotic cells indicate their suicide by specific membrane alterations and are subsequently recognized and phagocytosed *in vivo* either by neighboring cells or by professional phagocytes[5].

In contrast to *apoptosis*, *necrosis* or *accidental cell death* occurs particularly in pathological situations, and this term referring mostly to post-mortem events in tissues describes a passive, barely regulated form of cell demise. Cells undergoing *necrosis* display an early dysfunction of organelles, swell, and finally disintegrate after rupture of the cell membrane[2]. Apoptosis and necrosis often occur in a temporal sequence, and the fundamental distinction between apoptotic and necrotic cell death was blurred by the recognition that the same stimuli can induce either form of cell death, and furthermore by the finding that various intermediates between apoptotic and necrotic cell death exist[6-10].

A cautionary note has to be made to using the term *programmed cell death (PCD)*, since this definition is distinct from the designation apoptosis, although both are frequently used as synonyms. The expression *PCD* has been introduced in 1965 by the developmental biologist Lockshin and colleagues[11,12] and is a purely operational definition: (i) PCD occurs

in a developmental context, (ii) requires gene activation, and (iii) distinct cellular *death programs* are involved[13,14]. The latter argument is generally valid for both PCD and apoptosis, but examples for PCD without morphological features of apoptosis exist, and apoptosis can be executed in the absence of gene activation.

According to the above-mentioned pivotal functions of apoptosis in the developing as well as in the adult organism, a dysregulation of this process leading to either exaggerated or suppressed cell demise may underlie the pathogenesis of a number of diseases such as cancer, AIDS, and autoimmune or neurodegenerative disorders[15,16]. Distinct surface receptors were discovered about 10 years ago that transduce death signals, and the *death receptors* (DRs, 1.2.1) TNF-R1 and CD95 turned out as important triggers for apoptotic cell death: for instance, (i) DRs have been implicated in the disappearance of leukocytes in AIDS[17], (ii) CD95-deficient mice develop a severe lymphoproliferative disease[18,19], and (iii) DR-mediated apoptosis of hepatocytes is associated with the pathogenesis of various liver disorders (1.3). As a further example, apoptosis dysregulation has been discussed recently to contribute to multiple organ failure (MOF) in hyperinflammation foremost by three mechanisms, i.e. by enhanced apoptosis of parenchymal cells in various organs, by exaggerated lymphocyte apoptosis resulting in a state of immunosupression, and by an inhibition of neutrophil apoptosis giving rise to an accumulation of these potentially dangerous cells. Therefore, apoptosis-modulating pharmacological strategies are aimed to interfere with the dysregulation of apoptosis in sepsis, cancer and other disorders (reviewed in[15,16,20-23]).

1.2 Key components of apoptotic signal transduction

1.2.1 Death receptors

In general, physiologic cell death can be triggered either by gene activation (*programmed cell death*), by direct activation of intrinsic cell death programs in damaged or superfluous cells (*death by default*, 1.2.3), or *via* the activation of distinct death receptors (*instructive death*)[24]. All death receptors (DRs) belong to the rapidly expanding TNF/NGF (nerve growth factor) receptor family and represent type I membrane glycoproteins. Among the to date known six DRs (for overview see table 1), the signal transduction of TNF-R1 and CD95 has been studied most extensively in the past ten years (reviewed in[25-30]), and these two DRs are the most prominent triggers for apoptosis in the liver (1.3).

A. Apoptosis mediated by CD95. In 1989, the groups of Krammer and Yonehara independently recognized an unexpected cytotoxic activity of antibodies which were generated against tumor antigens[46,47]. After the discovery of the corresponding cytokine receptor independently termed APO-1[48] and Fas[49](official designation today: CD95), the respective ligand was purified and cloned in 1994[35,36].

receptor	consensus name	ligand	DR synonyms	DR references
DR1	**TNF-R1**	TNF	p55, TR60, CD120a	31-34
DR2	**CD95**	CD95L	APO-1, Fas	48,49
DR3	**APO-3**	APO-3L	TRAMP, LARD, WSL-1	37-39
DR4	**TRAIL-R1**	TRAIL	-	40,41
DR5	**TRAIL-R2**	TRAIL	APO-2, Killer, Trick 2	41-43
DR6	**DR6**	?	-	44

Table 1: Death receptors and their respective ligands*, compiled from*[26-29,45]*. The references given describe the cloning of the respective DRs.*

The receptor CD95 (synonyms: APO-1, Fas) is expressed in many tissues and cells, most abundant in thymus, heart, lung and liver[50]. In contrast to the widespread expression of CD95, CD95L production is principally restricted to the immune system, and most CD95L is found to be produced by cytotoxic T cells and natural killer (NK) cells[35]. Furthermore, immune-privileged areas and tumor cells produce substantial amounts of CD95L, presumably in order to withstand an attack of the immune system[24,51-54]. Both the receptor CD95 and its ligand CD95L exist in a membrane-bound as well as in a soluble form displaying distinct biological properties[55-59]. Apoptosis induced by the CD95 system plays an important role in regulating the cells of the immune system, especially T cell and B cells (reviewed in[54]). A further prominent role for CD95 outside the immune system is the maintenance of liver homeostasis (1.3).

A common characteristic of all DRs is the *death domain* (DD) in the cytoplasmatic tail, which represents a highly conserved 80 amino acid domain that transduces the apoptotic signal by protein-protein interactions. The term *death domain* was originally coined by Tartaglia *et al.* as a result of deletion studies on the TNF-R1[60]. Upon binding of CD95L to CD95 and trimerization of the receptor subunits, the DDs of CD95 and of the adapter protein FADD interact[61]. Subsequently, further molecules associate with the intracellular domain of CD95: FAP-1[62], Daxx[63], RIP[64], caspase-8 (see 1.2.2 B), viral or cellular FLIPs serving as a pseudo caspase-8 and thus antagonizing the apoptotic signal[65-68], and many others. Together, these proteins form a complex that is termed *death-inducing signaling complex*, DISC[69]. It turned out that the actual composition of the DISC is influenced by a number of factors, and it regulates the propagation of the CD95 apoptosis signal[65,70-72].

It has been first demonstrated for CD95 that the adapter molecule FADD can recruit and activate the upstream caspase-8 at the DISC level by physical interaction with another conserved sequence, the DED motive present on both FADD and caspase-8[73,74], thereby directly initiating the activation of the caspase proteases (1.2.2). Moreover, the involvement

of phosphorylation and kinase activation has been established in CD95 responses, but the biological significance of this aspect of CD95 signaling to date is not fully understood (reviewed in[75]).

B. Apoptosis triggered by TNF-R1. This receptor was discovered in 1985 by several groups and cloned about five years later[31-34]. The cytokine TNF, however, binds to two receptors (TNF-R1, TNF-R2), is a principal mediator of inflammation and can be produced by virtually all cell types. The biological response towards TNF is, compared to CD95L, far more diverse. Besides induction of cell death, TNF is able to mediate many biological processes such as growth, cachexia, cell adhesion, lipolysis, the production of acute phase proteins, and others (reviewed in[76,77]).

In line with this pleiotrophy, signaling mediated by TNF is of greater complexity compared to CD95 due to the following reasons (reviewed in[26,28,29]): (i) Hsu *et al.* described antother adapter molecule, **TRADD** (TNF receptor-associated DD)[78-80], which binds to the intracellular part of TNF-R1; TRADD then recruits FADD, which interacts and thereby activates pro-caspase-8 as mentioned for CD95[81,82]; furthermore, TRADD activates NF-kB *via* the DD-domain containing kinase RIP[83], the Jun-N-terminal (JNK) kinase pathway *via* TRAF2 (TNF receptor-associated factor 2)[84-87]; also, the cytosolic phospholipase A2 (PLA2)[88-90] and the acidic sphingomyelinase[91] are activated upon TNF-R1 activation (reviewed in[25,26,30]); (ii) TNF binds to two receptors, TNF-R1 and **TNF-R2**, the latter being not a DR[34,92]and presumably serving as a TNF recruitment mechanism that has been termed *ligand passing*[93]. However, TNF-R2 is described to induce apoptosis although it lacks a DD[94] and is further required for Con A-mediated hepatotoxicity *in vivo*[95]; (iii) recently, another signal transduction component of TNF-R1 has been revealed, the silencer of death domain (**SODD**) selectively binding to the DD of TNF-R1, a process which may be regulated by HSP70[85,96]; (iv) in contrast to CD95, **TNF-R1 internalization** is a prerequisite for apoptosis-propagating, but not other, signal transduction pathways[97]. However, the exact function of protein translocation in the induction of apoptosis is not understood[97-102].

C. Alternative death receptors: DR3-DR6. After the discovery and characterization of DR1 and DR2, four additional DRs have been described. DR3 and DR6 appear to be closely related to the TNF-R1 with regard to their NF-κB- and FADD-dependent caspase-activating capacity[37-39,103,104].

In particular, the cloning of the cytokine TRAIL and its receptors (for references see table 2) has revealed a novel and complex apoptosis-inducing system[16,26,29]. Additional to the two DRs TRAIL-R! and TRAIL-R2 (see table 1), several TRAIL-binding decoy receptors lacking a DD have been discovered[40, 41,105,106]. The biological role of the TRAIL-Rs has not been sufficiently established yet, but it appears that they abide to the principle of caspase-activation as established for CD95 (1.2.1 A). In contrast to a previous report[107], it was shwon recenty that both TRAIL receptors trigger apoptosis *via* FADD/caspase-8, and that this pathway can be inhibited by FLIPs[108-115].

The cytokine TRAIL holds an appealing potential for the treatment of cancer[16], since an unexpected differential sensitivity for TRAIL-induced apoptosis was observed in cancerous cells compared to normal cells[116,117]. However, it was also reported that TRAIL potently induces apoptosis in human hepatocytes, obviously enquestion a TRAIL-based anti-cancer strategy[118,119]. A detailed description of the TRAIL-Rs is provided in recent comprehensive reviews [16,26,29,113,120].

D. LPS can induce apoptosis, but not via DR activation. The known potential of bacterial products to directly induce apoptosis[121] has been challenged recently: in 1998, it was found that LPS-induced apoptosis involves FADD interactions and caspase activation, implying a conserved apoptotic signal transduction by so far unknown receptors[122-127]. Then, the toll-like receptors (TLRs) which mediate LPS responses have been discovered just recently, and this issue is of emerging interest[128-134]. Since also a cross-talk between the signaling of both receptor system, DRs and TLRs, is expected, it may be that *the light at the end of the tunnel might only be the light of the oncoming train.*

1.2.2 Caspases – the death proteases

In the early 90's, the study of cell death genes in the nematode *C. elegans* and the parallel finding that IL-1β is processed to its active form by a protease of a new family has lead to the discovery of mammalian caspase-1[135-138]. Since than, we have seen an unexpected rise in data concerning caspases, which is reflected by more than 4,000 articles published about this issue up to now (comprehensive reviews are recommended[16,139-141]). The name *caspase* eludes to their nature as cysteinyl aspartate-specific protease[142]. Caspases are regarded as the principal intracellular initiators and executors of apoptosis and caspases at the same time coordinately disrupt survival pathways and disassemble important architectural components of the cell. It is suggested that these events lead to the stereotypic morphological changes that characterize apoptosis, but the key substrates which are responsible for these changes have still to be found. Additionally, the nuclear DNA is degraded in a caspase-dependent manner early in apoptosis, marking the irreversible genetic death of the cell. Thus, a tight control of caspase activation is of vital importance for the cells' survival and therefore regulated in a complex manner.

A. Properties and classification of caspases. Caspases form a unique class of cysteine proteases and are characterized by the following properties: (i) caspases are present in all mammalian cells as latent inactive proenzymes (zymogens) with a size of 30 to 50 kDa containing three domains: the NH_2-terminal pro-domain, a large subunit (≈20 kDa) and a small subunit (≈10 kDa); (ii) as a post-translational modification/activation mechanism, procaspases are proteolytically processed by different pathways (see below) to form heterodimeric subunits which eventually assemble to active heterotetramers; (iii) caspases have a highly conserved active site region and use a unique catalytic mechanism; (iv) caspases cleave a defined subset of substrates after aspartic acid residues with the requirement of a tetrapeptide sequence as a minimum. However, caspase activation results

not necessarily in apoptosis, but is also implicated in other central biological processes such as T cell proliferation, differentiation, inflammation and long-term memory[143-148].

The 14 caspases known to date have been subdivided into three groups with regard to their structure, function, and cleavage specificity (table 2): **group I** caspases are implicated in the maturation of the pro-inflammatory cytokines IL-1β and IL-18 at the aminoacid sequence WEHD and do not have a significant role in apoptosis; caspases of **group II** cleave a number of death substrates during apoptosis at DxxD aminoacid sequences (see below) and thus have a pivotal role as central executioners in apoptosis; **group III** caspases are unique in their ability to become activated in protein complexes (see below) and possess comparatively long pro-domains with regulatory elements, e.g. DED (death effector domain) or CARD (caspase recruitment domain) motives. The recently discovered caspases 12, 13 and 14 cannot be categorized conclusively, since their preferred substrate specificities and their functions have not been determined yet.

B. Activation of group III caspases: apoptosis initiation. Activation of upstream group III caspases (also termed *initiator caspases*) occurs by a mechanism termed *recruitment activation* (reviewed in[4,30,141,181,182]): when the upstream pro-caspases 8 or 9 are recruited to the protein complexes DISC or apoptosome *via* interactions of either the DED or CARD domain, the respective pro-caspases can auto- or cross-activate. In the case of the DISC (1.2.1), a high local concentration of the pro-caspase-8 zymogen is sufficient to initiate the trans-activation of the zymogens[183-187]. For pro-caspase-9 activation, the reaction is mediated by a holoenzyme complex (apoptosome, 1.2.3) of about 700 kDa containing cyt c/dATP as cofactors, where pro-caspase-9 activates[188-194].

Also, some pro-caspases (e.g. pro-caspases 3 and 9) are able to auto-activate in the absence of protein complexes under certain conditions[195-199], and other proteases such as granzyme B or calpains can activate caspases (see below). Whereas the mechanisms of activating caspases 8 and 9 are well defined, the roles of caspase-10 and especially caspase-6 in apoptosis initiation are less apparent. It has been shown that inherited mutations of caspase-10 result in an autoimmune lymphoproliferative disease[200], and this caspase was reported to be recruited to CD95 and both TRAIL-Rs[170,201]. Likewise, caspase-12 seems to initiate a pro-apoptotic signal by a poorly understood mechanism that proceeds at the endoplasmic reticulum[202,203] although caspase-12 appears to be more related to group I caspases in terms of its structure[150].

Additionally, the scenario of apoptosis initiation includes compartmentalization and translocation of signaling proteins (reviewed in[204]). Pro-caspases as well as the active enzymes are localized within distinct cell organelles[205-207], e.g. sub-pools of inactive caspase-9 were found in the nucleus[208-210]. It has been demonstrated that caspases are activated upon TLR engagement, i.e. that the adapter protein RIP2 oligomerizes and thereby activates caspase-1, an interaction that is negatively regulated by the protein ICEBERG[128,211]. This activation reaction is very similar to the APAF-1/IAP/caspase-9 complex (see 1.2.3), indicating an evolutonary conserved caspase-activating machinery. Alternatively, it has been shown that caspase-11 is activating and thus upstream of caspase-1[212,213], and caspase-5 substitutes the murine caspase-11 in human cells[214].

function[1]	casp.	hu/mu[2]	synonyms	cleavage[3]	consensus[4]	references[5]
group I: cytokine maturation	**1**	hu/mu	ICE, IL-1β convertase	WEHD	**WEHD**	136,149,150,151
	4	hu	ICE_{rel}-II, TX, Ich-2	(W/L)EHD		150,152-154
	5	hu	ICE_{rel}-III, TY	(W/L)EHD		154,155
	11	mu	Ich-3	?		150,156
group II: apoptosis execution	**2**	hu/mu	Ich-1, Need2	VDVAD	**DxxD**	157, 158,150
	3	hu/mu	CPP32, Apopain, Yama	DEVD		138,159,160,150
	7	hu/mu	Mch3, ICE-Lap3, CMH-1	DEVD		161-163,150
group III: apoptosis initiation	**6**	hu/mu	Mch2	VEHD	**xExD**	164,150
	8	hu/mu	Mch5, MACH, FLICE	LETD		73,74,104,165
	9	hu/mu	Mch6, ICE-Lap6	LEHD		166,167,168,169
	10	hu	Mch4, FLICE-II	LexD		170,171
unknown	**12**	mu	-	?	**?**	150
	13	hu	ERICE	?		172
	14	mu	MICE	?		173-175

Table 2: Overview of the mammalian caspase family and [1]classification in three groups with respect to their functions, *adapted and compiled from[27,141,142,176-180]. With respect to the present literature, it is not possible to conclusively classify caspases 12, 13 and 14; [2]indicates whether a caspase is described for the human (hu) or murine (mu) system or both; [3]analysis of the cleavage site based on analysis of the cleavage specifities by combinatorial peptide library approaches[177,178,180]; "?" indicates unknown cleavage specificities; [4]"x" represents any amino acid; [5]references refer to the cloning of the respective caspase; underlined references indicate cloning of murine caspases.*

C. Activation and activity of group II caspases: cell death execution. By trans-activation, group III initiator caspases can cleave executor group II caspases at their respective xExD sequences. Thereby, a *caspase cascade* is triggered, including a number of caspase-caspase interactions[188,215-219]. Then, executor caspases (group II) cleave and thereby inactivate, disassemble, release or even activate a defined set of *death substrates*. Up to now, about 100 caspase substrates have been discovered, and many of the morphological and biochemical characteristics of an apoptotic cell are thought to be initiated by this highly specific proteolysis (reviewed in[4,27,220-222]). For instance, the Rip kinase and NF-κB become inactivated upon caspase cleavage[223,224], whereas the PAK2 kinase becomes activated by this mechanism[225]. As further examples, many structural proteins such as lamin or fodrin are

disassembled during apoptosis[226-228], and the fragmentation of nuclear DNA in multiples of 180 bp is executed by a DNAse which is indirectly activated by caspase-3[229-232]. Still, the search of *critical* caspase substrates is an open field for further investigations.

D. Redox sensitivity of caspases. Caspase are cysteine proteases and thus require a reduced cystein in their active site for full activity. Therefore, these proteases are highly sensitive towards oxidation by different mechanisms: thiol-modifying compounds such as dithiocarbamates, selenite or GSSG directly inhibit caspase activity, and also hydrogen peroxide can oxidize caspases *in vitro*, but obviously not under intracellular conditions[233-238]. However, pro-oxidative conditions were discussed to block apoptosis caspase oxidation (1.5). *Vice versa*, thioredoxin can reactivate oxidized caspase-3 in the µM range[239], and the reductant DTT is usually added to caspase activity assays in the mM range in order to prevent caspase autoxidation[240].

Furthermore, NO donors prevented apoptosis in some cell death paradigms[241-249], an effect that has been repeatedly linked to a direct S-nitrosation of the active site cysteine of caspases or pro-caspases[250-257]. However, this issue is under debate, since NO is pro-apoptotic in many experimental systems (reviewed in[258-260]), and data that argue against caspase S-nitrosation in hepatocytes were collected in the course of this thesis as outlined in the discussion section (6.3).

E. Are proteases distinct from caspases involved in apoptosis? Emerging evidence indicates that apoptosis can be mediated by alternative proteolytic pathways involving serine proteases, calpains, cathepsins and the proteasome (reviewed in[146,261-264]). In some cell death paradigms, apoptosis is entirely mediated by such alternative systems and occurs in the absence of caspase activation; alternatively, these pathways can interact with the caspase cascade. For instance, the cross-talk between **calpains** and caspases in cell death has been studied, and it was shown that calpains can induce apoptosis by caspase-12 activation[202,265] and furthermore cleave Bad, giving raise to a potent pro-apoptotic fragment[266]. Adding complexity, calpains can also proteolytically inactivate the initiator caspase-9 and thus block apoptosome-mediated apoptosis, and the calpain inhibitor calpastatin in turn is cleaved by caspases[264,267-269]. By the simultaneous release of **granzyme B** and perforin, cytotoxic T cells can induce death of target cells by two mechanisms[270]: granzyme B crosses the cell membrane of the target cell and can induce apoptosis *via* cleavage of caspase-3 and Bid[270-279], or can alternatively induce cell death directly in a caspase-independent fashion[280,281].

F. Caspase inhibitors and caspase knock-out mice. The dominant role of caspases in apoptosis has been elaborated by the use of pharmacological inhibitors, by overexpression of viral or endogenous inhibitory proteins and by targeted gene disruption, in particular by the generation of knock-out mice. Caspases can be efficiently inhibited by active-site mimetic peptide inhibitors which act as pseudosubstrates (reviewed in[282-284]). These relatively unspecific inhibitors are generated from a tri- or tetrapeptide sequence linked to a halomethyl ketone group, which can form a thiomethyl ketone with the active site thiol group of the caspase (irreversible suicide inhibitors, e.g. z-VAD-**fmk**); alternatively,

modified aldehyde peptides can be used (reversible inhibitors, e.g. Ac-DEVD-**CHO**). Depending on the experimental system investigated, the pharmacological inhibition of caspases may entirely block cell death e.g. in hepatocyte apoptosis (1.3.3), may delay or change the appearance of apoptosis[285], or switches the mode of cell death from apoptosis to necrosis[286-289]. However, non-specific effects of halomethyl ketone-derived inhibitors have to be considered in all these experimental approaches[290]. The therapeutic potential of caspase inhibitors in various disease conditions is enormous: for instance, caspase inhibitors were efficient to counteract ischemic organ injuries in five animal models (liver, heart, kidney, intestine, brain), they gave rise to promising results in animal models for neurodegenerative diseases, brain trauma and other disorders relying on exaggerated apoptosis rates (reviewed in[16,23]). Also, highly specific low-molecular weight non-peptide inhibitors have been introduced recently[291]. Since these compounds provide highly specific and potent anti-apoptotic tools especially due to their improved membrane-crossing properties, much effort is put in the development of this new generation of caspase inhibitors by at least five leading pharmaceutical companies[16].

Some cellular or viral gene products can inhibit caspases by protein-protein interactions (reviewed in[283,292]): (i) proteins of the broad **IAP** (*inhibitor of apoptosis*) family block caspases 3 and 9, and also caspase-9 activation at the apoptosome level[293-300]; (ii) the cowpox viral protein **CrmA** inhibits several caspases of group I and III in the picomolar range[301-303]; (iii) the baculoviral protein **p35** inhibits several caspases[302,304].

The critical role of caspases in apoptosis has been confirmed by the generation of various caspase-deficient mice, and this issue has been reviewed in detail[147,305-307]. Three examples are mentioned in brief: (i) due to developmental defects, **caspase-8-deficient mice** are not viable, but derived cell lines display prominent deficiencies in DR signaling[104]; (ii) **caspase-9 knock-out mice**[168,169] as well as APAF-1-deficient animals[308,309] die early in embryonic development primarily due to overgrowth of neuronal structures, and derived cells show distinct defects in apoptosome-mediated pathways; (iii) **mice deficient for caspases 1 or 11** do not display any developmental defects, but are less endotoxin-sensitive as a result of their disability to produce the proinflammatory cytokine IL-1β[212,310,311].

1.2.3 The role of mitochondria

Mitochondria have a Janus-headed function for the cell, since they are the energy producing organelles and thus provide the cell with ATP, but at the same time have the ability to kill the cell by triggering apoptosis. Today, it appears that besides the important role of DRs and caspases, mitochondria are the major initiators of apoptosis, and this issue has been reviewed extensively[209,312-318]. Upon a plethora of intrinsic or extrinsic death and survival signals, mitochondria integrate these informations and can rapidly provoke apoptosis *via* the release of several apoptogenic factors from the intermembrane space in the cytoplasm[319], i.e. caspases[210], cyt c[320-322], and AIF[323](figure 1); concomitantly, several non-apoptogenic proteins can be released can be released by mitochondira in such a situation[324].

For instance, redox stress[325] or ceramides[326] can induce the opening of a non-specific, high conductance permeability transition pore in the inner and outer mitochondrial membrane. This event causes a breakdown of the mitochondrial membrane potential ($\Delta\Psi$), and is frequently associated with mitochondrial swelling and uncoupling of the respiratory chain. This situation was termed *mitochondrial permeability transition* (MPT), and MPT has been described to occur in many pathophysiologic relevant circumstances[327-329]. Further, the pro-apoptotic protein Bax, a member of the Bcl-2 protein family, directly leads to cyt c translocation[330]. Likewise, truncated Bid (tBid) is formed upon cleavage of Bid by caspase-8[331,332], and tBid also mediates cyt c release from mitochondria[333](see also figure 1). Notably, cyt c release triggered by tBid or Bax occurs in the absence of MPT induction[334,335].

The best studied mitochondrial event in apoptosis induction is the translocation of the respiratory chain component cyt c from mitochondria into the cytoplasm [321,336,337]. The presence of cyt c in the cytoplasm connects the death sensor mitochondrion to caspases (figure 1), since cyt c together with APAF-1, pro-caspase-9 and dATP forms the apoptosome, a protein complex of approximately 700 kDa[193] in which active caspase-9 is formed after interaction of the respective CARD domains[120](see also 1.2.2, reviewed in[30]). At the other hand, the phylogenetically old flavoprotein AIF is released from mitochondria after MPT, and AIF can induce certain apoptotic nuclear alterations independent of caspases[232,338-340]. However, the detailed mechanism whereby these changes occur remain relatively require further elucidation[341].

Apoptosis induction by the mitochondrial-apoptosome pathway is controlled by multiple mechanisms (shown in figure 1): (i) most prominently, proteins of the broad **Bcl-2 family** both negatively (e.g. Bcl 2, Bcl-x_L) and positively (e.g. Bax, Bak) regulate this pathway, and these proteins additionally form hetero- and homodimers (reviewed in[4,336,342,343]); (ii) several **heat shock proteins** (HSPs) are known to possess an anti-apoptotic potential[344], and in particular HSP27[345-347], HSP70[192,348] and HSP90[349] have been recently reported to directly interfere with the apoptosome pathway, whereas the role of HSP60 in caspase-3 activation is less clear[350,351]; there is no general agreement as to the definite underlying mechanisms of HSP interference with the apoptosome pathway[352]; (iii) proteins of the **IAP family** such as XIAP or c-IAP1/2 negatively regulate the activation of caspases 3 and 9 by complex and not completely understood mechanisms[293-300]; (iv) as an additional level of regulation, the protein **Smac**[353,354]**/DIABLO**[355] sequesters IAPs, thus serving as a pro-apoptotic *antidot-antidot*; notably, Smac/DIABLO is translocated from the mitochondria together with cyt c, suggesting that the balance between these proteins and the IAPs determines the overall sensitivity of the cell towards apoptosis induction *via* the apoptosome pathway (figure 1, reviewed in[356]). The design of mitochondrion-targeted cytotoxic drugs was proposed recently as a novel strategy for e.g. overcoming apoptosis resistance of cancer cells[357,358].

1.2.4 Type I and type II apoptosis: two distinct pathways triggered by CD95

Based on the findings that DR-triggered apoptosis can be inhibited by Bcl-2[359] and amplified by Bid cleavage leading to mitochondrial cyt c release[331,360], the group of Peter presented in 1998 a concept which integrates DR- and mitochondria-mediated signaling pathways[361]. The original *two-pathway hypothesis* claimed that, depending on the cell type, CD95 activation directly activates a caspase cascade **or** additionally includes the mitochondrial pathway to eventually result in apoptosis. This helpful simplified scheme has found widespread acceptance in the literature[4], but has to be carefully applied to other DR-dependent models and was also under criticism (reviewed in[362]) mainly due to methodical concerns[71,363-365].

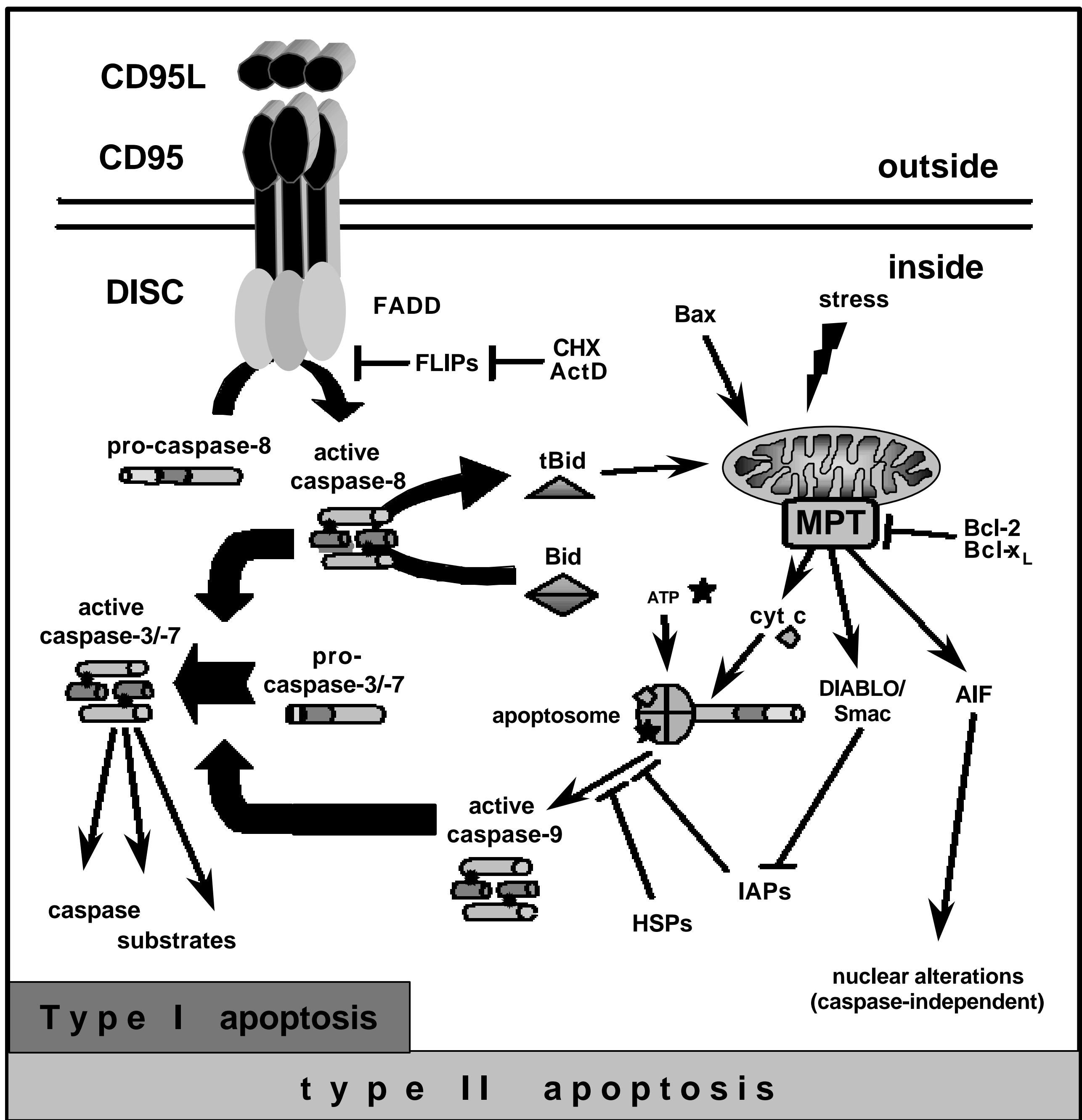
As outlined in figure 1, the first signaling events following CD95 trimerization and DISC formation are similar in type I and type II apoptosis, and in both cell types, FLIP proteins (1.2.1) can interfere at the DISC level and thereby inhibit caspase-8 activation[70,361]. Since FLIP proteins have a short half-life, FLIP levels substantially decrease when cells are treated with inhibitors of protein (CHX, cycloheximide) or RNA synthesis (Act D), possibly explaining the sensitization against CD95-mediated apoptosis by these compounds[72].

In the course of studying the DISC formation after treatment of cell lines with an activating anti-CD95 antibody, it was recognized that SKW6.4 and H9 cells (type I cells) are able to activate large amounts of caspase-8 at the DISC level, whereas Jurkat or CEM cells (type II cells) only process minute amounts of active caspase-8[361]. Further, the protein Bid is truncated by caspase-8 giving rise to tBid, which in turn triggers the release of cyt c, and the latter process is known to be sensitive towards inhibition by Bcl-2/Bcl-x_L (1.2.3). In this line, only type II cells depend on the mitochondrial *amplification loop*, and only type II apoptosis can accordingly be blocked by Bcl-2/Bcl-x_L. Importantly, these differences between type I and type II cells were also detected when the CD95L was used instead of an activating anti-CD95 antibody[71]. Apart from tBid, also cellular stress (e.g. ROI[325,366,367], ceramides[70,326,368]) and Bax can lead the concomitant release of cyt c, AIF and DIABLO/Smac from mitochondria (figure 1).

With regard to present data, this hypothesis can be extended as follows. (i) The strict classification of *cells types* in type I or II is not always conceivable, since also a single given cell type might be able to follow both CD95 pathways depending e.g. on its differentiation status: primary human T cells undergo type II apoptosis on day one *in vitro*, whereas they appear to stick to the type I paradigm after five days in culture (I. Schmitz, personal communication). (ii) It was discussed recently that a plethora of methodical variables and experimental conditions determines whether a cell would behave more like a type I cell or as type II cell in different settings; thus, a continuum of cell death pathways requiring apoptosome formation to different extents is suggested[362-365]. (iii) In this line, it was under debate whether diverse primary cells can be classified as type I or type II, and obviously, this strict classification is difficult due to the above-mentioned arguments; this difficulty is also apparent for the classification of hepatocyte CD95 apoptosis (1.3.2). (iv)

The formation of a functional apoptosome is essential for CD95 type II apoptosis and requires sufficient ATP/dATP levels[186,188,194,369-371]. Accordingly, when intracellular ATP concentrations are lowered e.g. by respiratory chain inhibitors, CD95 ligation in type II cells (e.g. Jurkat) results in cell death with typical necrotic features[10,372]. On the other hand, type I cells (e.g. SKW6.4) when depleted of ATP undergo still apoptosis, but late energy-requiring nuclear alterations are slightly affected[373]. As a further variable, the glutathione requirement of both CD95 pathways was comparatively studied in the present thesis (chapter 4).

***Figure 1: Two major apoptosis CD95 pathways**, adapted from[4,30,361]. For details, see text. DISC, death-inducing signaling complex; FADD, FLICE-associated death domain; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis protein; AIF, apoptosis-inducing factor; HSP, heat shock protein; tBid, truncated Bid.*



1.3 Death receptor-mediated apoptosis in the liver

Apoptosis is responsible for the cellular default demise of hepatocytes and thus important for hepatic tissue homeostasis and the maintenance of liver function. For instance, toxin-damaged[374] or senescent[375] hepatocytes are rapidly eliminated by apoptosis. An exaggerated or diminished apoptosis rate in the liver is therefore suggested to be involved in the pathogenesis of several hepatic disorders, i.e. hepatitis of viral or auto-immune origin, alcoholic hepatitis, Wilson's disease, hyperinflammatory liver failure, primary biliary cirrhosis, transplant rejection and toxic liver injury (reviewed in[376-382]).

In human patients, *circumstantial evidence* points to an involvement of the CD95L/CD95 and the TNF/TNF-R1 systems in several liver disorders (reviewed in[383-385]), e.g. an upregulation of CD95 expression and an increase of apoptotic hepatocytes was seen in fulminant liver injury and hepatitis B[386-388], an enhanced TNF/TNF-R1 expression and a concomitant increase in hepatocyte apoptosis was observed in alcoholic liver disease and in fulminant hepatic failure[389-391], and *vice versa*, an upregulation of CD95L was described in transformed carcinogenic hepatocytes[385,392,393]. Four examples can be given for a *causal involvement* of the two mentioned DRs in disease-related models, i.e. their auto- or paracrine overactivation can result in exaggerated hepatocyte apoptosis: **CD95** triggering (i) was shown to be important *in vivo* in a murine model of cholestatic liver damage and in bile salt-induced hepatocyte apoptosis *in vitro*[394,395], and (ii) was responsible for copper overload-induced hepatocyte death, which is regarded as an *in vitro* model for Wilson's disease, a copper overload disorder[387,396]. **TNF-R1** engagement mediated hepatic damage (iii) in a mouse model of alcoholic liver injury[397,398] and (iv) after intoxication of mice with the classical hepatotoxins amanitin or actinomycin[374]. Conclusively, the accurate regulation of DR-mediated apoptosis appears to play a significant role for the vitality of the liver, and DR signaling pathways may be a target for the prevention of exaggerated hepatocyte death in certain instances (1.3.4).

1.3.1 Murine models of death receptor-induced hepatic apoptosis and necrosis

To study the regulation of hepatic apoptosis and to test pharmacological intervention strategies, a number of mechanistically different mouse models for DR-mediated liver injury have been developed. As summarized in table 3, the following six models were used in this study:

A. Liver injury models involving hepatocyte apoptosis executed by caspases. In galactosamine (GalN)-sensitized mice, the injection of low-dose (2-10 µg/kg) lipopolysaccharide (**GalN/LPS model**) leads to endogenous production of TNF mainly by resident liver macrophages (Kupffer cells), and TNF then induces hepatocyte apoptosis. Likewise, recombinant TNF can be used (**GalN/TNF model**). In both cases, cell death is triggered *via* TNF-R1 and caspase activation; sinusoidal endothelial cells are not involved in

organ damage[377,379,399-401]. Notably, it is still unclear why a transcriptional arrest by e.g. GalN is a prerequisite for TNF-R1-induced apoptosis in the hepatocyte[402].

Independent of the TNF/TNF-R1 system[400], the application of the activating anti-CD95 antibody Jo-2 (**CD95 model**) in naive mice leads to lethal liver destruction within hours due to massive caspase-mediated apoptosis of hepatocytes[400, 403-405]. Importantly, this effect does not require a sensitization of the animals and is also seen when instead of αCD95, the CD95L is used or endogenously generated[406,407]. It was reported that also other organs than the liver are affected by αCD95[408,409], but this depends largely on the antibodies and mouse strains used and is of minor importance for the αCD95 antibody Jo-2[405]. CD95 is also highly expressed in sinusoidal endothelial cells (SEC)[410], and a prominent SEC damage precedes the onset of liver damage in this model[411].

B. High-dose treatment of naive mice with hepatotoxins. Two classical hepatotoxins of fungal origin, actinomycin-D and α-amanitin, are potent inhibitors of hepatic transcription that sensitize hepatocytes towards low levels of endogenously produced TNF (**Act D model, α-amanitin model**). By a paracrine action of locally produced TNF on TNF-R1 in the liver, caspase-independent, but morphologically clearly definable hepatocyte apoptosis including DNA fragmentation is initiated[374,412]. Likewise, high-dose GalN was reported to induce TNF-dependent liver damage in the absence of caspase activation[413]. It can be suggested that alternative protease pathways mediate cell death in these models (1.2.2 E).

model	HC apop.[1]	SEC dam.[2]	DEVD cleav.[3]	mediated by	endog. cytokines	references[4]
GalN/LPS	+	-	+	MΦ	TNF	401,429,433,434,435
GalN/TNF	+	-	+	-	-	429,434,435
CD95	+	+	+	-	-	403,411,436
Act D, α-amanitin	+	?	-	MΦ	TNF	374,412
Con A	+/-	+	+/-	MΦ, TC, NK	TNF, IFN-γ, IL-4	95,412, 414,416,417, 419,420,423
LPS shock	-	-	-	MΦ	TNF	401,427

Table 3: Overview of cytokine-mediated liver injury models which have been used in this study. *It is indicated whether [1]hepatocyte apoptosis or [2]sinusoidal endothelial cell death is implicated in the onset of liver injury, [3]whether DEVD-afc-cleaving activity of group II caspases occurs in the respective model; "+/-" indicates that contradictory data have been published (see text); further, immune cells (MΦ, macrophage; TC, T cells; NK, natural killer cells) and cytokines which mediate liver injury are indicated; [4]references are given for the first description of the model (underlined) and for all additional properties mentioned in the table.*

C. The Con A model. Concanavalin A (Con A) is a plant lectin that activates T cells *in vitro* and *in vivo*. When injected into mice, Con A triggers a selective liver injury[414] which depends on the release of the cytokines TNF[415,416], IFN-γ[417,418], and IL-4[419]. Further, the Con A model involves an early SEC damage[412,420] and requires a cross-talk between macrophages and T cells[421]. In the Con A model, both necrotic and apoptotic hepatocyte demise with or without a contribution of caspases have been described (see also 6.3)[95,379,414,416,422,423]. In contrast to the *in vivo* situation, stimulation of hepatocytes *in vitro* with Con A elicits cytotoxicity due to cytoskeletal disturbances with a distinct morphology[424].

D. Liver injury in the LPS shock model. Injection of high doses of LPS (2-20 mg/kg) in naive mice is a commonly used animal model for a systemic inflammatory response syndrome (SIRS, reviewed in [425,426]). Here, various organs are affected by an infiltration of activated neutrophils and an exaggerated cytokine production, and the mode of hepatocyte cell death is regarded to be primarily necrotic in this model, although it depends on TNF[401,427,428]. By injection of intermediate doses of 100-200 μg/kg LPS in GalN-sensitized mice, a liver injury model with features of both the GalN/LPS model (caspase-dependent hepatocyte apoptosis) and the LPS shock model (neutrophil infiltration) is obtained[429-432].

1.3.2 Physiological regulation of death receptor-mediated hepatocyte apoptosis

A. Immune modulation of DR-induced hepatocyte apoptosis. Hepatic apoptosis induced by TNF or CD95L may be regulated by a number of additional cytokines in the *in vivo* situation. For instance, the pro-inflammatory cytokine IL-1β was shown to prevent hepatic damage in the GalN/TNF model[241,399], whereas in the CD95 model, IL-1β was reported to have no effect[400] or to mediate hepatoprotection[437]; notably, *intracellular* IL-1β was also shown to act as an endogenous inhibitor of the CD95 pathway in cell lines[438]. Likewise, the hepatoprotective potential of IL-15 in the CD95 model[439] and of the immunomodulatory compound linomide in several liver injury models[440,441] cannot be conclusively explained. At the other hand, the IFN-γ-inducing factor IL-18 can enhance hepatic apoptosis under certain pro-inflammatory conditions[442-444], and chronic active hepatitis is induced in mice strongly overexpressing IFN-γ in the liver[445]. Thus, IL-18/IFN-γ may act as positive regulators of hepatocyte apoptosis *in vivo*, and also *in vitro*, hepatocyte apoptosis can be initiated by TNF plus IFN-γ[446,447].

B. The role of acute phase proteins. Under inflammatory conditions, the liver produces a number of different acute phase proteins which fulfill many protective and regulatory functions. In the GalN/LPS model, the acute phase protein and serine protease inhibitor α_1-antitrypsin was demonstrated to inhibit the release of TNF in the circulation and was suggested to prevent the onset of liver injury by this mechanism[448]. Later, it was shown that α_1-acid glycoprotein and α_1-antitrypsin both prevent hepatic apoptosis and lethality in

the GalN/TNF model, but not in CD95-triggered apoptosis[449-451]. However, there is no information available by which mechanism anti-apoptotic effects are mediated. Both proteins α_1-acid glycoprotein and α_1-antitrypsin do not prevent hepatocyte apoptosis in the respective *in vitro* models. Thus, the observed protection *in vivo* appears to be indirect and mediated e.g. by the induction of the platelet-activating factor (PAF) as suggested by Van Molle *et al.*[450,451].

C. Energy requirement of hepatocyte apoptosis. Besides glutathione (chapter 1.4.), the cellular ATP content is another apoptosis-related metabolic parameter[10,373] that can be modified selectively in the liver. When hepatic ATP is depleted by the use of different carbohydrates such as fructose[452], an inhibition of TNF-R1-triggered apoptosis, but a potentiation of CD95-mediated apoptosis was found *in vivo* and also in primary cultured hepatocytes *in vitro*[453]. Since fructose was previously shown to be cytoprotective in other experimental systems due to its antioxidative and other properties[454,455], it was clearly elaborated in this study[453] that prevention of TNF-R1-triggered apoptosis by carbohydrates occured as a result of their ATP-depleting capacity. The inhibition of TNF-R1 signaling was located upstream of mitochondrial function, indicating that a very early and so far unidentified step requires ATP in this model. It can be hypothesized that either the TNF-R1 DISC formation[69,96] or the internalization of TNF-R1[101,102] is affected under the condition of low ATP in the hepatocyte.

D. Nitric oxide in DR-mediated liver injury. The short-living molecule nitric oxide (NO) is produced by the enzyme NO synthase (NOS) and represents a potent biologic mediator with both pro- and anti-apoptotic properties[248,259,260,456-458]. In the liver, NO can be produced by the iNOS[459] and has a number of diverse functions[381,458,460-462]. In experimental systems, NO can be generated by NO-releasing compounds such as SNP (sodium nitropusside). With regard to hepatic apoptosis, the application of SNP prevented GalN/TNF-triggered hepatotoxicity[241], and a protective effect of various NO donors in TNF-induced hepatocyte apoptosis was described repeatedly by the group of Billiar[245,251,253,463,464]. It appears that this protective effect is confined to TNF-R1-mediated hepatocyte apoptosis (6.4), and the following mechanisms have been proposed to account for apoptosis inhibition by the above-mentioned group: upregulation of HSP70[463], cGMP-dependent mechanisms[244,465], prevention of mitochondrial dysfunction[251] and Bcl-2 cleavage[466], and S-nitrosation of caspases[245,252,253]. However, it has not been clearly demonstrated whether caspases can be S-nitrosated under intracellular conditions in the hepatocyte (see also 1.3.3 A and 6.4).

1.3.3 Does hepatic CD95-mediated apoptosis represent type I or type II?

The contribution of mitochondrial disturbances leading to MPT, the release of cyt c and the subsequent induction of apoptotic or necrotic cell death is known to occur in liver disorders such as hepatic ischemia/hypoxia or toxin-induced hepatocyte damage[327-329,467]. Likewise, the MPT is clearly involved in TNF-triggered cell death[468,469], also in the hepatocyte[379,470]. Here, it will be discussed why the situation for the CD95 system with regard to the mitochondrial pathway is still unclear.

As outlined in chapter 1.2.4, the signaling of CD95 includes the mitochondrial branch and the cyt c/apoptosome pathway in some cell types (type II cells), whereas CD95 induces apoptosis without any mitochondrial contribution in others (type I cells, see also figure 1). In the original paper by Scaffidi *et al.*[361], it was suggested that hepatocytes represent type II cells, since two earlier studies reported that Bcl-2-overexpressing mice are insensitive towards induction of hepatic damage by αCD95[471,472].

However, exclusively *in vivo* data are presented in these papers, and the animals died with and without liver-specific overexpression of Bcl-2 in one study after a very high dose of αCD95[471], whereas lethality was prevented in the second study as a result of Bcl-2 overexpression in several organs[472]. Moreover, Yin *et al.* showed recently that Bid-deficient mice are partially protected from αCD95-mediated effects, but primary cultured hepatocytes derived from these animals underwent apoptosis upon TNF or αCD95 treatment notwithstandingly[473,474]. In this line, own experiments with Bcl-2-overexpressing hepatocytes revealed no differences between wild type and overexpressing cells with regard to CD95-triggered apoptosis (chapter 5). As mentioned in 1.3.2 B, the metabolic depletion of ATP does not lead to an inhibition, but to an enhancement of hepatocyte apoptosis in the CD95 model *in vitro* and *in vivo*[453]. This suggests that the apoptosome pathway, which requires ATP, might not be involved in this cell death paradigm. In line with these arguments, it was clearly demonstrated by Hatano *et al.* recently that only TNF-R1-mediated apoptosis of primary cultured murine hepatocytes depends on MPT, whereas MPT only accelerates CD95 apoptosis; apparently, the induction of MPT is not a necessary event in the CD95 model *in vitro*[475], but occurs concomitantly with hepatocyte death *in vivo*[476]. Thus, hepatocytes appear to react upon CD95 engagement as type I cells *in vitro*, whereas a type II component might be operative *in vivo*. Given a possible role of sinusoidal endothelial cell damage for CD95-mediated apoptosis in the *in vivo* situation[410,411], these contradictory findings might be explained by a effect of Bcl-2/Bid on sinusoidal endothelial cells. Conclusively, CD95-triggered apoptosis in the *hepatocyte* itself appears to be precede independent of the mitochondrial branch.

1.3.4 Pharmacological and genetic intervention strategies against hepatic apoptosis

The inhibition of hepatic apoptosis is desirable in situations where exaggerated hepatocyte death contributes to the pathogenesis of e.g. fulminant liver injury or toxin-induced hepatitis. Therefore, the following experimental approaches were developed to control hepatic apoptosis:

A. Pharmacological interventions. Caspases are activated after receptor engagement in CD95- and in TNF-R1-mediated hepatic apoptosis[429,436,477,478], and it has been demonstrated in several studies that an inhibition of caspases by peptide-derived inhibitors (1.2.2 F) results in a complete prevention of receptor-mediated hepatocyte apoptosis *in vivo* and provides long-term survival of animals[479-481]. Also, caspase inhibitors were promising in the prevention of hepatocyte apoptosis in hepatic ischemia[482]. Recently, low-molecular-weight caspase inhibitors have been introduced that may be more specific for distinct caspases and provide better cell permeability[16,283,291]. The anti-apoptotic property of NO in the liver (1.3.2 C) can also be used for pharmacological intervention: the liver-specific NO-releasing prodrug V-PYRRO/NO has a potent hepatoprotective quality in the GalN/TNF model without affecting hemodynamics[244]; likewise, a NO-releasing Aspirin derivate mediated hepatoprotection in the Con A hepatotoxicity model[423]. A number of other compounds such as colchicine[483,484], linomide[441], and verapamil[485] turned out to be protective in DR-dependent liver injury models, but the detailed underlying mechanisms remain to be elucidated.

B. Genetical approaches. By gene transfer and generation of transgenic mice, several strategies with a conceivable therapeutic potential have been tested in animal models and primary hepatocytes: (i) mice overexpressing the anti-apoptotic proteins CrmA[486], SV40[487], or Bcl–2[471,472] were protected against DR-mediated hepatic damage; (ii) by injection of mice with an oligonucleotide antisense which inhibits CD95 expression in hepatocytes, liver injury upon CD95 treatment was entirely blocked[488]; (iii) the hepatoprotective mediator HGF (hepatocyte growth factor)[489,490] was anti-apoptotic *in vivo* when applied by adenoviral gene transfer[491]; in this line, a HGF gene therapy prevented liver cirrhosis in a rat model[492]; (iv) with an adenoviral vector carrying the iNOS gene, TNF-R1-mediated apoptosis in rat hepatocytes was inhibited[493]. Thus, the targeted upregulation, inhibition or repair of distinct genes involved in apoptosis regulation may provide a perspective for future intervention strategies.

1.4 Glutathione: metabolism and influence on cell death

1.4.1 Function and metabolism of glutathione

The tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine) represents the most abundant intracellular non-protein thiol. Thus, GSH serves as the major antioxidant and provides defense against xenobiotics as a phase II conjugation substrate[494-496]. As coenzyme and low-molecular weight thiol, GSH affects numerous central cellular functions such as metabolism, DNA and protein synthesis, transport, catalysis, cell growth, gene transcription, and apoptosis[497-499]. Furthermore, GSH ensures the maintenance of thiol moieties of proteins and the reduced forms of e.g. coenzyme A or ascorbic acid. Therefore, cells tightly regulate synthesis, utilization and export of glutathione. Under normal conditions, intracellular GSH concentrations are well maintained within the millimolar range[496]. The highest GSH concentrations in the organism are found in the alveolar fluid (about 30 mM), in the eye (about 25 mM) and within hepatocytes (about 10 mM), whereas e.g. the brain displays significantly lower GSH contents[500].

An overview of glutathione metabolism is outlined in figure 2 and reviewed in[495,496,501,502]. GSH is synthesized in the cytoplasm from glutamate, cysteine and glycine by two consecutive reactions of the ATP-dependent enzymes γ-glutamylcysteine synthase (γGCS) and GSH synthase (GS), the former enzyme being sensitive towards inhibition by BSO (see also 1.4.2). GSH synthesis is limited by substrate availability, usually cysteine being the limiting substrate, and furthermore regulated by a non-allosteric feedback inhibition by GSH with a K_i of 1.5 mM[502].

The destiny of GSH is characterized by the following three main pathways (figure 2): (i) GSH can be oxidized to glutathione disulfide (GSSG, also referred to as *oxidized glutathione*) by non-enzymatic reactions with peroxides and protein disulfides, or by the reactions of GSH peroxidases (GPx), selenium-dependent enzymes that consume peroxides (ROOH) using GSH as an electron donor. Subsequent to the superoxide dismutase/catalase system, these reactions represent a dominant antioxidant defense of the cell. Glutathione is maintained predominantly in its reduced form by the action of the GSSG reductase (GR) at the expense of NADPH as cosubstrate. As a result of this effective recycling reaction, GSSG concentrations are kept quite low within the cell, i.e. the GSH/GSSG ratio is usually found to be >100[503]. (ii) As the second major GSH-consuming reaction, diverse GSH S-transferases (GST) conjugate a variety of xenobiotics to generate a large set of thioethers (phase II reactions), which are usually committed to detoxification and elimination as mercapturic acids. GSH S-conjugates as well as GSSG are actively extruded by the cell. This energy-requiring process is mediated by transmembrane proteins belonging to the recently discovered multidrug-resistance protein (MRP) family[504,505]. These GSH-consuming reactions can be also exploited to create an experimentally desired depletion of GSH (1.4.2). (iii) GSH can be furthermore degraded by the membrane-bound enzyme γ-glutamyl transpeptidase (γ-GTP), i.e. the γ-glutamyl moiety of GSH is cleaved by this membrane-

bound enzyme outside the cell. Then, transpeptidation reactions follow which generate γ-glutamyl amino acids, that can be imported in the cell and reutilized for GSH synthesis[496].

Besides these three major fates of GSH, an enormous variety of biologically important GSH derivates with complex chemistry is generated within the cell, e.g. S-nitroso-glutathione (GSNO), thiolesters, the glutathionyl radical and protein-bound GSH[506]. When GSH synthesis is entirely blunted by inborn γGCS or GS deficiency, hemolytic anemia, cataract formation and an impaired central nervous system function result from exaggerated oxidative stress by chronic GSH deficiency[496,498]. However, this circumstance is in general compatible with human life, implying that all above-mentioned functions of GSH are not exclusive, but redundant. In this line, recent reports concerning the generation and characterization of GS-heavy subunit and γ-GTP knock-out mice which are both GSH-deficient revealed that these animals have severe developmental defects and are not viable. Notably, cell lines derived from GS-heavy subunit-deficient mice were viable and divided not only after supplementation with GSH, but also after addition of NAC. This suggests that the presence of GSH as such is not required for cell division and metabolism[507,508].

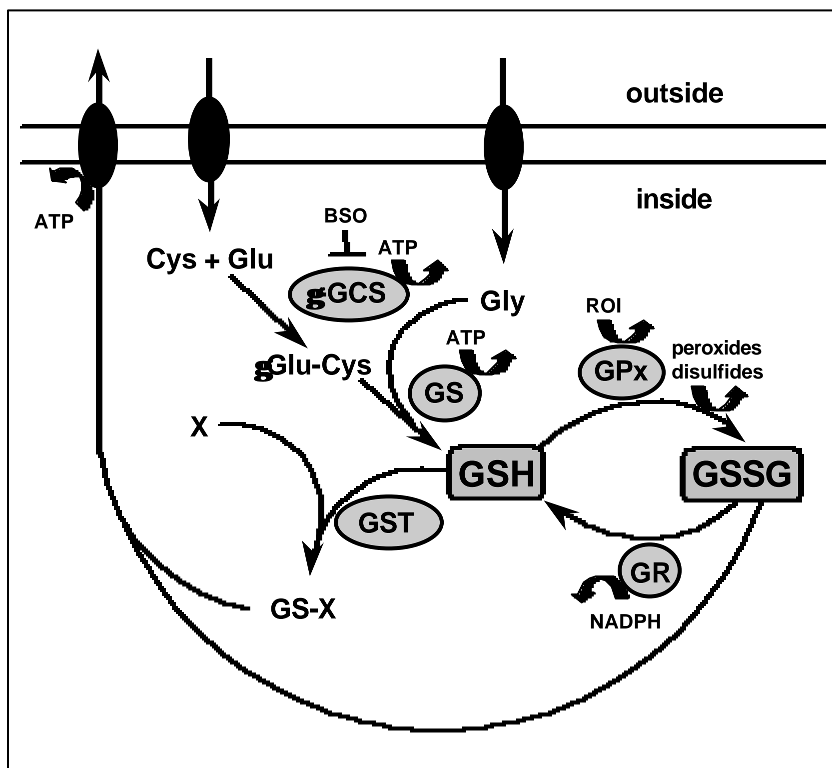


Figure 2: Outline of glutathione metabolism. *Additional abbreviations used: GPx, GSH peroxidases; GR, GSSG reductase; g-GCS, g-glutamylcysteine synthase; gGlu-Cys, g-glutamylcysteine; GST, GSH S-transferase; GS, GSH synthase, NADPH, nicotin-amid dinucleotide phosphate; X, xenobiotics or GSH de-pletors (CDNB, DEM, pho-rone).*

Adapted from[495,496,501,502,506].

1.4.2 Experimental approaches to modulate intracellular glutathione levels

To study the role of glutathione in biological systems, it is often desired to lower or enhance intracellular GSH pools. In general, an intracellular GSH deficiency can be experimentally created by four mechanisms: (i) A frequently used approach is the irreversible **inhibition of glutathione synthesis** by buthionine sulfoximine (BSO), which affects all organs and depletes GSH in the cytoplasm as well as in mitochondria. Repetitive treatment of cultured cells or animals over a period of hours to days is required to lower GSH by BSO. Treatment of mice with BSO for longer terms leads to mitochondrial swelling, diverse organ damage, and, most remarkably, brain damage[496,502,509]. (ii) By **enzymatic GSH depletion**, α,β-unsaturated carbonyl compounds such as diethyl maleate (DEM), cis-chloro dinitrophenol (CDNB) or phorone preferentially deplete GSH *via* enzymatic conjugation by GSH S-transferases (figure 2), followed by excretion of these conjugates[509,510]. In this case, preferable cytoplasmatic and nuclear, but only to a lesser extent mitochondrial GSH is depleted[511]. Since a depletion of GSH by GSH S-transferase substrates directly stimulates GSH synthesis which in turn consumes ATP[512](1.4.1), it is a common approach to combine these GST substrates with a low concentration of BSO to achieve a swift, sustained and non-toxic GSH depletion. (iii) GSH can be **chemically oxidized** and thereby depleted *via* GSSG export by substances such as Diamide[506] or by an indirect mechanism with phenolic redox-active compounds[513,514]. (iv) **Starvation** results in a gradual decrease of intracellular GSH due to the lack of the GSH precursor cysteine[496,498,502]. It is important to address a side effect of the GSH depletor DEM, i.e. this compound impairs protein synthesis in the liver, which is not the case when GSH is depleted by phorone[515]. Also, the NO synthase requires glutathione, e.g. GSH depletion by DEM inhibited iNOS induction in macrophages[516-519]. These frequently neglected limitations of the tool DEM have a significant impact on the interpretation of published work (6.2.3).

To elevate GSH levels, which may be of therapeutic value in some instances, two principles are to mention (reviewed in[496,502]): (i) GSH synthesis can be enhanced by supply of **GSH precursors** such as cysteine, NAC, 2-oxothiazolidine-4-carboxylate (OTC) or GSH itself, which is rapidly degraded in biological systems[496,520]. However, these approaches are foremost confined by the fact that the feedback inhibition of the glutathione synthase limits the GSH enhancement by this mean. Second, cysteine is toxic to cultured cells because it autoxidizes rapidly to cystine, and GSH is not efficiently taken up by cells[496]. Accordingly, it has been clearly demonstrated that the uptake of NAC does not necessarily lead to GSH enhancement in the liver[521]. Protection against CD95-triggered apoptosis by NAC has been attributed to its GSH-repleting potential[522], although it has been elaborated earlier in an elegant study by the group of Orrenius using both D- and L-NAC that this anti-apoptotic effect is definitely not due to the GSH-raising property of NAC[523]. Furthermore, NAC itself does interfere with an enzymatic GSH determination assay, since NAC rapidly oxidizes DTNB (unpublished observation), leading to false-positive GSH determinations by the cycling method (3.10). (ii) **Mono- or diesters of GSH** rapidly enter the cell, and GSH is

subsequently released by the action of esterases in an ATP-independent fashion. Furthermore, no feedback inhibition of the glutathione synthase limits the rise in GSH in this case, thus making GSH esters the most useful tool[496,524,525]. At last, it should be considered that the operative term *total glutathione* expressing the total amount of free GSH + 2x GSSG is frequently used, also in this study. However, this denotation does not comprise important pools of bound glutathione, and e.g. protein-bound glutathione is of paramount importance for cellular function, but difficult to determine[506].

1.4.3 Glutathione and hepatocyte death

The ubiquitous tripeptide GSH represents the most abundant intracellular thiol and thus protects the hepatocyte from oxidative damage (1.4.1). Additional to the important functions that GSH has in non-parenchymal hepatic cells[526], the pivotal role of hepatocyte GSH is addressed here.

The intracellular GSH concentration of the hepatocyte can vary considerably, i.e. it can dramatically decrease (i) as a result of drug metabolism, (ii) as a subject of pronounced circadian alterations; (iii) after oxidative stress, (iv) in certain disease states, and (v) due to inherited deficiencies in GSH synthesis[498,500,527,528]. Thus, low GSH levels are observed e.g. during sepsis, acetaminophen intoxication, chronic alcohol consumption, and in acute Wilson´s disease[498,527,529-532].

GSH-depleting compounds usually do not induce redox-stress in the liver *per se* and are non-toxic in most cases[533]; given the abundance and importance of GSH, this is rather unexpected. However, when (i) the concentration of both main redox buffers of the hepatocyte, i.e. GSH and thioredoxin, is experimentally decreased at the same time, this condition resulted in severe liver necrosis[534]; (ii) copper overload in hepatocytes depletes GSH and induces cell death, a mechanism that is important in Wilson's disease[396,530,535-537]; (iii) when glutathione depletion by DEM reaches a critical value, hepatocyte necrosis may proceed, presumably by induction of lipid peroxidation[538-540]. However, DEM may exert additional side-effects, especially in *in vivo* models (1.4.2).

Depletion of GSH predisposes hepatocytes to oxidative injury, with the consequence that the liver toxicity of many xenobiotics in humans and animals is greatly enhanced under this condition. Numerous examples for the deleterious consequences of GSH depletion in toxic liver injury and hepatocyte necrosis, caused for example by carbon tetrachloride, acetaminophen, captopril, anthraquinone, or allylalcohol, underline the importance of an intact thiol status for the maintenance of cellular functions[494,500,527,541-545]. Low GSH increased furthermore bile-salt induced necrotic cell death[546], and the redox-active compound butylated hydroxytoluene (BHT) was reported to exert hepatotoxicity when hepatic GSH was depleted by BSO[547]. *Vice versa*, a pharmacological enhancement of hepatic GSH stores by e.g. GSH esters (1.4.2) renders the liver less vulnerable and protects against many direct hepatotoxins[494,498,524,541,548]. For instance, GSH ester enhanced hepatic GSH by 66%[524] and thereby protected the liver from BCNU or cyclophosphamide

toxicity[549]. In all of these instances, hepatocyte death occurs primarily by *necrosis*, whereas the GSH dependence of hepatocyte *apoptosis* is less well characterized.

Many data concerning inflammatory liver injury and GSH have been published, especially with regard to ischemia/reperfusion and TNF-mediated processes. It is well established that oxidative stress, ensuing GSSG oxidation and lipid peroxidation mediate liver damage in ischemia/reperfusion, as it has been demonstrated in isolated rat liver models[550,551]. The cellular source of ROI was shown to be neutrophils and Kupffer cells[552], which has been put into doubt later[553, 554]. Notably, GSH was suggested to contain a therapeutic potential in ischemia/reperfusion[555]. Also in a murine model of neutrophil-mediated liver damage (GalN/LPS model), it was shown that glutathione peroxidase knock-out mice are more susceptible to hepatic damage[556], arguing for ROI as mediators in this particular model. In the two above-mentioned models, TNF plays a major role[379, 435, 557, 558]. Notably, TNF directly induces ROI in hepatocytes[559], and TNF at the same time triggers the upregulation of the heavy subunit gene of the γ-glutamylcysteine synthetase and thereby elevates hepatocyte GSH by 80%[560], possibly serving as a feedback mechanism limiting oxidative stress. Thus, liver damage exerted by TNF appears to often involves ROI as a common mediator[379], and in these cases, the antioxidative quality of GSH has a beneficial effect.

On the contrary, it has been demonstrated earlier that the onset of liver damage in the GalN/TNF and GalN/LPS models depends on hepatic GSH[561]. At that time, the pivotal role of apoptosis in these models has not yet been elaborated[562], and caspases were not even discovered. Therefore, the present thesis addresses this issue with an emphasis on apoptotic signal transduction.

At last, the role of intracellular compartmentalization of GSH within the hepatocyte has to be mentioned. Hepatocytes actively concentrate GSH within the nucleus, both under normal conditions and after GSH depletion with DEM or BSO[563]. Ethanol primarily depletes mitochondrial GSH *via* its metabolite acetaldehyde[564]. This circumstance resulted in a dramatic decrease of the hepatic GSH/GSSG ratio and lipid peroxidation[532]. Furthermore, mitochondrial GSH depletion by ethanol lead to a sensitization of rat hepatocytes towards TNF-mediated apoptosis[565]. Thus, GSH depletion by ethanol has a different effect on DR-triggered apoptosis of hepatocytes to that achieved with GSH S-transferase substrates, because e.g. phorone depletes primarily the cytosolic GSH compartment[566](1.4.2).

1.5 Regulation of apoptosis by oxidative stress and glutathione

Reactive oxygen species (ROI) are continuously generated in the living cell by normal physiology, and the antioxidative systems of the cell usually control oxidative stress and thus prevent cellular damage. Accordingly, oxidative stress usually occurs as a result of an imbalance between ROI generation and antioxidative defense. At the other hand, it has been recognized about ten years ago that ROI and other intracellular redox-active molecules play a role as physiologically important second messengers at relatively low concentrations[567]. Redox signaling can be mediated either by ROI such as superoxide or peroxides, by nitric oxide (NO) or by thiol-disulfide couples, e.g. GSH/GSSG[325]. With regard to the previous chapter, it is important to recall the difference between a situation of low intracellular GSH, which does not necessarily induce redox stress, and primary redox stress, which may in turn lead to GSH depletion or changes in the GSH/GSSG ratio (1.4). In this chapter, the bifunctional role of redox and glutathione alterations regarding apoptosis is addressed.

1.5.1 Redox stress and glutathione extrusion: common mediators of apoptosis?

Whether or not redox stress is an integral part of the apoptotic process is still controversial and has been under debate for many years (reviewed in[325,499,568-570]). Evidence for that concept comes from (i) the fact that apoptosis is often accompanied by signs of oxidative stress; (ii) experiments showing an inhibition or suppression of apoptosis by antioxidants in many experimental systems, e.g. in apoptosis induced by TNF, p53, NO, staurosporine and other compounds; (iii) the finding that oxidants or oxidant-promoting compounds in lower concentrations triggered apoptosis; (iv) the demonstration that a depletion of the cells' main redox buffer GSH induced or potentiated apoptosis in many instances, e.g. in fibroblasts[538], endothelial cells[571] or neurons[572], which may be explained by an indirect enhancement of oxidative stress under this condition[568]. Ceramide generation, activation of kinases and activation of p53 have been proposed as downstream effectors of ROI that activate apoptosis pathways (reviewed in[570]).

However, the notion that ROI trigger apoptosis was challenged by Raff and colleagues, reporting that apoptosis can proceed also in low-oxygen environment[573]. At the other hand, it was argued that apoptotic oxidative stress is not necessarily directly mediated by ROI formation, but rather achieved by an active extrusion of cellular GSH: a decrease in intracellular GSH often precedes or accompanies apoptosis, e.g. in CD95-triggered apoptosis of Jurkat T cells[574] and other cells[575-580]. Concerning the kinetics of GSH extrusion, it was reported in HepG2 cells to occur prior to the activation of apoptotic machinery[576], while in Jurkat T cells it was shown to take place downstream of caspase activation[581]. However, it was also stated that GSH depletion and apoptosis execution are not directly linked in one of these studies[580].

Recent studies suggest that GSH depletion as an integral part of apoptotic signaling is clearly confined to the mitochondrial branch[582], implicating that the proceeding of DR-

triggered apoptosis is not dependent on GSH depletion in most cases. In particular, it was shown that GSH depletion alone can result in the release of cyt c[583]. Thus, e.g. ROI generation in TNF-induced cell death was inhibited by high GSH resulting in prevention of apoptosis[584,585]. However, it was clearly shown by the group of Nagata that CD95-triggered apoptosis does not depend on ROI production[586], and this pathway can even be inhibited by ROI[581]. Thus, there is no general agreement as to the role of redox stress or GSH extrusion as a *common* apoptosis mediator.

1.5.2 Pro-oxidative apoptosis inhibition by NO, ROI, and GSH depletion

Increasing evidence argues for a dichotomal role of redox stress with respect to cell demise: in some paradigms of cell death, a *protective* (i.e. anti-apoptotic), and not an aggravating effect of pro-oxidative conditions was reported.

To date, the following examples are to mention: (i) **S-nitrosation** of protein-SH groups represents an oxidation reaction, which is known to regulate a number of cellular proteins[249]. In some experimental systems, NO donors provided protection from apoptosis, which has been proposed to occur *via* a direct interaction of NO with caspases and pro-caspases (1.2.3 and 7.2); (ii) **superoxide anion** (O_2^-) prevented CD95-mediated apoptosis of leukocytes[581]; (iii) **various oxidants** such as disulfiram displayed an anti-apoptotic effect in CD95-triggered T cell apoptosis[233-235] and in the GalN/TNF hepatotoxicity model[587]; (iv) **lowering intracellular GSH** by the use of BSO or GSH-S transferase substrates (DEM, phorone, see 1.4.2) prevented NO-induced apoptosis of macrophages[588] as well as DR-mediated hepatocyte apoptosis *in vivo* (this study)[412,430,589]. In this line, it has to be mentioned that in some instances, antioxidants such as NAC, or vitamin E, have been shown to enhance or mediate apoptosis (reviewed in[590]).

Depletion of GSH can also result in a shift from apoptosis towards cell death with necrotic features in some systems[591,592], indicating that downstream mechanisms were impeded by this circumstance, while cell death still occured. Thus, it appears that dual redox regulation acts on apoptosis: oxidative stress can trigger upstream pathways (1.5.1), whereas it may at the same time suppress downstream effector mechanisms such as caspases[235]. Few examples have been reported for pro-oxidative apoptosis inhibition.

1.5.3 Redox-related signaling in apoptosis: caspases, Bcl-2, and NF-κB

Obviously, the distinct effects on apoptosis by redox manipulations in different experimental systems must be attributed to the differential redox sensitivity of apoptotic signaling components. Three mechanisms are prominent in this context and are briefly discussed below.

A. Caspases are prone to inhibition by redox stress. Evidently, caspases are highly sensitive towards inhibition by redox stress, since they require a reduced cysteine for activity (1.2.2). However, the proof that a nitrosation, S-oxidation or glutathionylation has occurred inside a living cell and could mediate apoptosis inhibition is still lacking. But there is evidence that oxidative stress or NO exposure can inhibit apoptosis by preventing caspase *activation*[238](this study, figures 11 and 12). It was demonstrated that in staurosporine- or STS-treated Jurkat T cells, the release of mitochondrial cyt c was delayed and the *activation* of procaspases 3 and 7 to the active proteases was precluded in the presence of NO. Stimulated cells died nonetheless by necrosis[9,10]. Triggering of caspase-dependent apoptosis by oxidative stress or NO has been freuquently described and indicates that caspases within the living cell may be relative resistant towards *oxidative inactivation*. For instance, neutrophils produce substantial amounts of ROI and undergo apoptosis at the same time[594]. But why are caspases frequently activated together with ROI production? A possible explanation is that activation of oxidative mechanisms in the execution phase of apoptosis provides a built-in mechanism to inactivate extracellular caspases after secondary cell lysis, thus preventing the damage of neighboring cells[325].

B. Bcl-2 regulates apoptosis via glutathione alterations. Besides the well-characterized antiapoptotic functions of Bcl-2 mentioned in chapter 1.2.3, this protein appears to prevent cell death by a less characterized antioxidative pathway (reviewed in[325,595]). In 1993, it was shown that (i) expression of Bcl-2 in a neuronal cell line prevented cell death triggered by glutathione depletion[596], and (ii) that Bcl-2 as an integral part of inner mitochondrial membrane can directly prevent lipid peroxidation and ROI- or menadione-induced cell death[597]. In this line, overexpression of Bcl-2 in HeLa cells rised intracellular GSH by about 2-fold by inhibiting methionine-dependent GSH efflux[598]. Accordingly, protection by Bcl-2 overexpression was repeatedly linked to elevated GSH levels, and this resistance was overcome by depletion of intracellular GSH[599-602].

Notably, Bcl-2 overexpression redistributed GSH readily to the nucleus, which coincidenced with a severe cytosolic GSH depletion[603]. This finding provides evidence for an additional role of Bcl-2 as a nuclear channel regulator[595]. Furthermore, the mitochondrial permeability transition pore (MTP, see 1.2.3) is also regulated by Bcl-2 and highly thiol-sensitive[595]. The thiol-modifying compound monobromobimane blocked the MTP, and direct MTP opening due to low GSH was described in ebselen induced apoptosis[604]. Therefore, the relative importance of the mitochondrial and thus Bcl-2-dependent branch can determine whether an experimental system is influenced by the above-mentioned mechanisms.

C. NF-κB: a redox-sensitive transcription factor. This transcription factor regulates central cellular functions such as stress response, cell growth and cell survival, and is activated upon a wide variety of stimuli including cytokines (e.g. TNF, IL-1, IFN-γ), ROI, PMA, UV light and others (reviewed in[605-608]). NF-κB is composed of two subunits (p50, p55), and activated upon phosphorylation and release of its inhibitor I-κB[609]. Notably, the DNA-binding activity of NF-κB is abolished by oxidation, whereas it is fully active in a reduced environment and thus depends on glutathione and thioredoxin[605,610,611]. In addition to NF-κB, other transcription factors display a similar redox sensitivity, e.g. fos, jun, p53, and AP-1[570].

In most experimental systems and especially in the hepatocyte, NF-κB is crucial for signaling cytoprotective mechanisms[612-615]. For TNF-R1-induced apoptosis, the molecular mechanisms of NF-κB mediating cell survival have been addressed[295,616]. Most conspicuous, mice deficient in the RelA component of NF-κB displayed a massive degeneration of the liver by exaggerated apoptosis[612]. Accordingly, complete inhibition of NF-κB in hepatocytes is sufficient to trigger apoptosis[614]. In conclusion, the redox-sensitivity of NF-κB may be important for its anti-apoptotic role in a number of cell death paradigms[607,609].

1.6 Objectives of the thesis

Triggering of either of the two death receptors TNF-receptor-1 (TNF-R1) or CD95 in the liver activates hepatic caspases, followed by hepatocyte apoptosis and acute liver failure. This mode of cell demise is also associated with human liver disorders and represents a hallmark of inflammatory liver disease. Thus, a better understanding concerning the modulation of cytokine-mediated hepatic apoptosis is important for future therapeutic intervention strategies in liver disease. Hepatic glutathione represents the major defense mechanism against *toxic* liver injury, and a depletion of glutathione results in an enhanced toxicity of many classical hepatotoxins. However, the role of glutathione in *hyperinflammatory* liver destruction is less clear. The regulation of death receptor-triggered cell demise by variations of hepatic glutathione levels was investigated in murine liver injury models involving *apoptosis* as well as *necrosis* as the primary mode of cell death. Furthermore, the impact of glutathione variations on type I CD95 apoptosis in the cell line SKW6.4 was elaborated. In particular, the following questions were addressd in the present thesis:

1. **Do glutathione levels vary in death receptor-triggered hepatic apoptosis, and how can intreheptic glutathione concentrations be modulated *in vivo* to study the impact on hepatocyte death?**
2. **How does depletion of hepatic glutathione influence the induction of hepatocyte apoptosis by either CD95 or TNF-R1 and subsequent liver damage, and can the onserved alterations be reversed by a repletion of glutathione or by the use of antioxidants?**
3. **Can a redox-based modulation of apoptosis-transducing hepatic caspases explain the observed hepatoprotective effect of glutathione deficiency?**
4. **Does glutathione affect the apoptosome-mediated pathway of caspase activation initiated by cytochrome c/dATP in liver cytosol *ex vivo*?**
5. **Do glutathione variations affect the formation of the CD95 death-inducing signaling complex (DISC) and thereby the activation of upstream caspases in SKW6.4 cells?**
6. **How does a depletion of glutathione influence caspase-independent, apoptotic liver injury models, and is hepatoprotection based on immune modulation and cytokine release alterations, or directly effective on the target cell level?**
7. **Which hepatic cell populations are affected by glutathione deficiency in Con A-mediated, T cell-dependent liver injury? Is the observed hepatoprotection directly linked to animal survival in the Con A model or in other models investigated?**

2 Materials and methods

2.1 Chemicals

Phorone was obtained from Aldrich (Steinheim, Germany), glutathione monoethylester from Calbiochem (La Jolla, CA, USA), benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (z-VAD-fmk) from Bachem (Heidelberg, Germany), D-galactosamine (GalN) from Roth (Karlsruhe, Germany), 1-cis-chloro-2,4-dinitrobenzene (CDNB) and Epon® from Fluka (Buchs, Switzerland), LPS (*Salmonella abortus equi*) from Metalon (Wusterhausen, Germany), acetaminophen from EGA (Steinheim, Germany), N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (DEVD-afc) and Pefablock® from Biomol (Hamburg, Germany), protein A sepharose from Sigma (Deisenhofen, Germany) and the molecular weight marker from Boehringer Mannheim (Mannheim, Germany). Pentobarbital (Narcoren®) was purchased from Sanofi Withrop (München, Germany). The dyes Hoechst-33342 and SYTOX were bought from Molecular Probes (Eugene, OR, USA). All other reagents not further specified were purchased from Sigma (Deisenhofen, Germany).

2.2 Antibodies and recombinant enzymes

Activating anti-CD95 antibody (Jo2) and polyclonal IgG-horseradish peroxidase-coupled secondary antibody (goat anti-mouse) were purchased from PharMingen (San Diego, CA, USA), and procaspase-3 polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY, USA). The anti-FADD mAb was purchased from Transduction Laboratories (Lexington, KY, USA). The anti-caspase-8 mAb C15 (mouse IgG2b)[617], the anti-Flip mAb NF6 (mouse IgG1)[65], and the anti-APO1 mAb (agonistic anti-CD95, IgG3κ, used for CD95 immunoprecipitation)[46] were kindly provided by the laboratory of Prof. P.H. Krammer (DKFZ, Heidelberg). Recombinant human caspase-3 was a gift from Dr. R.V. Talanian (BASF, Worcester, MA, USA). IFN-γ and recombinant murine TNF were kindly provided by Dr. G.A. Adolf (Bender & Co., Vienna, Austria). Antibody pairs (specific rat anti-murine mAb) for cytokine determinations (3.7.3) were purchased from Pharmingen (San Diego, CA, USA), except for the TNF ELISA (capture: polyclonal ovine anti-mouse TNF antibody, in-house preparation, immunoglobulin G fraction, 20 mg/ml; detection antibody: polyclonal anti-mouse TNF antibody from Endogen, Boston, MA, USA). Other recombinant enzymes not further specified were purchased from Boehringer Mannheim (Mannheim, Germany) or Sigma (Deisenhofen, Germany).

2.3 Cell culture materials

Cell culture plates (24 and 96 well), petri dishes and other plastic materials were purchased from Greiner (Frickenhausen, Germany). Culture media were from Biochrom (Berlin, Germany) and Whittaker (Heidelberg, Germany), and collagen was obtained from Serva (Heidelberg, Germany). Penicillin, streptomycin and FCS were bought from Gibco BRL Life Technologies (Eggenstein, Germany).

2.4 Animals

Specific pathogen-free male BALB/c mice (approximately 25 g, from the in-house animal breeding station of the University of Konstanz) were maintained under controlled conditions (22°C and 55% humidity, constant day/night cycle of 12 h) and fed a standard laboratory chow. All animals received humane care in concordance with the NIH guidelines as well as with the legal requirements in Germany. Mice were starved overnight before the onset of experiments, which generally commenced at 8 a.m.

2.5 Isolation and culture of mouse hepatocytes

Isolation of hepatocytes from 8 weeks old mice was performed by the two step collagenase perfusion method of Seglen[618] as modified by Klaunig[619,620] and Leist[621]. After isolation, hepatocytes were plated in 200 µl RPMI 1640 medium containing 10% heat-inactivated FCS in collagen-coated 24 well plates at a number of 8 x 10^4 hepatocytes per well. Cells were allowed to adhere to culture plates for 4 h before the medium was exchanged for RPMI 1640 without FCS. Adherence and incubations were carried out in a humidified atmosphere at 37°C, 5% CO_2, 40% O_2 and 55% N_2.

2.6 Culture of cell lines

The human B lymphoma cell line SKW6.4 (ATCC, Manassas, VA, USA) and the T cell line Jurkat (clone E6-1, ATCC) were grown in suspension in RPMI 1640 supplemented with 10% heat-inactivated FCS and 100 µg/ml penicillin/streptomycin. Cells were maintained in 75 cm^2 flasks in a humidified atmosphere at 37°C, 5% CO_2, 21% O_2 and 74% N_2 and were passaged routinely every two to three days in a ratio of 1:10 or 1:5 depending on cell density. For experiments, cells were centrifuged (1,000 g, 4°C, 5 min), resuspended in medium, plated in 96 well plates (100 µl cell suspension, 10^5 cells/well) and incubated with the compounds indicated (given in 100 µl/well, final volume: 200 µl/well) under the same conditions described for cell maintenance.

2.7 Animal experiments

2.7.1 Treatment schedules

After treatment with liver injury-inducing compounds, animals were sacrified by lethal anesthesia to obtain samples at different times as described (2.7.2). Alternatively, the survival of animal was monitored over a period of at least three months.

- ***Liver injury induced by anti-CD95:*** hepatic apoptosis mediated *via* CD95 was induced by application of agonistic anti-CD95 antibodies in doses of 3 µg/mouse (8 hour model) or 6 µg/mouse (for induction of lethal liver damage) given i.v. in a volume of 300 µl 0.1% HSA/saline.

- ***GalN/TNF-induced liver injury:*** TNF was given i.v. in a dose of 2 µg/kg in 300 µl 0.1% HSA/saline, and the aminosugar GalN (700 mg/kg, given in 300 µl saline, i.p.) was administered 30 min before TNF to block hepatic transcription (8 hour model). For survival studies, TNF was used in a lethal dose of 5 µg/kg.

- ***GalN/LPS-induced liver injury:*** after sensitization with GalN as mentioned above, LPS was administered i.p. in a volume of 300 µl sterile saline in a dose of 5 µg/kg (8 hour model), or in a dose of 10 µg/kg for induction of lethal liver damage.

- ***LPS shock model:*** LPS was injected i.p. in a dose of 10 mg/kg. For investigation of LPS-induced liver injury, animals were sacrificed after 20 hours. In the case of survival studies, mice were observed over a period of 72 h.

- ***Con A-induced liver injury:*** T cell-dependent liver injury was induced by Con A according to Tiegs *et al.*[414]. Con A was injected i.v. into naive mice in a volume of 300 µl pyrogen-free saline at a dose of 25 mg/kg (8 hour model) or 50 mg/kg for the induction of lethal liver damage.

- ***Liver injury induced by high-dose fungal toxins:*** the transcriptional inhibitors α-amanitin (3 mg/kg) and actinomycin D (Act D, 2 mg/kg) were given i.p. in a volume of 300 µl sterile saline (20 hour model).

- ***Glutathione depletion:*** The GSH depletors phorone (62.5-500 mg/kg) and 1-cis-chloro-2,4-dinitrobenzene (CDNB, 100 mg/kg) were injected dissolved in 300 µl vegetable oil intraperitoneally (i.p.), either 1 h prior to challenge with GalN/TNF, acetaminophen, αCD95, or Con A, or delayed 1 hour after challenge to avoid interference of the solvent with LPS, Act D, or α-amanitin at the site of injection. The GSH-synthesis inhibitor L-buthionin-S,R-sulfoximin (BSO, 890 mg/kg, -10 h and -1 h before challenge) was administered i.p. in 300 µl endotoxin-free saline.

- ***Further compounds:*** The irreversible, non-specific caspase inhibitor z-VAD-fmk was given in a dose of 10 mg/kg at t = -1 h, and additionally 5 mg/kg at t = +1/+3 h in a volume of 300 µl solvent (1% DMSO, 39% H_2O and 60% 0.1% HSA/saline, i.p.). Glutathione monoethylester (GSH-ME, 1 g/kg), sodium nitropusside (SNP, 5 mg/kg) and acetaminophen (175 mg/kg) were injected i.p. in 300 µl pyrogen-free saline, α-tocopherol (200 mg/kg) was injected i.p. dissolved in 300 µl vegetable oil. Butyl-hydroxytoluene (BHT, 200 mg/kg) was given orally dissolved in vegetable oil. N-acetyl cysteine (NAC, 1000 mg/kg), reduced glutathione (GSH, 0.5 mmol/kg) and catalase (10^6 U/kg) were given i.v. in 300 µl pyrogen-free saline.

2.7.2 Sampling of material

At the timepoints indicated, mice were euthanized by i.v. injection of 150 mg/kg pentobarbital plus 0.8 mg/kg heparin, and blood samples were obtained:

- To assess the extent of liver damage, blood was withdrawn by cardiac puncture and subsequently centrifuged (5 min, 14,000 g, 4°C). ALT, AST and SDH enzyme activities were measured in the plasma as described below (2.11.1).
- Blood samples for cytokine determinations (2.7.3) were obtained either from the tail veins using heparinized syringes, or alternatively by cardiac puncture as described above, subsequently centrifuged (5 min, 14,000 x g, 4°C) and stored at -80°C.

After blood withdrawal, livers were perfused for 10 s with cold perfusion buffer (PB, 50 mM phosphate buffer pH 7.4, 120 mM NaCl, 10 mM EDTA), immediately excised and processed as follows:

- Slices of the large anterior lobe were frozen in liquid nitrogen and stored at -80°C until the measurement of caspase-3-like activity (2.11.3), caspase-3 Western blot (2.13) or the preparation of nuclear extracts for NF-κB mobility gelshift assay (2.7.4).
- For *ex vivo* activation of caspases, whole livers (approximately 1.5 g) were immediately processed as described in 2.9 in detail.
- To analyze hepatic DNA fragmentation (2.7.5), slices of the large anterior lobe were disintegrated by Dounce homogenization in a ratio of 1:10 in PB and centrifuged (20 min, 14,000 g, 4°C).
- For determination of glutathione (2.10), liver and other organ samples were immediately freeze-clamped with pliers pre-cooled in liquid nitrogen and stored at –80°C.
- For liver histology (2.8), liver specimen were immediately cut into 1 mm thick slices and fixed (2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for electron microscopy; phosphate-buffered neutral 4% formalin solution for light microscopy).

2.7.3 Cytokine determinations

All enzyme-linked immunosorbent assays (ELISAs) were performed on flat-bottomed high-binding polystyrene microtiter plates (Greiner, Nürtingen, Germany). Streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA, USA) and the peroxidase chromogen substrate tetramethylbenzidine (TMB liquid substrate system, Sigma, Deisenhofen, Germany) were used to detect the immunocomplex. IL-1β was determined using a commercially available ELISA kit (Endogen, Boston, MA, USA), and the antibody sources for other ELISAs are given in 3.2. The detection limits of the ELISAs were 10 pg/ml for TNF and IFN-γ, 30 pg/ml for IL-2, 10 pg/ml for IL-4, and 15 pg/ml for IL-1

2.7.4 NF-κB mobility gelshift assay (EMSA)

Nuclear extracts were prepared from frozen liver sections using a modification of the method of Schreiber *et al.*[622]. Briefly, tissue samples were homogenized in a hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM Pefablock®). The homogenate was incubated for 10 min on ice and centrifuged (10 min, 1,000 g, 4°C). The cell pellet was suspended in 1.4 ml ice-cold buffer A, and 90 µl 10% solution of NP-40 solution was added followed by 10 sec of vigorous vortexing. The suspension was incubated on ice for 10 min and centrifuged (30 sec, 12,000 g, 4°C). The supernatant was removed and the nuclear pellet was extracted with 200 µl of hypertonic buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Pefablock®) by shaking at 4°C for 30 min. The extract was centrifuged (10 min, 12,000 g, 4°C), and the supernatant was stored at -80°C. A double-stranded oligonucleotide probe containing a consensus binding-sequence for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Promega, Heidelberg, Germany) was 5'-endlabeled with γ^{32}P-ATP (3000 Ci/mmol, Amersham, Braunschweig, Germany) using T4 polynucleotide kinase (Promega, Heidelberg, Germany). Samples of nuclear protein were incubated at room temperature in a 15 µl reaction volume containing 10 mM Tris-HCl pH 7.5, 5 x 10^4 cpm radiolabeled oligonucleotide probe, 2 µg poly(dIdC), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, and 0.5 mM DTT for 20 min. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4.5% non-denaturing polyacrylamide gel in 0.25 x TBE at 100 V. The gel was autoradiographed, and the specificity of the DNA/protein complex was confirmed by competition with a 100-fold excess of unlabeled NF-κB sequence (5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3', Promega, Heidelberg, Germany).

2.7.5 Hepatic DNA fragmentation

The 20% homogenate (in perfusion buffer) was centrifuged at 13,000 g for 20 min. DNA was precipitated from 450 µl supernatant by addition of 1 ml ethanol (-20°C) plus 50 µl sodium acetate (3 M) and stored at -20°C for further analysis on agarose gels. Ethanol-precipitated low molecular weight DNA was treated with RNase (1 mg/ml, 1 h, 50°C) and proteinase K (10 mg/ml, 18 h, 50°C). The DNA fragments were then purified by phenol/chloroform extraction and analyzed by electrophoresis on a 1% agarose gel.

2.8 Light and electron microscopy

Cell culture: To determine the amount of necrosis or apoptosis in lymphoid cells (2.6), cultures were stained with a mixture of the membrane permeate dye Hoechst 33342 (500 ng/ml) and the membrane impermeate dye SYTOX (500 nM) for 5 min at 37°C. Than, the amount of normal, necrotic (damaged/SYTOX-permeable membrane, normal nuclei) and apoptotic (impermeable membrane, condensed nuclei) were scored under a fluorescence microscope.

Liver histology: for light microscopy, liver specimen samples (2.7.2) were fixed in phosphate-buffered, neutral 4% formalin and subsequently embedded in paraffin. Five-micrometer sections were cut and stained using routine methods (hematoxilin and eosin, 5 ± 2 µm thick). For transmission electron microscopy (TEM), the liver samples were stored in 2.5% glutaraldehyde in buffer (0.1 M phosphate buffer pH 7.4) for 2-3 days prior to further processing. Specimens were postfixed with osmium tetroxide, dehydrated in graded alcohol, and embedded in Epon. Ultrathin sections (60-80 nm) were cut on a Reichert ultramicrotome (Leica, Glattbrugg, Switzerland) and contrasted with uranyl acetate and lead citrate. Stained sections were reviewed in a Phillips CM 10 electron microscope (Dietikon, Switzerland) operating at 60 KV.

2.9 Cytochrome c/dATP-induced caspase activation assay

Immediately after excision of liver specimen (2.7.2), extracts from whole livers were prepared by Dounce homogenization in 600 µl/liver hypotonic extraction buffer (HEB; 50 mM PIPES pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM Pefablock® and pepstatin, leupeptin and aprotinin, 1 µg/ml each, modified from[623]) and subsequently centrifuged (15 min, 14,000 g, 4°C). The interphase was again centrifuged (15 min, 14,000 g, 4°C) to obtain clear cytosolic extracts (approximately 250 µl/liver, 80 mg/ml protein). Aliquots (30 µl) were immediately frozen in liquid nitrogen and stored at -80°C. After thawing, the protein content was calibrated to 80 mg/ml, and triplicates (5 µl) of each sample were diluted with 5 µl HEB containing 200 µM cyt c, 20 mM dATP and other substances as indicated on round-bottomed microtiter plates. Than, caspase-activation was carried out at 37°C for 30 min. The reaction was stopped by addition of substrate buffer (60 µM fluorogenic substrate DEVD-afc in 50 mM HEPES pH 7.4, 1% sucrose, 0.1% CHAPS,

10 mM DTT or as indicated) by the automatic dispenser device of the fluorometer plate reader Victor^2 (Wallac Instruments, Turku, Finland), and DEVD-afc cleavage (caspase-3-like activity) was determined using the fluorometer plate reader Victor^2 as described below (2.11.3).

2.10 Determination of glutathione

The amount of total glutathione (GSx = GSH + 2 x GSSG) in liver tissue was quantified according to the enzymatic cycling method originally described by Tietze[624]. Briefly, freeze-clamped sections of liver tissue (2.7.2) were homogenized in 1% sulfosalicylic acid, and the supernatants were separated from precipitated proteins by centrifugation (5 min, 14,000 g, 4°C). The supernatants and standards (0-5 µM GSSG) were diluted in 0.1 M HCl containing 10 mM EDTA, and total glutathione was quantified with an ACP 5040 analyzer (Eppendorf, Hamburg, Germany).

These measurements were confirmed by quantification of reduced (GSH) and oxidized (GSSG) glutathione with the HPLC-method by Asensi *et al.*[503]. In this case, the freeze-clamped tissue samples were homogenized in 6% perchloric acid containing 2 mM bathophenanthroline-disulphonic acid, and additionally 20 mM N-ethylmaleimide for the determination of GSSG. After centrifugation (5 min, 14,000 g, 4°C), supernatants were analyzed by HPLC as described in detail[503].

2.11 Measurement of enzyme activities

2.11.1 Liver enzyme activities in plasma samples

The extent of liver damage was assessed by measuring plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) activity with an EPOS 5060 analyzer (Netheler & Hinz, Hamburg, Germany) according to the method of Bergmeyer[625] and calculated as U/l plasma.

2.11.2 Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) was determined in hepatocyte cell culture supernatants (S) and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100 according to Bergmeyer[625]. The percentage of lactate dehydrogenase release was calculated from the ratio of S/(S+C).

2.11.3 Caspase-3-like activity

The specific activity of caspase-3-like proteases was measured using the artificial tetrapetide substrate DEVD-afc. The cleavage assay was carried out on microtiter plates (Greiner, Nürtingen, Germany) according to the method originally described by Thornberry[240]. Samples (10 µl, approximately 1 mg/ml protein) were obtained from **(i) cell culture experiments** (2.5/2.6) after cell lysis (freeze-thaw in lysis buffer: 25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Pefablock and pepstatin, leupeptin and aprotinin, 1 µg/ml each, 0.1% Triton X-100) and subsequent centrifugation (15 min, 14,000 g, 4°C); **(ii)** ***in vivo*** **experiments**, where cytosolic extracts from liver tissue were prepared from frozen liver samples (2.7.2) by Dounce homogenization in hypotonic extraction buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Pefablock and pepstatin, leupeptin and aprotinin, 1 µg/ml each) and subsequently centrifuged (15 min, 14,000 g, 4°C). The clear supernatant was also used for the caspase-3 Western blot (3.13) or diluted 1:5 in extraction buffer and stored at -80°C for the DEVD-afc cleavage assay; **(iii)** the cyt c/dATP **caspase activation assay** (2.9), **(iv)** or **recombinant caspase-3** (diluted to 30 ng/ml protein in a buffer containing 50 mM HEPES pH 7.4, 1% sucrose, 0.1% CHAPS, 20% (v/v) glycerol) was directly used. In each case, the samples were diluted 1:10 with substrate buffer (60 µM fluorogenic substrate DEVD-afc in 50 mM HEPES pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT or as indicated) at a final volume of 100 µl, and blanks contained 10 µl extraction buffer plus 90 µl substrate buffer. Generation of free 7-amino-4-trifluoromethylcoumarin (afc) at 37°C was kinetically determined by fluorescence measurement (excitation: 385 nm; emission: 505 nm) using the fluorometer plate reader Victor^2 (Wallac Instruments, Turku, Finland). Protein concentrations of the corresponding samples were estimated with the Pierce-Assay (Pierce, IL, USA), and the activity was calculated using serially diluted standards (0-5 µM afc). Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

2.12 Immunoprecipitation of the CD95 DISC

The formation of the CD95 DISC in SKW6.4 cells was studied *via* immunoprecipitation of the receptor by the method described by Kischkel *et al.*[69] and Scaffidi *et al.*[626]. SKW6.4 cells were maintained in 50 ml Falcons in a number of 10^7 cells/sample with or without addition of the indicated concentrations of GSH depletors. After 1 hour, cells were either first treated with 2 µg/ml anti-APO1 mAb for 10 min at 37°C. After addition of 50 ml ice-cold PBS and short centrifugation (1,000 g, 4°C, 5 min), the cell pellet was lysed (lysis buffer: 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and small peptide inhibitors, 1% Triton X-100, 10% glycerol). Serving as an unstimulated negative control, one sample of cells were lysed in lysis buffer before supplementation with anti-APO1. After centrifugation of the lysates (15 min, 14,000 g, 4°C), the CD95 DISC was precipitated for 2 hours at 4°C from supernatants with protein A sepharose. After immunoprecipitation, the beads were washed five times with 20 volumes of lysis buffer and subsequently subtracted to SDS-PAGE separation (2.2.7).

2.13 Western blotting

Depending on the protein that should be detected, samples (20-60 µg protein/lane) were loaded on a 12% or 15% polyacrylamide gel, separated under reducing conditions, and subsequently blotted on a Hybond nitrocellulose membrane (Amersham-Buchler, Braunschweig, Germany) in a Bio-Rad semi-dry blotter at 0.8 mA/cm^2 for 60 min. Homogenous transfer to nitrocellulose membrane was controlled by Ponceau red staining. Blots were blocked for at least 1 hour with 2% BSA in PBS/Tween (PBS + 0.05% Tween-20), washed with PBS/Tween and then incubated with the following primary antibodies for 16 h at 4°C: rabbit anti-human procaspase-3 polyclonal antibody (1:500 in PBS/Tween, 5% non-fat dry milk; this antibody is cross-reactive with the 32 kDa murine pro-caspase); anti-FADD mAb (1:250 in PBS/Tween, hybridoma cell supernatant); anti-caspase-8 mAb C15 (1:20 in PBS/Tween, hybridoma cell supernatant); anti-Flip mAb NF6 (1:10 in PBS/Tween, hybridoma cell supernatant); and anti-CD95 mAb (1:500 in PBS/Tween, 5% non-fat dry milk). After washing in PBS/Tween and incubation with a polyclonal IgG-horseradish peroxidase coupled secondary antibody (1:20,000 in PBS/Tween), the blots were developed by chemiluminescence method (ECL, Amersham-Buchler, Braunschweig, Germany) following the manufactures protocol.

2.14 Statistics

All data are generally given as means ± SD. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test of the control *vs*. other groups. Statistical analysis that included all *vs*. all comparisons were done by the Tukey multiple comparison test. The statistic program InStat® (GraphPad software, USA) was used for statistics, and a p value <0.05 was considered significant.

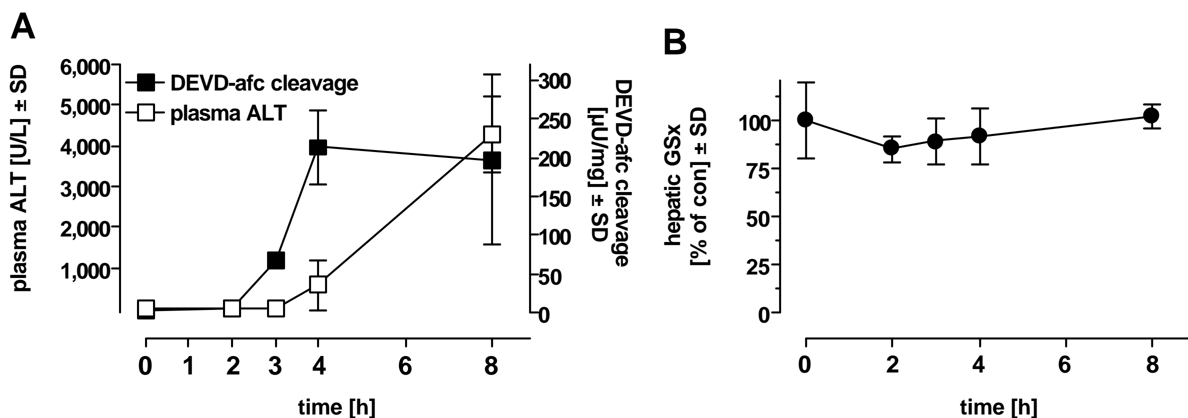
3 CD95-mediated hepatic apoptosis and glutathione

3.1 Do hepatic glutathione levels vary in CD95-mediated hepatic apoptosis?

It was reported that GSH efflux is an important event in apoptosis, also in cell death triggered by CD95 (1.3.1). Furthermore, a severe depletion of hepatic GSH stores occurs e.g. in sepsis, after intoxication with hepatotoxins, and in acute Wilson´s disease[498,527,529-532]. Therefore, it was of interest whether hepatic GSH levels are affected by massive hepatocyte apoptosis induced by the activating anti-CD95 antibody (αCD95) Jo2.

In a timecourse experiment, mice received 3 μg/animal of αCD95, and liver samples were taken by freeze-clamping at different times. The enzymatic activity of caspase-3-like proteases, i.e. caspase-3 and caspase-7 in the liver[478], was used as a quantitative biochemical parameter for the detection of caspase-dependent hepatocyte apoptosis *in vivo*[429,436,478,589]. Caspase-3-like activity became detectable at 3 hours and peaked at 4 hours, whereas the onset of liver enzyme release was determined at 4 hours (figure 3 A). Even after 8 hours, when the ALT release was exceedingly high (4,250 ± 950 U/l ALT), the amount of total hepatic glutathione (GSx = GSH + 2 x GSSG) remained largely at control levels, i.e. about 35 nmol GSx/mg protein (figure 3 B). These data indicate that hepatocytes maintain high GSH levels during the process of CD95-mediated active cell death, which is in contrast to what has been reported for Jurkat T cells[574-576]. Since GSH is kept high also in TNF-R1-mediated hepatic apoptosis[627], it is suggested that DR-dependent hepatocyte apoptosis *in vivo* depends on sufficient intracellular GSH, which was further investigated.

Figure 3: Kinetics of caspase-3-like activity, ALT release and levels of total glutathione (GSx) in



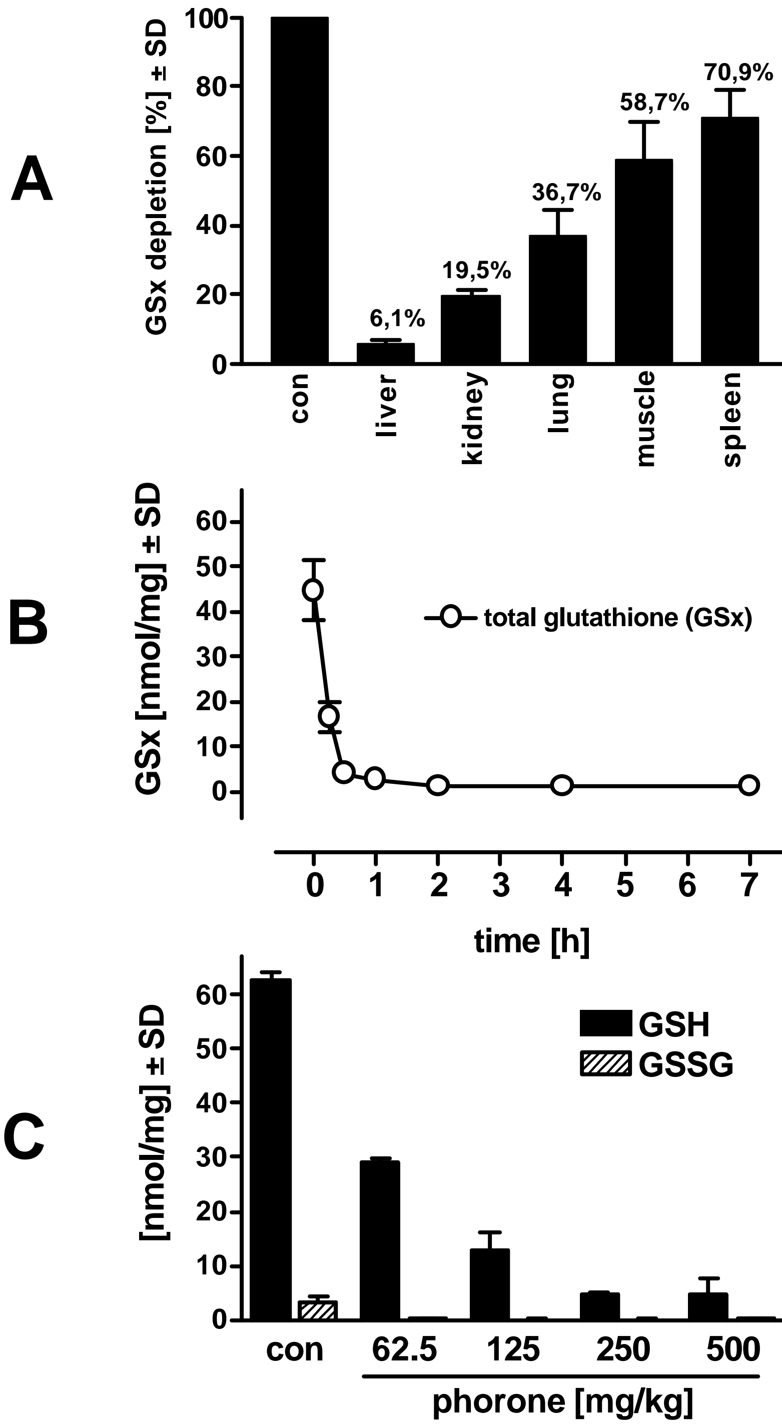
CD95-mediated hepatic apoptosis. *Five groups of mice were injected with* ***a****CD95 (3 µg/animal), and* ***(A)*** *the timecourse of hepatic caspase-3-like protease activity (DEVD-afc cleavage), of ALT release in the plasma, and* ***(B)*** *of total glutathione (GSx) in liver samples taken by freeze-clamp technique were determined. Values are depicted as means ±SD from three animals per group.*

3.2 Characterization of phorone-induced hepatic glutathione variations

As a non-toxic tool for lowering hepatic glutathione, the glutathione depletor phorone was used (1.4.2). The differences between diverse tissues, the kinetics, and the dose-response of GSH *versus* GSSG depletion caused by phorone were elaborated.

Under our experimental conditions, a depletion of glutathione below 20% one hour after injection of mice with 250 mg/kg phorone was only seen in the liver (6.1%) and in the kidney (19.5%), whereas other organs displayed lower sensitivity towards this GSH S-transferase substrate (figure 4 A). Furthermore, treatment of mice with the highest applicable dose of phorone (500 mg/kg) led to an immediate drop of total hepatic glutathione content to under 10% of the initial level within one hour, and this depletion was stable for several hours (figure 4 B). The experiment shown in figure 4 C displays the dose-dependence of both GSH and GSSG depletion in the liver one hour after phorone treatment. The amount of GSSG found in control livers was far below 5% of total glutathione confirming earlier findings[503]. In livers from phorone-treated mice, the following percentages of GSSG were found at the different doses used: 0.6% at 62.5 mg/kg and 125 mg/kg; 0.14% at 250 mg/kg and 0.29% at 500 mg/kg, respectively (figure 4 C). Thus, GSSG does not accumulate within hepatocytes during glutathione, indicating that no redox stress was induced by this treatment, which is in line with a recent study[533].

***Figure 4: Characterization of glutathione depletion induced by phorone**. (**A**) One group of mice was injected with 250 mg/kg phorone, another group was treated with vehicle control (oil). Organ samples were taken by freeze-clamp technique after one hour, and the content of total glutathione (GSx) from each organ control group was set at 100%, and the GSx depletion is shown as % of control for each organ. (**B**) After treatment of animals with 500 mg/kg phorone, the time course of total glutathione in liver samples taken by freeze-clamp technique was determined. (**C**) The content of hepatic GSH and GSSG were determined by HPLC from liver samples taken by freeze-clamp technique one hour after treatment of mice with different doses of phorone as indicated. Values are means ±SD from three animals per group.*


A
GSx depletion [%] ± SD
100
80
60
40
20
0
70,9%
58,7%
36,7%
19,5%
6,1%
con
liver
kidney
lung
muscle
spleen
B
GSx [nmol/mg] ± SD
60
50
40
30
20
10
0
total glutathione (GSx)
0
1
2
3
4
5
6
7
time [h]
C
[nmol/mg] ± SD
60
50
40
30
20
10
0
GSH
GSSG
con
62.5
125
250
500
phorone [mg/kg]

3.3 Hepatoprotection by phorone treatment in CD95-mediated liver injury

The impact of glutathione depletion on the CD95 model was investigated. A dose of 3 µg/animal of the activating anti-CD95 antibody (αCD95) caused severe damage to hepatocytes as assessed by the release of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) in the plasma 8 hours after challenge (figure 5 A). Administration of the glutathione-S transferase substrate phorone one hour prior to αCD95 injection dose-dependently prevented hepatocyte death. A dose of 125 mg/kg phorone was sufficient to achieve significant protection from CD95-mediated liver injury, and at the greatest dose used, i.e. 500 mg/kg, protection was almost complete as documented by a reduction of plasma ALT by >95%. When comparing this dose-dependence to the GSx depletion data shown in figure 4 C, it is obvious that a significant protection from liver injury was observed at a GSH depletion of about 80% compared to control values. Moreover, the alternative depletor DEM and the glutathione synthesis inhibitor BSO both conferred protection against CD95-mediated liver injury (89% and 77% protection, respectively, at the basis of ALT release in the plasma). The survival rate of mice exposed to a lethal αCD95 dose was further tested (figure 5 B). After injection of 6 µg/animal αCD95, control mice developed fulminant hemorrhagic liver destruction and died within 6 hours as described previously[400,403]. All animals that had received 250 mg/kg phorone one hour before challenge survived an observation period of 3 months.

The histological examination of liver tissue samples confirmed these outcomes. In livers from mice which had received 3 µg/animal αCD95, a collapse of the normal sinusoidal liver architecture, especially in the midzonal area, was seen 8 hours after application (figure 5 C). Further major pathological signs were diffuse congestive hemorrhagic foci and ubiquitous apoptotic nuclei. In contrast, liver specimens from a comparable area taken from mice injected with phorone prior to CD95 activation (figure 5 D) displayed a morphology that was indiscernible from that of untreated healthy mice (without figure). Thus, the morphological observations correlate well with the findings based on clinical chemistry, suggesting that there were no secondary effects in the liver due to phorone treatment. In conclusion, CD95-mediated apoptotic liver damage appears to be strongly dependent on sufficient intracellular GSH levels. This fact is in obvious contrast to liver damage due to a number of necrosis-inducing hepatotoxins, since GSH depletion strongly enhances hepatoxicity in this case[494,541].

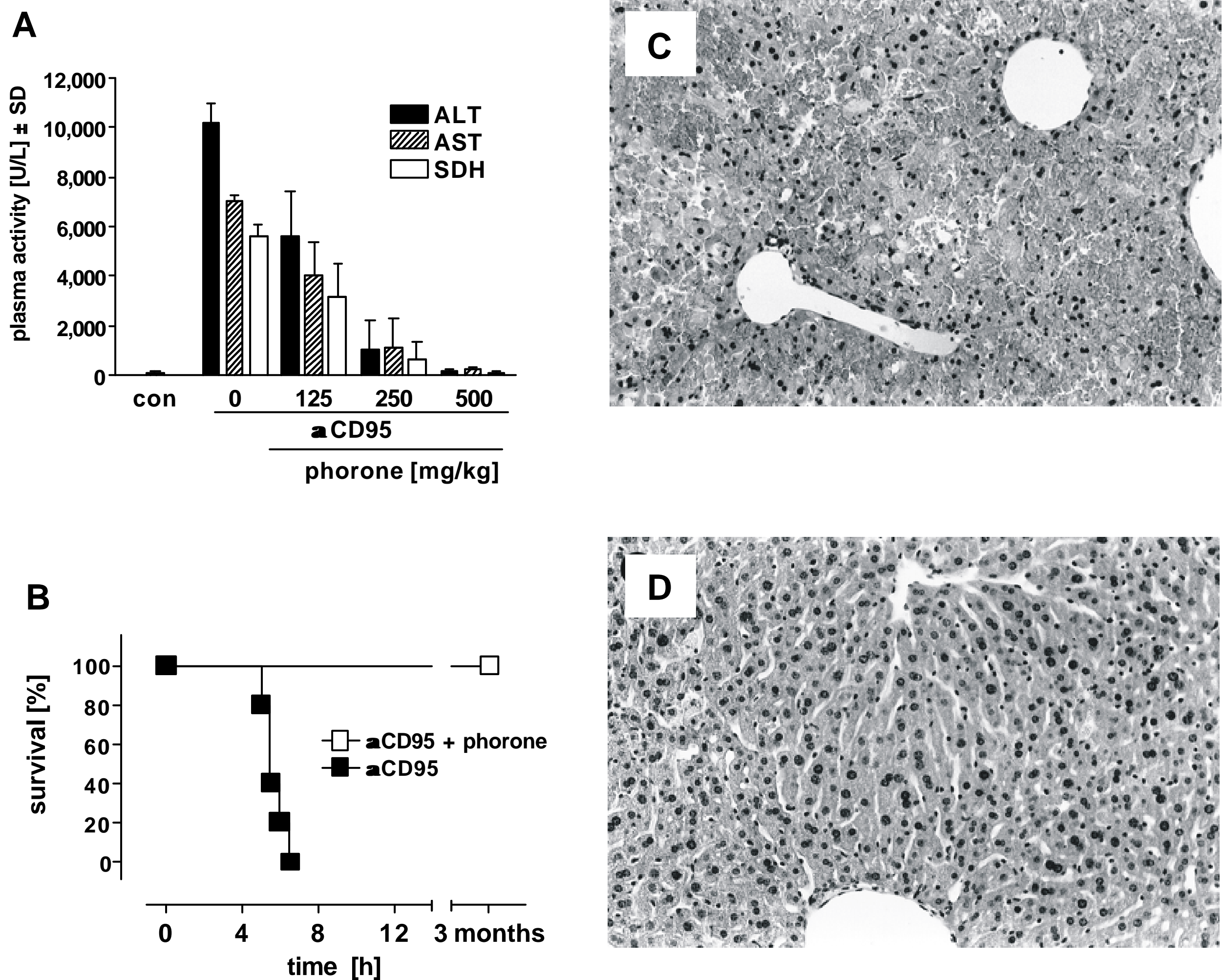


Figure 5: Inhibition of CD95-mediated liver damage and prevention of mortality by phorone pretreatment. *(**A**) Four different groups of **a**CD95-injected mice (3 µg/animal) received different doses of phorone as indicated 1 hour before **a**CD95 application. Eight hours after challenge, blood was sampled, and the plasma activities of ALT, AST and SDH were determined. Values are depicted as means ±SD from three animals per group. Asterisks (*) indicate a p value <0.05 compared to **a**CD95-treated controls based on ANOVA analysis followed by the Dunnett multiple comparison test. (**B**) Liver damage was induced with 6 µg/animal **a**CD95 in two groups (n = 5/group) of mice, one of which was additionally treated with 250 mg/kg phorone 1 hour prior to **a**CD95 challenge. The survival rate was monitored for the time indicated. (**C, D**) Liver sections were prepared for morphological examination 8 hours after intervention from livers of (C) **a**CD95-treated mice (3 µg/kg) and (D) mice additionally pretreated with 500 mg/kg phorone 1 hour before **a**CD95 injection. Photographed at an original magnification of 50 x.*

3.4 Is the prevention from apoptosis reversed by glutathione repletion?

In order to examine potential effects of phorone unrelated to depletion of glutathione, it was of interest whether the repletion of the depleted hepatic glutathione pool could restore the hepatocytes' ability to undergo apoptotic cell death. Figure 6 A demonstrates that the glutathione content of livers depleted by 92% following phorone treatment could be restored to 42% of control values after treatment with the permeable glutathione donor glutathione monoethylester[524,525]. As a functional consequence of thiol repletion, mice significantly regained sensitivity towards CD95-mediated liver injury compared to animals in the depleted group (figure 6 B). In control experiments, no hepatotoxicity of phorone plus glutathione ester without CD95 stimulation, nor an enhancement of liver injury when the ester was given alone before αCD95 challenge was noticed (without figure). The restoration of glutathione levels was incomplete, and consequently the extent of liver injury did not reach that of naive controls. In order to check whether GSH could be functionally substituted with other antioxidants, several substances that are known to protect in pro-oxidative liver injury models in mice were additionally examined[469,628-630]. Given in a similar regimen as the glutathione monoethylester one hour after phorone and one hour before αCD95 treatment, none of the tested antioxidants significantly restored sensitivity towards CD95-mediated liver injury (figure 6 C), only GSH slightly restored liver damage. Thus, oxidative stress is an unlikely cause for the interruption of apoptotic signaling due to phorone. Taken together, these observations not only provide evidence for the reversibility of the protection in the presence of the compound phorone, but furthermore demonstrate the specificity of this regulation for glutathione.

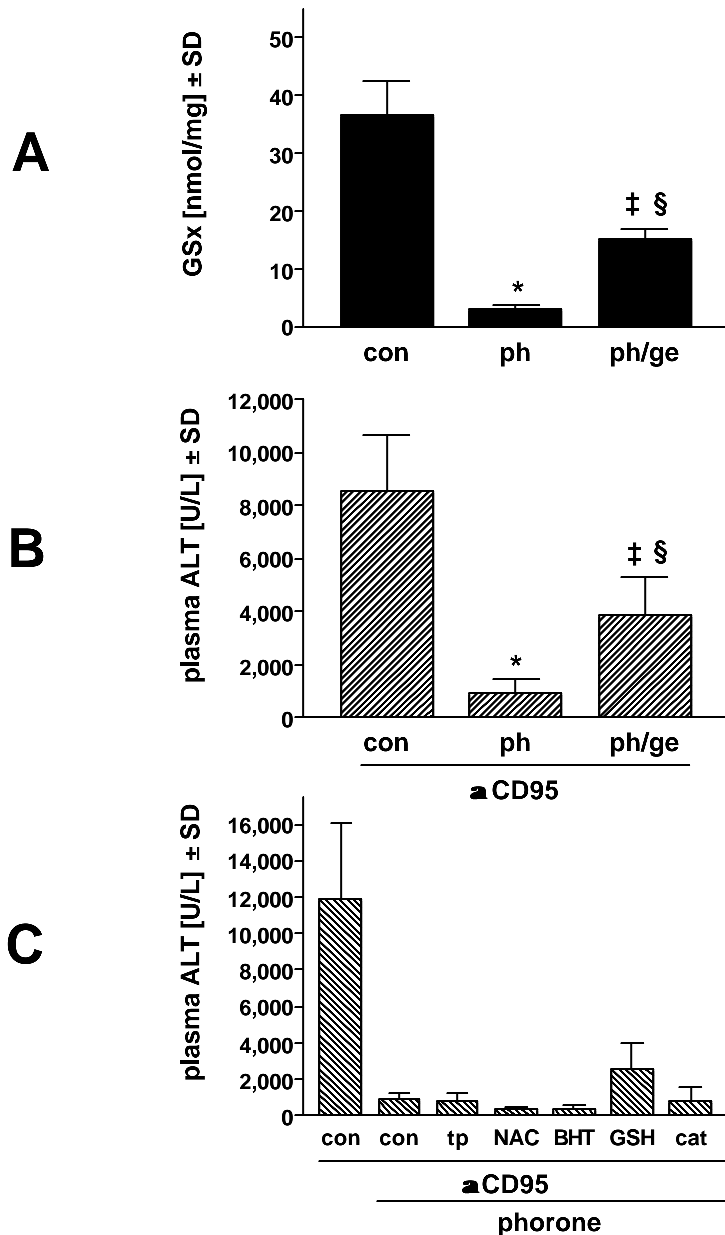


Figure 6: Repletion of hepatic glutathione by injection of glutathione monoethylester restores susceptibility of phorone-treated mice towards CD95-mediated liver injury. *(A) The total hepatic glutathione content (GSx) of control mice (con), of mice treated with 250 mg/kg of phorone 2 hours prior to sampling of material (ph), and of mice that had received phorone and in addition 1 g/kg glutathione monoethylester 20 minutes before liver withdrawal (ph/ge). Values are depicted as means ±SD from three animals per group. (B) In three parallel groups, mice were injected with aCD95 (3 µg/animal). Phorone and glutathione monoethylester were injected before challenge with a similar treatment regimen as in (A). The extent of liver damage was assessed 8 hours after aCD95 injection by measurement of plasma ALT activities. Values are depicted as means ±SD, n = 6/group. A p value <0.05 based on ANOVA analysis followed by the Dunnett multiple comparison test is indicated (*, ph vs. con; ‡, ge/ph vs. con; §, ge/ph vs. ph).* ***(C)*** *Animals were treated with the same regimen with respect to phorone and aCD95 as in* ***(B)****, but received the antioxidants N-acetyl cysteine (1000 mg/kg), free glutathione (0.5 mmol/kg), a-tocopherol (200 mg/kg), butyl-hydroxytoluene (200 mg/kg), and catalase (10^6 U/kg) 1 hour before challenge. Values are depicted as means ±SD, n = 3/group.*

3.5 Low glutathione prevents caspase-3 activation and DNA fragmentation

The influence of GSH depletion on caspase-3-like activity, caspase-3 activation and on fragmentation of nuclear DNA into n x 180 bp, a hallmark of apoptotic cell death executed by a DNase which is in turn activated by caspase-3[232,631,632], was now investigated.

In livers of mice injected with αCD95, a rapid activation of hepatic caspase-3-like proteases was found after 3 hours which peaked at about 4 hours (figure 3 A). When animals were pretreated with phorone, this increase in hepatic caspase-3-like activity after administration of αCD95 was completely abrogated in a dose-dependent manner at this timepoint (figure 7 A). In order to relate the caspase-3-like activity changes to protein processing, the activation of procaspase-3 was further examined by Western blot analysis (figure 7 B). The inactive p32 pro-form was detected in liver samples from control animals (lane I), whereas the p32 fragment was undetectable in livers of mice 6 hours after treatment with αCD95 (lane II). The additional 29 kDa fragment that was also detected in all samples by the antibody may correspond to procaspase-3 after removal of the pro-domain as recently proposed[478]. In liver tissue from mice treated with 500 mg/kg phorone prior to αCD95 injection (lane IV), the p32 pro-form was found in an amount indistinguishable from that in control tissue (lane I), indicating that this executor caspase was not activated by upstream caspases when GSH was depleted. As expected in the absence of caspase-3 activation, the cleavage of hepatic DNA (DNA laddering) 6 hours after induction of apoptosis with αCD95 (figure 7 C, lane 2-5) was also prevented by phorone with a similar dose dependence as the inhibition of caspase activity in figure 7 A. These findings demonstrate that depletion of GSH completely interrupts apoptotic CD95 signaling pathways in hepatocytes upstream of group II caspases.

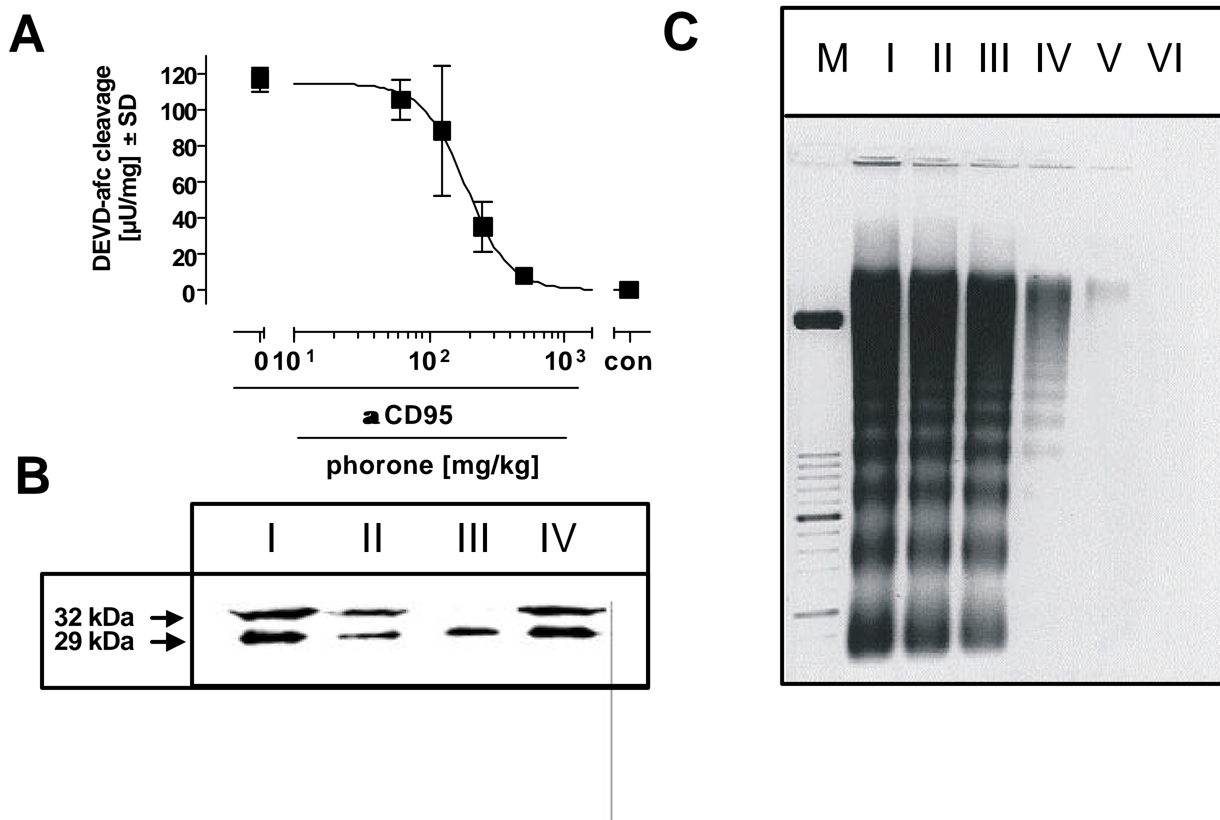


Figure 7: Inhibition of caspase-3-like activity, caspase-3 processing and DNA fragmentation by phorone pretreatment. *(**A**) Liver samples were taken from 5 groups of mice 4 hours after challenge with 3 µg/animal **a**CD95 and from untreated controls. Caspase-3-like protease activity was determined in cytosolic extracts. Four groups additionally received different doses of phorone (62,5-500 mg/kg) 1 hour before **a**CD95 challenge as indicated. Values are depicted as means ±SD from three animals per group. (**B**) In a parallel experiment, liver samples were taken 6 hours after **a**CD95 challenge and the processing of procaspase-3 was analyzed by Western blot. (I) Untreated control animal, (II) 3 µg/animal **a**CD95, (III/IV) pretreatment of mice with phorone (250 and 500 mg/kg, respectively) 1 hour before **a**CD95 challenge. (**C**) In a similar experimental setting, DNA fragmentation in liver specimen was analyzed by gel electrophoresis. (M) marker, (I) 3 µg/animal **a**CD95, (II-V) pretreatment with phorone (62.5, 125, 250, 500 mg/kg) 1 hour before **a**CD95 challenge, and (VI) untreated control animal.*

3.6 Does a direct caspase inhibition by low GSH explain hepatoprotection?

Since caspases are known to be sensitive towards oxidation of their active site cysteine moiety including the formation of mixed disulfides[233,240], a possible GSH-dependent modulation of caspase activity was investigated.

First, the GSH dependence of the proteolytic activity of recombinant human caspase-3 (3 ng/ml) was studied, which was inactive in substrate buffer devoid of reducing agents (figure 8 A, con). In the presence of a physiologic concentration of GSH (5 mM), activity was restored, whereas shifting the GSH/GSSG-ratio in the assay buffer by adding an increasing percentage of GSSG resulted in a stepwise attenuation of proteolytic activity. This experiment demonstrates that caspase activity *in vitro* strictly depends on the availability of a reducing agent such as GSH, and that GSSG can further modulate the specific activity of caspases.

Hepatic caspase-3-like proteases are activated at about three hours after αCD95 application *in vivo* (figure 8 B), whereas the time course of changes in total glutathione following phorone injection is very rapid (figure 4 B). Thus, the phorone-induced loss of liver glutathione occurred before caspase-3-like proteases would have been activated due to CD95 engagement in the experiments shown in figure 5. Therefore, phorone treatment was shifted in order to intervene when hepatic caspase-3-like proteases had already been activated. Essentially complete protection from liver damage was observed when phorone was administered simultaneously with or one hour after αCD95 challenge (figure 8 C). When given 2 hours later, however, only partial protection was observed (67% reduction of plasma ALT). Similar results were obtained when mice were treated with the broad-spectrum caspase inhibitor z-VAD-fmk instead of phorone, but curative treatment 2 hours after αCD95 injection lead to a comparatively better protection (89% reduction of plasma ALT) than observed with phorone. This can be attributed to kinetic phenomena, since z-VAD-fmk acts directly on caspases, while GSH depletion *via* phorone takes about 30 minutes to be completed (figure 4 B). At 3 hours, when caspase-3-like proteases were already active (figure 8 C), neither z-VAD-fmk nor phorone significantly inhibited liver injury. These experiments demonstrate that both a disturbance of the hepatic glutathione status and direct caspase inhibition entirely protect the liver in the CD95 model, but only before caspase-3-like proteases become activated. This conclusion points to a block of CD95 signaling at the level of caspases 8 or 9, which was further studied (3.7, chapter 4).

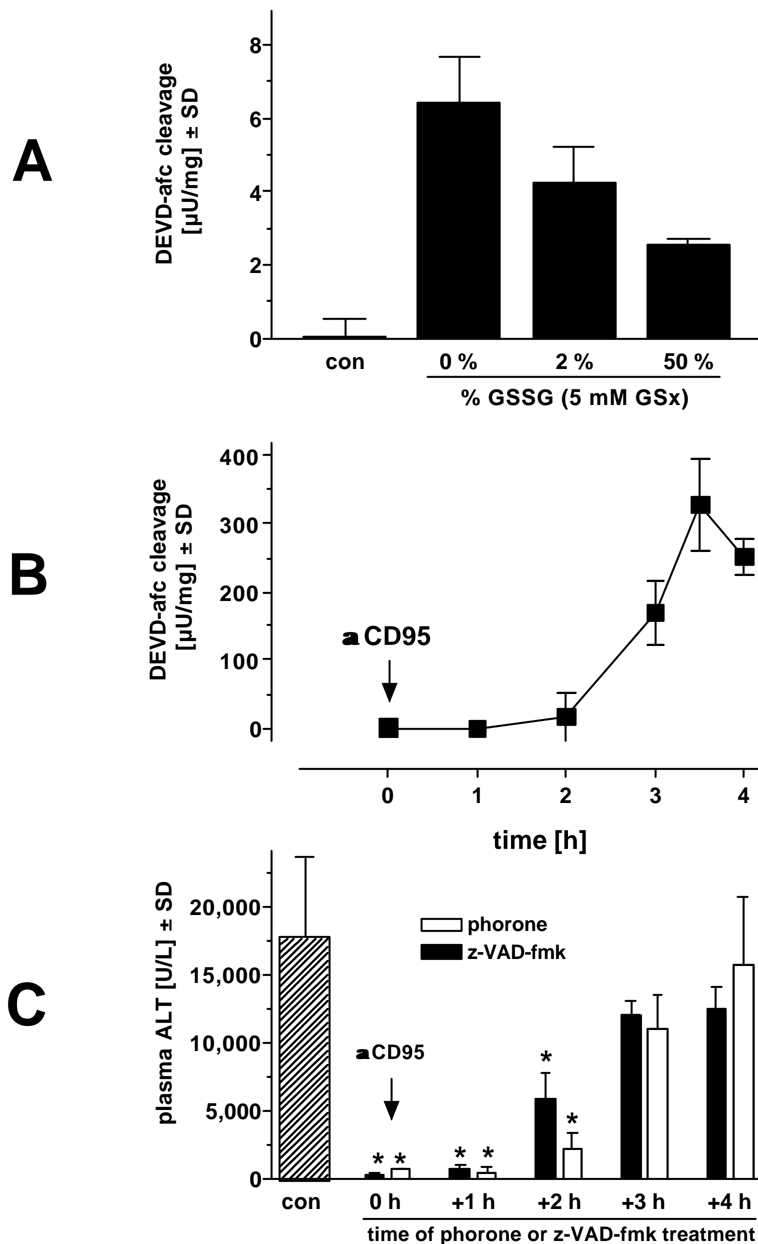


Figure 8: Thiol-dependent activity of caspase-3, CD95-induced activation of hepatic caspase-3-like proteases and its relation to delayed protection by phorone or z-VAD-fmk. *(**A**) The DEVD-afc cleaving activity of recombinant human caspase-3 (3 ng/ml) was measured without reducing additives (con), and 10 minutes after addition of 5 mM total glutathione. Additionally, the GSH/GSSG-ratio was varied up to 50% GSSG as indicated. Values are depicted as means ± SD of triplicates. (**B**) The timecourse of hepatic caspase-3-like protease activation upon aCD95 injection (3 µg/kg) was followed. Values are depicted as means ± SD from three animals per group. (**C**) Mice were injected with 3 µg/animal aCD95 (con), and 8 hours later, the release of ALT in the plasma was determined. Five groups of mice additionally received phorone (500 mg/kg) or the caspase inhibitor z-VAD-fmk (30 mg/kg) at timepoints indicated (0-4 hours) relative to the aCD95 challenge. Asterisks (*) indicate a p value <0.05 compared to aCD95-treated controls based on ANOVA analysis followed by the Dunnett multiple comparison test. Values are depicted as means ± SD from three animals per group.*

3.7 Is cyt c/dATP-induced caspase activation *ex vivo* dependent on GSH?

Besides the assembly of the DISC[113,184,633], the apoptosome formation represents the second well-characterized mechanism of upstream (group III) caspase activation[188,190,194,369,370]. The mitochondrial/apoptosome pathway has been shown to be activated also in receptor-mediated apoptosis (1.2.4), which has also been postulated for CD95-induced hepatic apoptosis (1.3.3).

Several groups have used a cyt c/dATP-mediated caspase activation assay[623,634] based on cell cytosol for studying e.g. caspase-caspase interactions or biochemical determinants of caspase-9 activation[215,345,346,348,635,636]. Here, caspase activation by cyt c/dATP and the influence of z-VAD-fmk, low GSH and NO was investigated in liver cytosol using an *ex vivo* approach.

First, the activation rate of caspase-3-like proteases by cyt c/dATP in liver cytosol prepared from naive mice was characterized. In figure 9 A, the time dependence of the reaction is displayed. From 20 min after addition of cyt c/dATP onwards, DEVD-afc cleaving activity increased linear, and all further activation assays were run for a period 30 minutes. In the absence of cyt c/dATP, no activity was detectable (figure 9 A), and we observed that addition of the reductant DTT in the reaction mixture up to 10 mM did not significantly change caspase activation (without figure). It is apparent that the concentrations of the various components of the apoptosome limits the rate of the activation reaction. In agreement with this assumption, an effective activation of liver cytosols was possible at final protein concentrations of >32 mg/ml (figure 9 B). Thus, cytosols were adjusted to a fix final concentration of 80 mg/ml (40 mg/ml in the activation reaction) for experiments further shown. Figure 9 C shows the concentration response curve of both ATP and dATP, and EC_{50} values were calculated as 0.42 and 1.08 mM for dATP/ATP, respectively. This difference might reflect the greater stability of dATP compared to ATP[637], and 5 mM dATP was used for further experiments to achieve a maximal activation rate. The concentration response curve of cyt c allowed to estimate an EC_{50} value of 21 µM (figure 9 D), and 100 µM were used for further experiments. This EC_{50} value for cyt c is approximately 5-fold higher compared to reconstituted systems using cytosols derived from cell lines. Finally, this activation assay was particularly sensitive towards inhibition by the non-specific irreversible caspase inhibitor z-VAD-fmk in the activation reaction (IC_{50}: 17 nM, figure 9 E). This IC_{50} value for z-VAD-fmk is far lower than that towards recombinant caspase-3 (100 nM), but close to the IC_{50} value for z-VAD-fmk seen for inhibition of CD95-triggered caspase-3 activation in primary cultured hepatocytes (11 nM)[638].

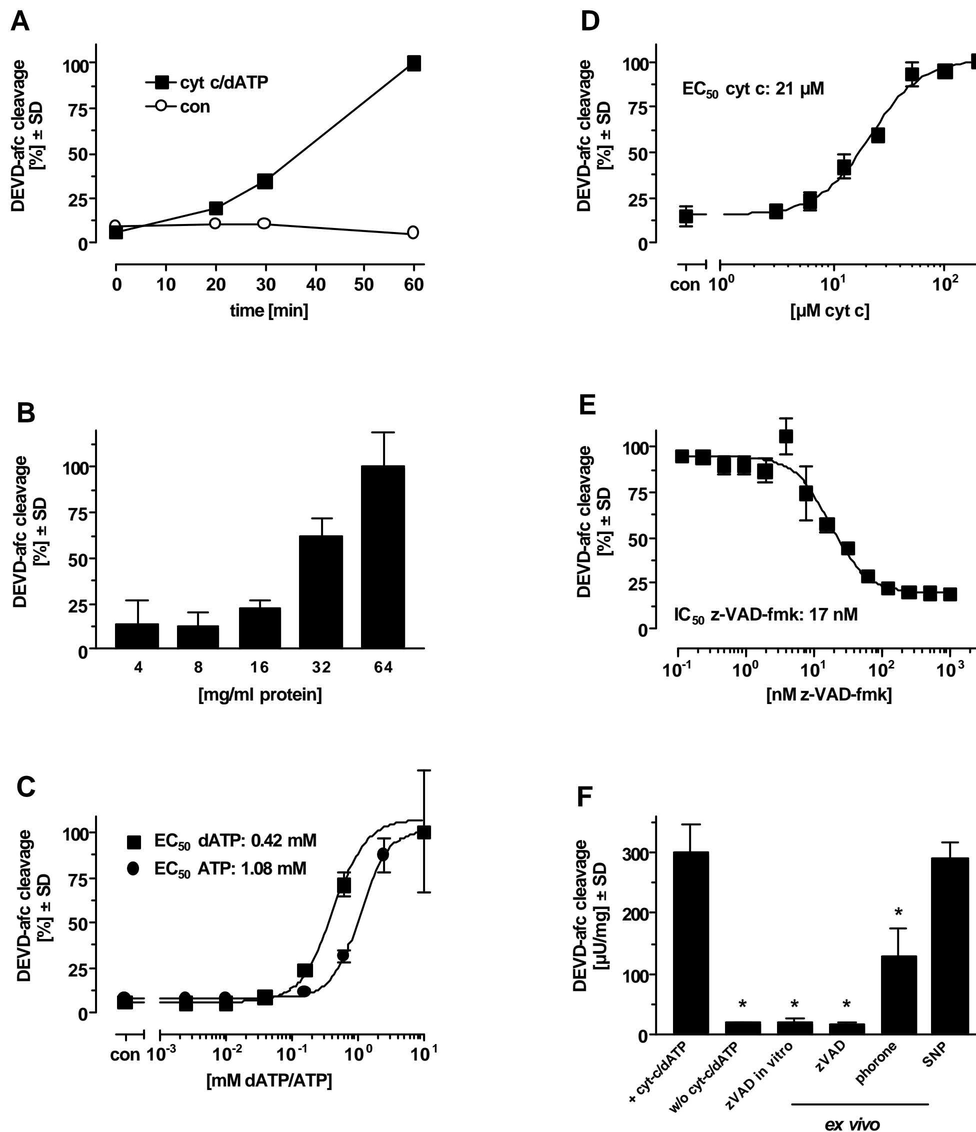


Figure 9: Activation of hepatic caspase-3-like proteases by cyt c/dATP in liver cytosol. *Liver cytosolic extracts were prepared in a hypotonic buffer as described in the methods section. Five microliters of the cytosol was coincubated with varying concentrations of cyt c and dATP/ATP in a final volume of 10 µl, and DEVD-afc cleaving activity was subsequently determined (final volume: 100 µl).* ***(A)*** *Time dependency of caspase activation. After addition of 100 µM cyt c/2 mM dATP, the activation reaction was stopped by addition of 90 µl DEVD-afc-containing assay buffer after the timepoints indicated and the DEVD-afc cleavage was determined.* ***(B)*** *Activation of caspases is dependent on the protein concentration. Cytosols with the indicated final protein concentrations were incubated with 100 µM cyt c/5 mM dATP, and 30 min later, DEVD-afc cleavage was measured.* ***(C)*** *In the presence of 100 µM cyt c, the concentration of ATP or dATP were varied in the activation reaction as indicated (40 µg/µl final protein concentration).* ***(D)*** *In a similar experiment, the cyt c concentration was varied as indicated (5 mM dATP, 40 µg/µl final protein concentration).* ***(E)*** *Sensitivity of the activation reaction towards inhibition by z-VAD-fmk (100 µM cyt c, 5 mM dATP, 40 µg/µl final protein concentration). Data are means ± SD from triplicate experiments (A-E).* ***(F)*** *Effect of z-VAD-fmk, phorone and SNP treatment of mice on caspase activation ex vivo. Control mice (bars 1-3) and animals receiving the caspase inhibitor z-VAD-fmk (10 mg/kg i.v.), phorone (500 mg/kg i.p.) or SNP (5 mg/kg i.p.) for 1 hour (bars 4-6) were euthanized. Subsequently, liver cytosols were prepared, adjusted to 80 mg/ml protein concentration and used for the activation assay. In vitro, z-VAD-fmk was added in a concentration of 10 µM. Data are means from 3 animals/group, and Asterisks (*) indicate a p value <0.01 compared to positive control (bar 1) based on ANOVA analysis followed by the Dunnett multiple comparison test.*

Finally, an *ex vivo* experiment was done to address whether liver cytosol from differently treated mice displayed differences in the activation assay. Under optimal conditions as described above, a physiological caspase-3-like activity of 300 μU/mg protein was induced within 30 minutes in control liver homogenates by addition of cyt c/dATP (figure 9 F). Again, the reaction was completely blocked when z-VAD-fmk was present in the system. Serving as a methodological control, liver cytosol derived from animals that had been pretreated with a hepatoprotective dose of z-VAD-fmk[481] 1 hour prior to liver excision was also not activable in this assay (bar 4). Moreover, liver specimens were prepared from mice that had received a dose of phorone providing complete protection against CD95-induced apoptosis (figure 5). Notably, glutathione-depleted cytosols displayed a significantly suppressed activation rate (-57% DEVD-afc cleavage), which was fully restorable by the addition of 10 mM DTT to the activation reaction (not shown). Also, cytosols from mice treated with the NO donor SNP in a dose that entirely prevents TNF-induced hepatic apoptosis[241] was tested, since S-nitrosation of procaspases was proposed to account for the antiapoptotic function of NO[254,639,247]. However, caspase-3-like proteases in respective liver samples were fully activated by cyt c/dATP (figure 9 F). These data suggest a prerequisite of an intact intracellular glutathione status for an undiminished activation of caspases *via* the apoptosome, which might contribute to the antiapoptotic and hepatoprotective effects of depleted glutathione described in this study (see 6.2.1).

4 Depletion of glutathione interrupts CD95 type I apoptosis in SKW6.4 cells

To evaluate mechanistic aspects of apoptosis inhibition by low GSH, the CD95 DISC (death-inducing signaling complex) formation was investigated in an appropriate experimental system. Upon CD95 triggering, the lymphoid B cell line SKW6.4 undergoes CD95 type I apoptosis[70,361]. Instead of primary cultured hepatocytes, this cell line was chosen for DISC immunoprecipitation due to the following reasons: (i) the immunoprecipitation of the CD95 DISC has been established in SKW6.4 cells[361,626]; (ii) in contrast to hepatocytes, SKW6.4 cells can be effectively GSH-depleted by GSH S-transferase substrates (4.1) and are thereby protected from CD95-induced apoptosis (4.2); (iii) as outlined in the introduction (1.3.3), it can be argued from the present literature that CD95-mediated hepatic apoptosis represents type I apoptosis; (iv) in agreement, Bcl-2-overexpressing hepatocytes were sensitive towards stimulation by αCD95 in our hands (HC derived from Bcl-2-overexpressing mouse: 62.8% LDH release; control HC from a C57/B6 mouse: 54.3% LDH release; 100 ng/ml αCD95 for 20 h); (v) Not all antibodies for the detection of murine DISC-associating proteins in Western blot are available yet.

4.1 Glutathione depletion and glutathione repletion in SKW6.4 cells

Initially, several compounds were tested for their capacity to lower intracellular total glutathione (GSx) in SKW6.4 cells. Incubation with the glutathione synthesis inhibitor buthionine-sulfoximine (BSO) lead to a sustained, but slow decrease in intracellular GSx (figure 10 A). The water-soluble GSH S-transferase substrates diethylmaleate (DEM) and cis-chloro-2,5-dinitrobenzoate (CDNB) depleted intracellular GSx when given in combination with BSO to below 10% of the initial value within 1 hour (figure 10 A). This depletion occurred without induction of toxicity within 4 hours in the concentrations used (DEM: toxic >2 mM, CDNB: toxic >20 µM). The fact that DEM has to be applied in the mM range, whereas CDNB efficiently depletes GSx in the µM range reflects the different K_M for GSH S-transferases.

For repletion of intracellular GSx, the highly cell-permeable glutathione donor glutathione monoethylester (GSH-E) was used[496]. In an attempt to achieve maximal GSx repletion, various experimental settings were compared. In figure 10 B, the most effective incubation is shown, i.e. depletion of GSx for 1 hour (CDNB concentrations as indicated) followed by 1 hour repletion with 10 mM GSH-E. Note that the repletion was complete and highly significant at a concentration of 2.5 µM CDNB (89% of control), whereas at higher CDNB concentrations, only an incomplete, but still significant repletion was observed (60% of unrelated control). Thus, the amount of intracellular GSx in SKW6.4 cells can be experimentally adjusted with the above-mentioned compounds without affecting cell viability.

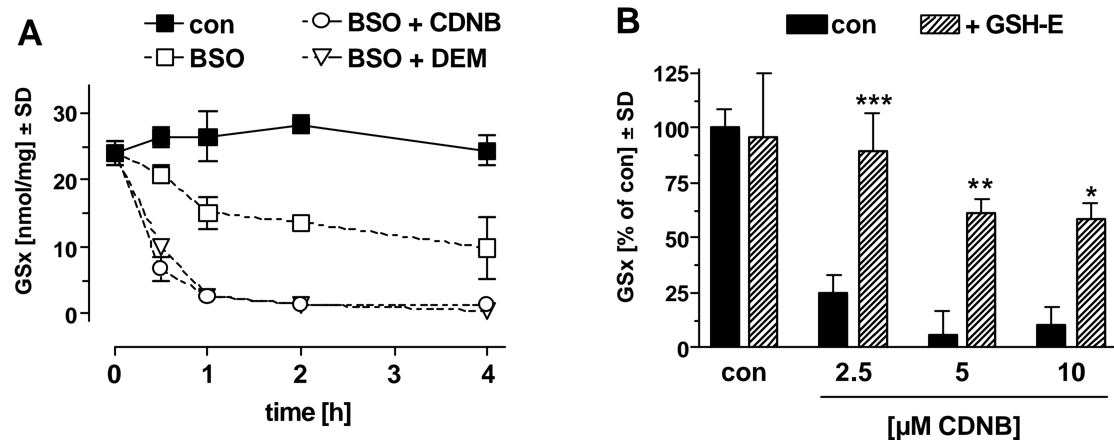


Figure 10: GSH depletion and repletion in SKW6.4 cells. *(**A**) SKW6.4 cells (10^5/well) were incubated with 0.2 mM BSO, 0.2 mM BSO +5 µM CDNB, or 0.2 mM BSO +1 mM DEM. After different timepoints, samples for the determination of total glutathione (GSx) and protein were obtained, and the amount of intracellular GSx was calculated as nmol GSx/mg protein. (**B**) Cells were GSH-depleted by the indicated concentrations of CDNB in the presence of 0.2 mM BSO, and 1 hour later, 10 mM GSH ester (GSH-E) was added. Two hours after addition of CDNB, cells were washed 3 times in PBS, and the GSx content was determined (expressed as % of untreated control). Values are depicted as means ±SD from three wells. Asterisks (*/**/***) indicate p values of <0.05/<0.01/<0.001 for CDNB +GSH-E-treated cells compared to CDNB-treated controls (ANOVA analysis followed by the Tukey multiple comparison test).*

4.2 Deficiency of glutathione inhibits CD95 type I apoptosis

Next, it was investigated whether CD95 type I apoptosis in SKW6.4 cells would also be influenced by glutathione variations. As shown in figure 11 A, exposure of cells to a CD95-activating antibody (CH-11) for 4 hours resulted in 70% apoptotic cells as determined by Hoechst/SYTOX exclusion staining. When cells were depleted of GSx by DEM or CDNB for one hour and subsequently treated with CH-11, apoptosis was blocked, i.e. a decrease in the apoptosis rate to <15% was observed under low GSx.

Apoptosis upon DR stimulation is initiated by caspase-8 and executed by caspase-3-related proteases[29,74,141], and caspases are redox-sensitive proteases[233,235,237,239,250,594]. Therefore, caspase-3-like proteolytic activity upon CD95 stimulation was first examined. Four hours after CD95 engagement, a high activity of DEVD-cleaving caspases was determined (figure 11 B, filled squares). In the presence of DEM alone or DEM plus BSO, caspase activity was suppressed at this time to 42% or <20%, respectively. Since the DEVD-afc cleavage assay was carried out in the presence of high concentrations of a reductant (10 mM DTT) which would reactivate directly oxidized caspases[237,250,640], the reduction in caspase-3-like activity represents a decrease in executioner caspase *activation* rather than *activity* itself. In this line, Western blot analysis of caspase-8 activation after CD95 triggering revealed that the active caspase-8 p18 fragment was not detectable in cytosolic extracts upon glutathione depletion by DEM (figure 11 C). Therefore, the CD95 DISC formation in SKW6.4 cells was eventually analyzed to evaluate upstream signaling events (figure 12).

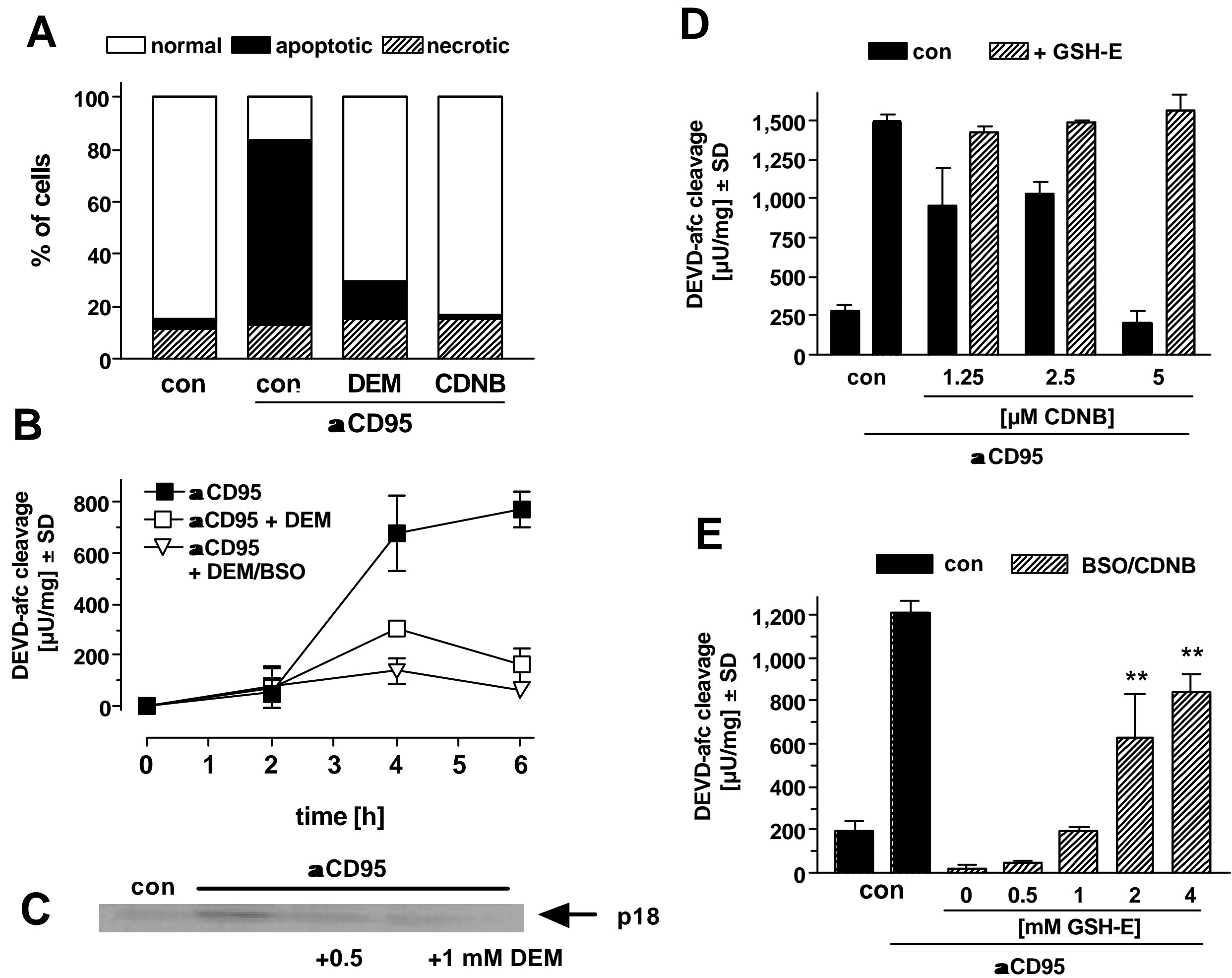


Figure 11: Inhibition of CD95-induced apoptosis by GSH depletion in SKW6.4 cells and restoration of cell death by GSH repletion. ***(A)*** *SKW6.4 cells (10^5/well) were GSH-depleted by a combination of 0.2 mM BSO and 1 mM DEM or 10 µM CDNB, respectively. One hour later, cell death was induced by addition of 100 ng/ml aCD95 (CH-11), and after 4 hours, the percentage of occurring apoptotic or necrotic cell death was determined after staining the cells with H-33342 plus SYTOX (3 independent determinations/incubation).* ***(B)*** *In a parallel experiment, cells were exposed to aCD95 (100 ng/ml), BSO (0.2 mM) and DEM (1 mM) as indicated, and the caspase-3-like activity was determined in cytosolic extracts prepared at the times indicated.* ***(C)*** *Western blot analysis of the active caspase-8 fragment (p18). Cytosolic extracts from SKW6.4 cells were prepared in an experiment as described in (B).* ***(D)*** *Cells were GSH-depleted by the indicated concentrations of CDNB, and 10 mM GSH ester (GSH-E) was added one hour later. Two hours after CDNB, apoptosis was induced by aCD95 (100 ng/ml CH-11). Four hours later, the caspase-3-like activity was determined in cytosolic extracts.* ***(E)*** *In a similar experiment, cells were GSH-depleted with 0.2 mM BSO/5 µM CDNB, and GSH was repleted by the indicated concentrations of GSH-E. A p value <0.01 based on ANOVA analysis followed by the Dunnett multiple comparison test is indicated (** vs. untreated control). Values are depicted as means ± SD from three wells (B, D, E).*

Additionally, CD95 type II apoptosis in Jurkat T cells was comparatively studied with respect to GSx depletion. In these cells, CD95 apoptosis occurs involving the mitochondrial branch of apoptotic signaling[361]. GSx depletion was achieved with the same non-toxic concentrations of BSO and DEM/CDNB as described for SKW6.4 cells (figure 11 A). In contrast to type I apoptosis, cell death in this case switched from apoptosis to necrosis. Ensuing CD95 ligation, 65% of Jurkat cells underwent apoptosis, which was suppressed to 11% after preincubation with 1 mM DEM. Instead, 86% necrotic cell death was now seen under GSx depletion. This switch in the mode of cell death was accompanied by a marked decrease in caspase-3-like activity under low GSx (1,795 ± 65 µU/mg to 105 ± 50 µU/mg 4 hours after CH-11). Thus, the block of CD95 signaling by GSx depletion appears to occur particularly in type I apoptosis and therefore may be located at the DISC level.

4.3 Repletion of glutathione restores activation of group II caspases

Glutathione-depleting compounds may bind to thiol residues and thereby inhibit caspases, as it has been shown for strong thiol-modifying agents such as dithiocarbamates[233]. Therefore, a possible direct interaction of DEM or CDNB with caspases was addressed by two experimental approaches. First, the impact of GSx repletion on CD95-induced caspase activation was examined. Caspase-3-like activity 4 hours after CD95 engagement was inhibited in a concentration-dependent fashion by CDNB (figure 11 C). When high GSx levels were maintained by the use of 10 mM glutathione ester as elaborated above (figure 10 B), caspase activity could be completely restored to initial control values. When SKW6.4 cells were depleted by 5 µM CDNB and different concentrations of the ester were applied before CD95 ligation (figure 11 D), it turned out that a concentration of 2 mM ester was sufficient to significantly restore activation of executioner caspases. In addition to this finding, both glutathione depletors CDNB (up to 20 µM) and DEM (up to 4 mM) did not reduce caspase-3-like activity when directly added *in vitro* to the cleavage assay in the absence of DTT. Conclusively, these data imply that the glutathione depletors used in this study do not directly interfere with caspases, but rather act on CD95-mediated apoptosis *via* the depletion of intracellular glutathione.

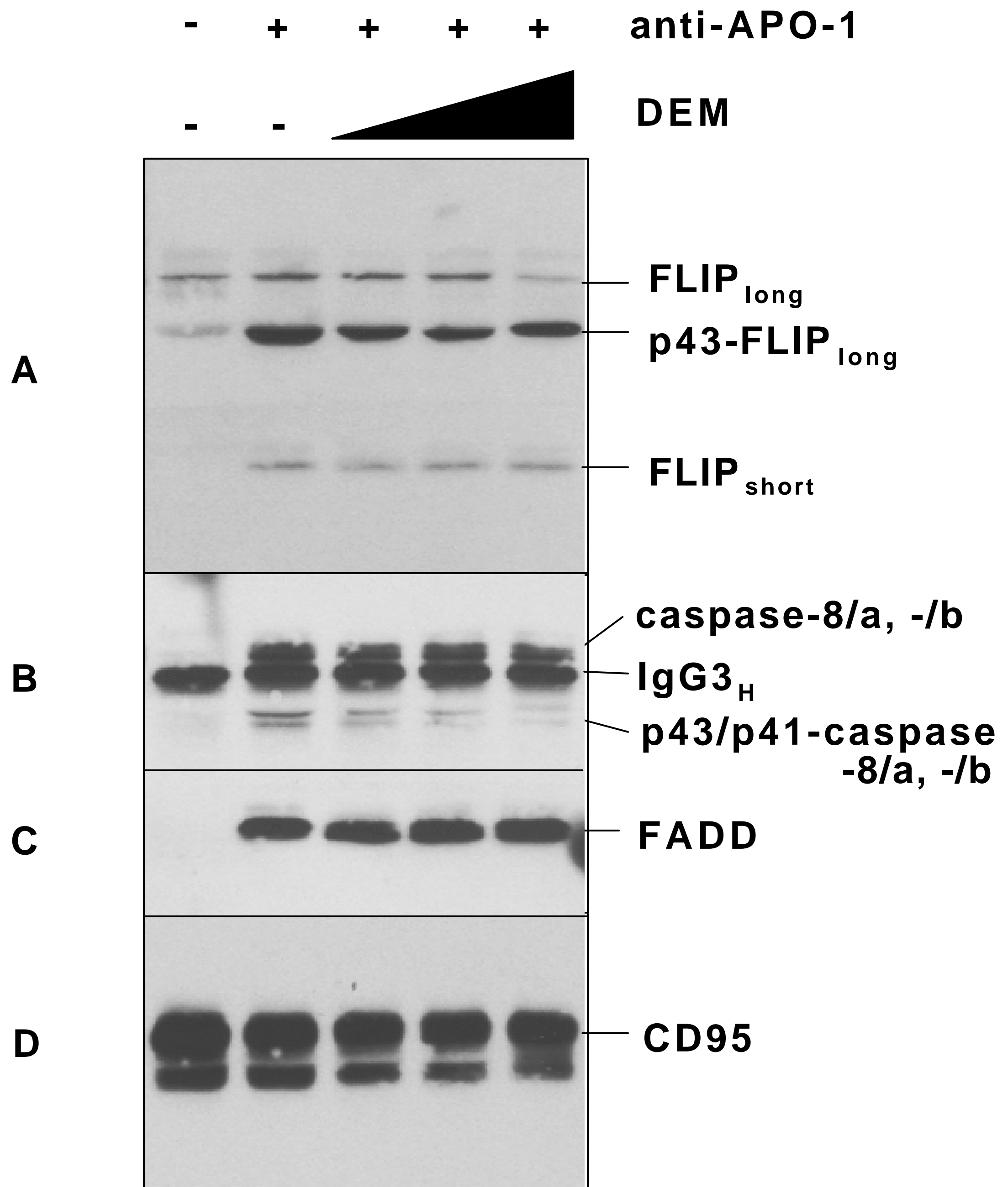
4.4 Does glutathione depletion by DEM influence CD95 DISC formation?

The formation of the DISC comprises the recruitment of the proteins FADD (Fas-associated death domain), FLIP (FLICE-inhibitory protein) and eventually caspase-8 to the intracellular death domain of CD95[641]. All of these components contain several cysteine residues, and we speculated whether a depletion of intracellular GSH might hamper the formation of the DISC and by this mechanism prevent the initiation of apoptosis.

After maintenance of SKW6.4 cells for 1 hour in medium containing BSO plus 0.25, 0.5 and 1 mM DEM, the DISC was immunoprecipitated with the antibody anti-APO1 as described[70,626]. Western blots of the precipitated proteins revealed that even in the presence of up to 1 mM DEM, both forms of FLIP (short/long FLIP), FADD and both caspase-8 subspecies were still recruited to the receptor CD95 (figure 12 A-C). Also, the amount of precipitated CD95 itself did also not change under different GSx levels (figure 12 D). These data indicate that the DISC formation took place also under the condition of low GSx.

Turnover of both caspase-8 proenzyme isoforms at the DISC precedes further processing of the p43/p41 fragments in the cytoplasm[617]. Notably, this first step in caspase-8 maturation was suppressed in a concentration-dependent fashion after glutathione depletion (figure 12 B). At a concentration of 1 mM DEM which entirely suppressed apoptosis (figure 11 A) and activation of executioner caspases (figure 11 B), caspase-8 cleavage was not detectable at the DISC level. This is furthermore in line with the experiment demonstrating the absence of active p18 caspase-8 in the cytoplasm after exposure of the cells to DEM (figure 11 C). Conclusively, these data show that in SKW6.4 cells, a depletion of intracellular glutathione by GSH S-transferase substrates can halt CD95 type I apoptosis at the level of caspase-8 proenzyme processing at the DISC.

Figure 12 (next page): Immunoprecipitation of the DISC after GSH depletion in SKW6.4 cells. *Cells (10^7 per sample) were treated with BSO (0.2 mM) plus DEM (0.25, 0.5 and 1 mM, respectively) as indicated, and CD95 was imunoprecipitated with 2 µg/ml anti-APO1 antibody (+) after 1 hour for 10 min. Lane 1 shows the negative control with anti-APO1 bound to protein A sepharose after cell lysis as described in methods section (-). After washing of sepharose beads, adherent proteins were loaded on 12% SDS page electrophoresis and subsequently subtracted to Western blot analysis.* ***(A)*** *Detection of p46, p43 and p36 c-FLIP isoforms using NF6 anti-c-FLIP antibody.* ***(B)*** *The same blot was stripped and subsequently developed with C15 anti-caspase-8 mAb, which detects both caspase-8 isoforms (zymogens: p54/p55, cleaved caspase-8: p43/p41). The secondary antibody also detected $IgG3_H$ of anti-APO1 used for immunoprecipitation.* ***(C)*** *FADD was detected using an anti-FADD mAb, and* ***(D)*** *CD95 was immunodetected serving as loading control by an anti-CD95 mAb.*


- + + + + anti-APO-1
- - DEM
A
B
C
D
FLIP long
p43-FLIP long
FLIP short
caspase-8/a, -/b
IgG3 H
p43/p41-caspase -8/a, -/b
FADD
CD95

5 Glutathione depletion prevents apoptotic and necrotic death receptor-triggered hepatocyte demise

5.1 Low glutathione inhibits TNF-R1-, caspase-dependent liver injury

In the chapters 3 and 4, the GSH dependence of CD95-induced apoptosis and several mechanistic aspects were covered. In this chapter, the GSH dependence of further liver injury models will be described. Firstly, the effect of the GSH depletor phorone in TNF-R-dependent models of acute inflammatory liver injury involving caspase activation was investigated. As shown in table 4, the caspase-3-like activity in liver tissue from control animals was under the detection limit of the DEVD-afc cleavage assay. In contrast, in both TNF-R1-mediated models, i.e., in GalN/LPS or GalN/TNF, a greatly increased caspase-3-like activity was observed at 6 hours. This pivotal event in TNF-R1-signaling was entirely abrogated in animals that were pretreated with a dose of phorone which depletes hepatic GSH stores by 90% within one hour (figure 4 B).

Likewise, the histological examination of liver specimens from mice injected with GalN/LPS (8 h) revealed massive hepatocyte apoptosis, as judged by the frequent appearance of nuclear fragmentation and hyperchromatic nuclear membranes (figure 12 A) as described[399,401]. At this late timepoint, also necrotic foci, mild neutrophil infiltration, erythrocyte agglutination and a complete destruction of the sinusoidal structure of the liver were observed. In phorone-treated animals, signs of liver injury elicited by GalN/LPS were absent (figure 12 B). Accordingly, the ALT release was at control levels in mice that had received phorone in addition to GalN/LPS or GalN/TNF (table 2). In the GalN/LPS-model, the systemic release of TNF was increased 3-fold in the phorone-treated group compared to controls (without figure), which might be specific for GSH deficiency[642]. A similar hepatoprotection upon GSH depletion was observed in the CD95 model (table 4), as elaborated in chapter 4. As a further methodical control, the hepatotoxicity of acetaminophen occurring in the absence of caspase-3-like activity was increased 16-fold in phorone-treated mice compared to naive mice (table 4, see also 1.4.3).

Model	phorone	ALT plasma activity (U/l) ± SD	DEVD-afc cleavage (μU/mg) ± SD
control	-	15 ± 5 (3)	≤ 20 (3)
	+	15 ± 5 (3)	≤ 20 (3)
acetaminophen	-	230 ± 200 (3)	≤ 20 (3)
	+	3,790 ± 1,290 (3)	≤ 20 (3)
GalN/LPS	-	6,930 ± 1,070 (3)	480 ± 20 (3)
	+	20 ± 10 (3)	≤ 20 (3)
GalN/TNF	-	9,680 ± 4,300 (3)	420 ± 20 (3)
	+	760 ± 670 (3)	60 ± 20 (3)
αCD95	-	9,950 ± 2,900 (12)	120 ± 20 (3)
	+	600 ± 700 (12)	≤ 20 (3)
Act D	-	9,800 ± 880 (3)	≤ 20 (3)
	+	550 ± 400 (3)	≤ 20 (3)
α-amanitin	-	3,850 ± 1,800 (3)	≤ 20 (3)
	+	340 ± 40 (3)	≤ 20 (3)
Con A	-	5,950 ± 3,350 (12)	≤ 20 (3)
	+	350 ± 550 (12)	≤ 20 (3)
LPS	-	2,560 ± 1,140 (6)	≤ 20 (3)
	+	100 ± 20 (6)	≤ 20 (3)

Table 4: Prevention of hepatotoxicity and hepatic caspase-3-like activity by phorone treatment in different models of cytokine-mediated liver injury. *Mice were injected with acetaminophen (175 mg/kg), GalN/LPS (700 mg/kg; 5 μg/kg), GalN/TNF (700 mg/kg; 2 μg/kg),* ***a****CD95 (2 μg/animal), Act D (2 mg/kg),* ***a****-amanitin (3 mg/kg), Con A (25 mg/kg), or LPS (10 mg/kg), and additionally treated with phorone (250 mg/kg) as indicated at various time points (acetaminophen; GalN/TNF;* ***a****CD95; Con A: t = -30 min; GalN/LPS; LPS: t = +1 h; Act D;* ***a****-amanitin: t = +2 h). Mice were sacrificed for the evaluation of plasma ALT levels at 8 hours (acetaminophen; GalN/TNF; GalN/LPS;* ***a****CD95; Con A) or 20 hours (Act D;* ***a****-amanitin; LPS), and for the determination of caspase-3-like activity at 4 (****a****CD95), 6 (GalN/TNF; GalN/LPS), 8 (control, acetaminophen, Con A) or 20 hours (Act D;* ***a****-amanitin; LPS). The numbers of animals/group are indicated in parentheses.*

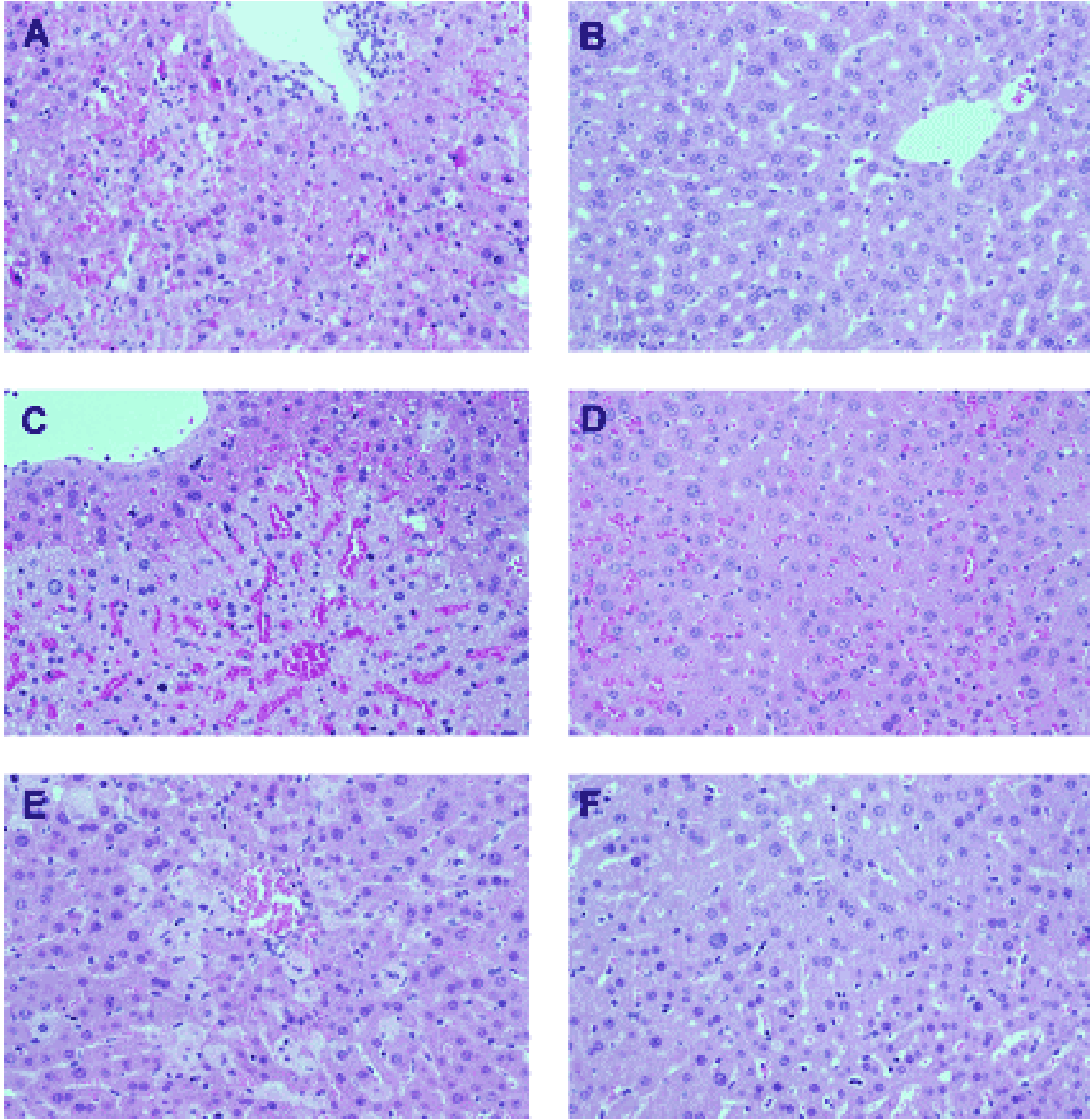


Figure 13: Histopathology of apoptotic and necrotic liver damage (left panel) and prevention by phorone treatment (right panel) in mice. *Animals received **(A/B)** GalN/LPS (A, 700 mg/kg; 5 µg/kg, 8 hours), and one group was additionally treated with phorone (B, 250 mg/kg, t = +1 h); **(C/D)** Con A (25 mg/kg, 8 hours), one group treated with phorone (D, 250 mg/kg, t = +3 h); **(E/F)** high-dose LPS (10 mg/kg, 20 hours), one group treated with phorone (F, 250 mg/kg, t = +1 h). Photographed at an original magnification of 200 x.*

5.2 Indirect triggering of TNF-R1 by high-dose hepatotoxins: apoptosis is independent of caspases, but prevented by glutathione deficiency

It was shown that the toxicity of α-amanitin or actinomycin D (Act D) involves a local effect of TNF on TNF-R1 of hepatocytes. By this mechanism, hepatocytes underwent apoptosis as assessed by morphological evidence and DNA laddering[374]. Since the contribution of caspases in these models had not been examined, it was first investigated whether caspase-3-like proteases become active after administration of α-amanitin or Act D. No increase in DEVD-afc-cleaving activity at any timepoint examined was detected (without figure), and even 20 hours after treatment of mice, no respective enzyme activity was observed (table 4). To exclude a possible contribution of caspases other than DEVD-afc-cleaving enzymes, a pharmacological approach was chosen, i.e. repetitive treatment of mice with a high dose of the irreversible, non-specific caspase inhibitor z-VAD-fmk (10 mg/kg at t = -1 h, 5 mg/kg at t = +1/+3 h). While this inhibitor completely abrogated liver injury elicited by administration of αCD95 in a parallel control experiment (7,860 ± 270 U/l ALT 8 h after 2 µg/animal αCD95, 35 ± 10 U/l ALT 8 h after 2 µg/animal αCD95 +z-VAD-fmk-treatment, n = 3/group), z-VAD-fmk had no impact on liver toxicity elicited by Act D (1,180 ± 260 U/l ALT 8 h after 2 mg/kg Act D, 1,040 ± 110 U/l ALT 8 h after 2 mg/kg Act D +z-VAD-fmk-treatment, n = 3/group). These data show that apoptotic signaling in these models is not transmitted through caspase activation, but by alternative signaling pathways[379].

Again, the extent of liver injury after administration of α-amanitin or Act D, was dramatically decreased by >90% in mice that had been pretreated with the GSH depletor phorone (table 4). This experiment demonstrates that also in these caspase-independent, TNF-R1-mediated liver injury models, the toxicity of classical hepatotoxins is attenuated under conditions of low hepatic GSH.

5.3 Depletion of glutathione modulates the cytokine release and NF-κB activation in the Con A model

Intravenous injection of concanavalin A (Con A) in naive mice induces the polyclonal activation of T cells, which release pro-inflammatory cytokines (e.g. TNF, IFN-γ, GM-CSF), followed by acute inflammatory liver injury that resembles immune-mediated hepatitis in humans[414,421]. Mice pretreated with the glutathione depletor phorone were protected against liver injury induced by Con A, as demonstrated by suppression of the ALT release by 94% 8 hours after Con A challenge (table 4). As described[95], no activation of hepatic caspase-3-like enzymes was detectable, and animal lethality in this model was not affected by phorone-mediated hepatoprotection (see 5.5, figure 17)

Phorone preferentially depletes hepatic glutathione, but, to a lesser extent, can also affect the glutathione content of other organs and cells[509]. A drop in total glutathione in spleen cells isolated after injection of phorone was shown by our group [412,643], and it was further reported that glutathione alterations affect various lymphocyte functions *in vitro* and *ex vivo*[497,644,645]. Therefore, it was investigated whether phorone treatment affects the systemic release of cytokines in the Con A model. Examining the time course of IFN-γ release, it turned out that the release of this pivotal cytokine[417] was strongly suppressed in animals treated with Con A plus phorone compared to Con A-treated control mice (figure 14 A), i.e. an inhibition to 19% of control levels at 3 hours. In phorone-treated mice, a remarkable delay in the release of TNF was noticed (figure 14 A), which is produced by macrophages and T cells in this model[416,421]. As a functional consequence, this delay was paralleled by a delayed activation of the transcription factor NF-κB in the liver (figure 14 B). NF-κB activation was detectable 1 hour after Con A treatment in response to TNF as described[646], but not before 3 hours in mice that additionally received phorone and thus were completely protected from liver damage. Furthermore, the release of IL-2, a marker for T cell proliferation, and of IL-4, whose release is also crucial in the Con A model was investigated[419,647]. Similar to TNF, the release of both IL-2 and IL-4 was delayed, but after 3 hours, there was no difference in the circulating levels of these cytokines between Con A-treated mice and animals that received Con A and phorone (figure 14 C).

To elucidate whether this immunomodulation by phorone treatment is solely responsible for the protection observed, the time of phorone injection relative to the Con A challenge was varied. However, even when phorone was given 3 hours after Con A, the prevention of liver damage was almost complete, i.e. a reduction by 97% compared to Con A controls. Moreover, a significant reduction of ALT release was still seen by phorone application at +5 hours (figure 14 D). Thus, it appears that GSH depletion in the Con A model severely affects T cell response *in vivo* and delays NF-κB activation in the liver. Nevertheless, hepatocytes are protected under these circumstances, and even delayed administration of the GSH depletor interrupts cell death.

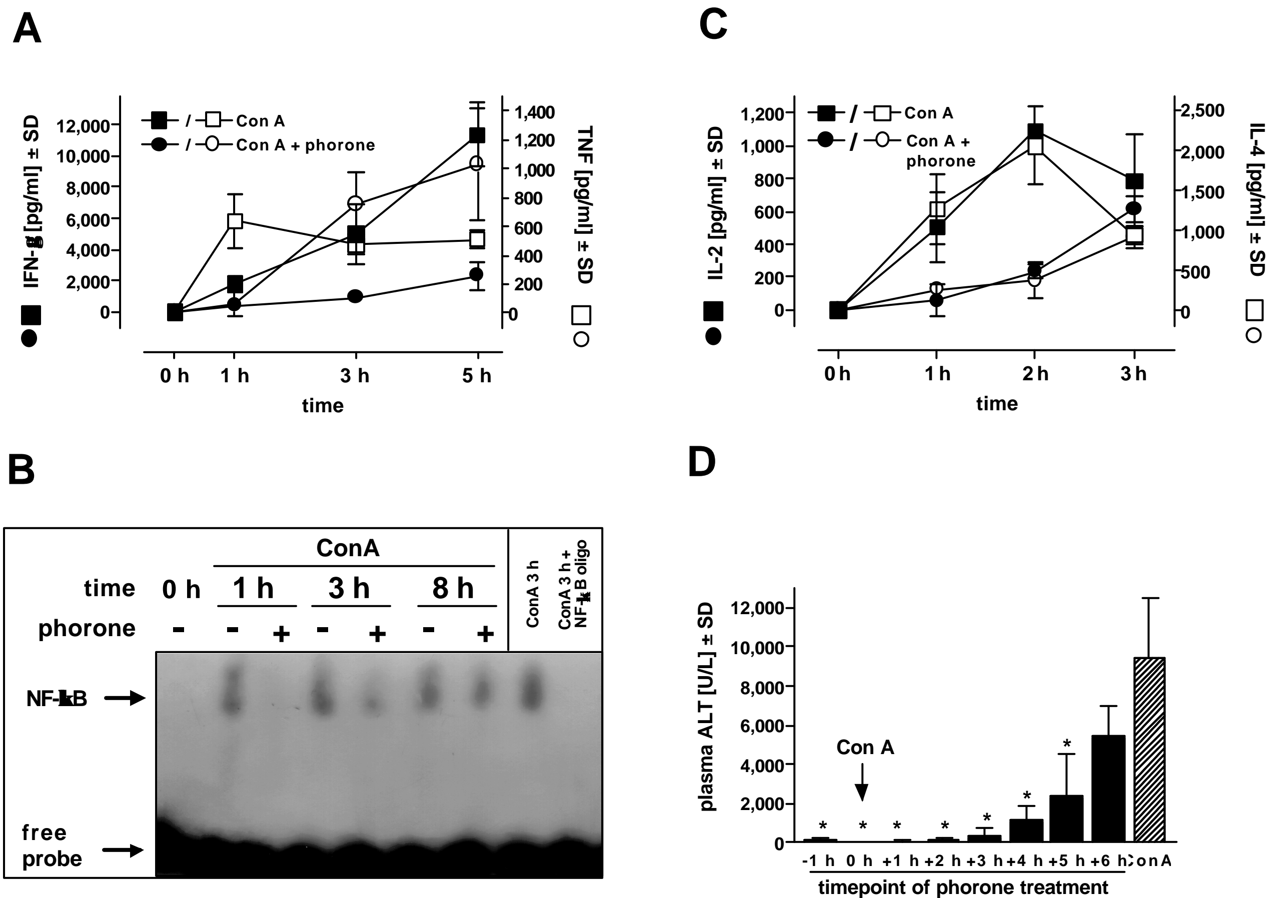


Figure 14: Influence of glutathione depletion on Con A-induced cytokine release, NF-kB activation, and curative protection by phorone in murine Con A-mediated liver injury. *Mice were injected with Con A (25 mg/kg) with and without pretreatment with phorone (t = -1 hour); **(A)** the systemic release of IFN-g and TNF was followed over a period of 5 hours (n = 3/group); **(B)** the activation of NF-kB was assessed at the timepoints indicated in Con A-treated, Con A + phorone-treated and in a control mouse as indicated; as a control, the specificity of the DNA/protein complex was competed with an excess of unlabeled NF-kB sequence; **(C)** the systemic release of IL-2 and IL-4 was followed over a period of 3 hours (n = 3/group); **(D)** Mice were treated with Con A (25 mg/kg), and 8 hours later, the release of ALT in the plasma was determined. Eight groups of mice additionally received phorone (250 mg/kg) at the timepoints indicated relative to Con A challenge; n = 3/group, Con A control: n = 9, and Asterisks (*) indicates a p value <0.05* versus *Con A-treated controls (ANOVA followed by the Dunnett multiple comparison test).*

5.4 Con A model: direct inhibition of endothelial and hepatocyte cell death

From the data presented above (5.3), a direct inhibition of cell death at the target cell level, i.e. a protection of hepatocytes and/or endothelial cells by low GSH is suggested to occur in the Con A model. This phenomenon was histologically elaborated in detail: 8 hours after Con A injection, large areas, especially in the periportal zone, displayed pronounced sinusoid destruction (figure 13 C). The histology of the damaged areas resembled that of ischemic liver injury, including pyknotic nuclei, hepatocyte necrosis, and virtual disappearance of sinusoidal endothelial cells (SEC), and severe erythrocyte agglutination

had taken place. In contrast, liver specimens from phorone-treated mice were histologically indiscernible from control livers. Notably, specimen taken from mice that received phorone 3 hours after Con A injection displayed erythrocyte agglutination, but no necrotic foci, and SEC were still present in these samples (figure 13 D). The examination of the sinusoidal structure by electron microscopy supported these findings: in contrast to control liver specimens (figure 15 A), SEC in livers from Con A-treated mice (8 hours) detached from neighboring hepatocytes. Some sinusoids appeared entirely denuded, and agglutinating erythrocytes filled the sinusoids and were found also in the space of Disse (figure 15 B). However, there were no hepatocytes that displayed typical morphological features typical for apoptosis present. Pretreatment of mice with phorone entirely abrogated the destruction of sinusoids (figure 15 C). Delayed administration of phorone 3 hours after Con A selectively prevented SEC damage (figure 15 D), and, as a consequence, hepatocyte death as judged by the reduction of ALT release (figure 14 D), but failed to inhibit erythrocyte agglutination (figure 13 D). These data conspicuously demonstrate that in the Con A model, an experimentally induced deficiency of glutathione influences the immune response, but also directly prevents SEC and hepatocyte death.

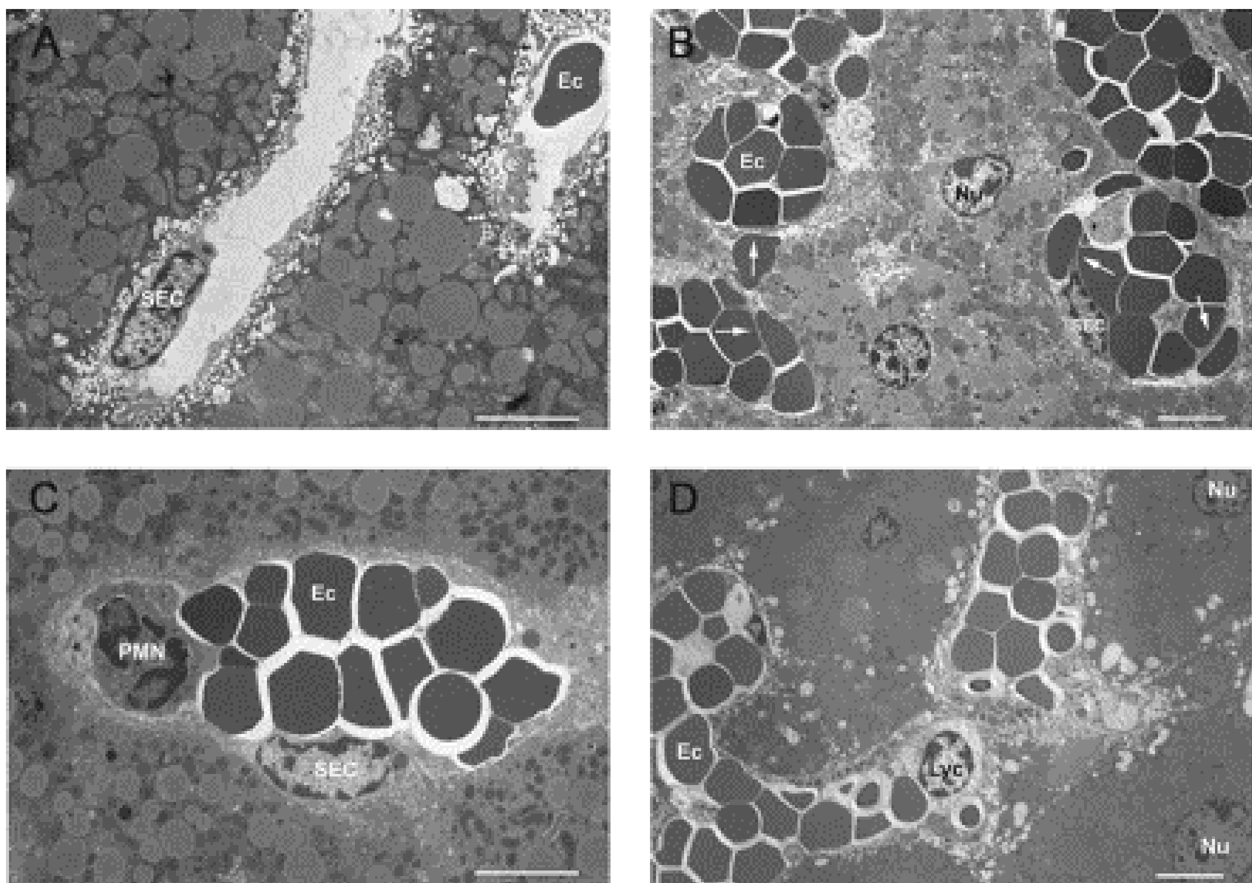


Figure 15: Transmission electron micrographs of hepatic sinusoids in murine Con A-mediated liver injury. ***(A)*** *Untreated control;* ***(B)*** *Con A (25 mg/kg), 8 hours;* ***(C)*** *Con A, pretreatment with phorone (-1 hour, 250 mg/kg);* ***(D)*** *Con A, injection of phorone 3 hours after Con A. The bar in each figure represents 6 µm; SEC, sinusoidal endothelial cell; detached SEC are marked with arrows; Ec, erythrocyte; Nu, hepatocyte nucleus; PMN, polymorphonuclear granulocyte; Lyc, lymphocyte.*

5.5 Low glutathione prevents liver damage, but not lethality, in LPS shock

The influence of hepatic glutathione alterations in a commonly used murine model of hyperinflammatory shock, i.e. administration of high-dose lipopolysaccharide (LPS) to naive mice was investigated. Here, liver injury occurs by necrosis of hepatocytes, despite its dependence on TNF[401, 427,648](1.4.1). Mice were injected with 10 mg/kg LPS, and the histological examination of liver specimen taken 20 hours later demonstrated a typical, dispersed single cell necrosis of hepatocytes, which did not display any zonation (figure 13 E). The nuclei of necrotic cells appeared karyolytic, pronounced hepatocyte membrane lysis occurred, and a mild infiltration of granulocytes was observed. In mice that had received LPS and phorone, no necrotic hepatocytes were found, and the overall histology of the liver appeared normal (figure 13 F). Likewise, the ALT release in mice treated with phorone and LPS was entirely abrogated in contrast to LPS-treated animals, and this model obviously does not involve the activation of hepatic caspase-3-like caspases (table 4). The observed protection was not due to inhibition of cytokine release, since plasma peak level concentrations of TNF and IL-1 were not significantly altered in phorone-treated mice (figure 16). Thus, a direct inhibition of cell death at the target cell level is mediated by low GSH also in this liver injury model.

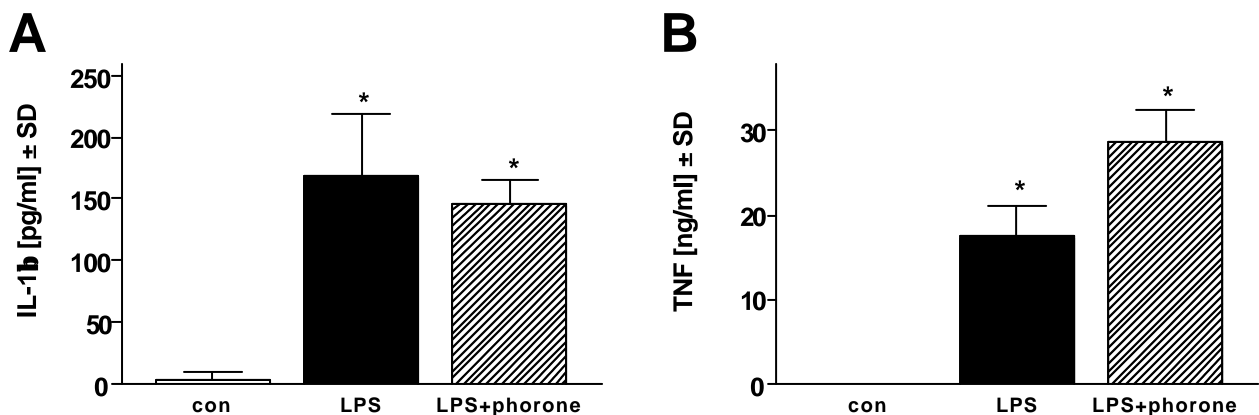


Figure 16: Circulatory release of IL-1b and TNF after LPS treatment of mice. *Mice received 10 mg/kg LPS, and one group additionally was treated with phorone 1 hour after LPS. The release of (**A**) IL-1b and (**B**) TNF 2 hours after challenge was determined by ELISA. n = 3/group, P <0.05* versus *untreated controls based on ANOVA, followed by the Dunnett multiple comparison test. LPS* versus *LPS +phorone was considered not significant.*

Finally, the survival outcome in four animal models was compared (figure 17). Hepatoprotection by phorone treatment entirely abrogated lethality in two TNF-R1-dependent apoptotic models (GalN/LPS, GalN/TNF), as described for CD95-mediated liver injury (figure 5 B). In contrast, mortality in the Con A model and in LPS shock was not influenced at all by the hepatoprotective effect of phorone (figure 17 B), indicating that liver damage in these two experimental settings is, in contrast to models of apoptotic, caspase-dependent liver injury models, not directly linked to animal lethality.

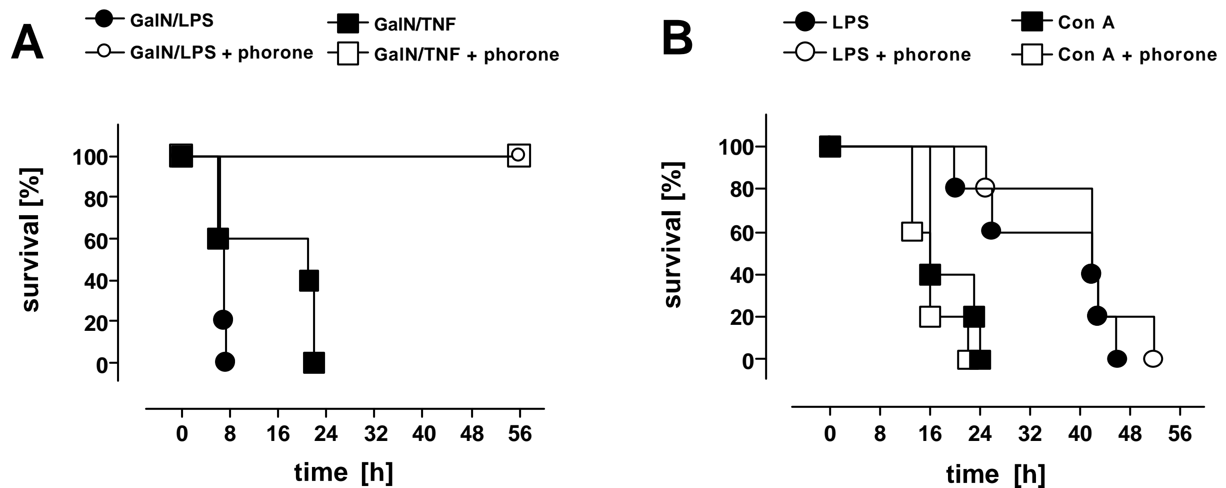


Figure 17: Prevention of lethality by phorone in murine GalN/LPS- and GalN/TNF-mediated liver damage, but not in the LPS shock and Con A model. ***(A)*** *Animals were injected with GalN/LPS (700 mg/kg; 10 µg/kg) or GalN/TNF (700 mg/kg; 5 µg/kg), respectively, and two additional groups received phorone (250 mg/kg, t = -1 h for GalN/TNF, t = +1 hour for GalN/LPS). The survival time was monitored for the time indicated.* ***(B)*** *Mice were treated with either Con A (50 mg/kg), or high-dose LPS (10 mg/kg), and two additional groups received phorone (250 mg/kg, t = -1 h for Con A, t = +1 hour for LPS). The survival time was monitored for the time indicated.*

6 Discussion

6.1 Glutathione deficiency in necrotic *versus* apoptotic liver damage

In this study, several hyperinflammatory liver injury models were investigated with regard to their glutathione dependence, the mode of cell death, and the activation of caspases. In the LPS shock and the Con A model, the possible role of immunosuppression was also examined, since cellular glutathione levels are known to influence the immune response[497,644,645]. When GSH was depleted, the onset of liver injury was blocked in *all* models investigated *at the target cell level*, i.e. the hepatocyte (summarized in table 5/6.4). Additionally, it was demonstrated that the structure of sinusoidal endothelial cells was preserved in the GSH-depleted state in the Con A model.

It is well documented that liver parenchymal cells when depleted of GSH are greatly sensitized towards a variety of different toxins which in most cases induce *non-apoptotic* cell death (1.4.3). The following two complementary mechanisms are held responsible for the enhanced hepatotoxicity after GSH depletion of e.g. acetaminophen, menadione, or carbon tetrachloride: (i) in GSH-depleted liver tissue, xenobiotics and their phase I metabolites escape conjugation with GSH via GSH S-transferases and accumulate within hepatocytes. These reactive metabolites are able to inactivate essential macromolecules of the cell by covalent modification; (ii) oxidative stress triggered by xenobiotics rapidly exhausts the antioxidative power of the GSH peroxidase/GSSG reductase system resulting in lipid peroxidation and loss of membrane integrity[494,495]. This perspective resulted in the paradigm that glutathione deficiency of the liver is associated exclusively with adverse qualities.

Antioxidants such as GSH also were protective in various models of *apoptotic* cell death, and this phenomenon has been attributed to an interference with apoptotic signaling[649-652](1.5.1). For instance, increased intracellular glutathione levels protected against CD95-mediated T cell apoptosis[522,653], and hepatocyte apoptosis due to oxidative stress was consistently enhanced by GSH depletion[544]. Moreover, the anti-apoptotic and hepatoprotective protein Bcl-2 has been proposed to act as an antioxidant[471,573,597,654], and the contribution of reactive oxygen species to TNF-mediated hepatocyte death has been investigated intensively[584,655]. Furthermore, a marked active glutathione efflux was noted in various experimental models of apoptosis including CD95-mediated cell death[381,574-576,583,656]. Thus, there is no general agreement as to a defined role of oxidative stress in apoptosis, and the physiological significance of glutathione and its export under these conditions is unknown.

The findings of the present study demonstrate that variations in the cells' main thiol buffer, i.e. the intracellular GSH concentration, can completely interrupt DR-dependent hepatocyte apoptosis and necrosis *in vivo*. Phorone was chosen as a non-hepatotoxic model compound to efficiently deplete GSH by enzymatic conjugation, because it is neither redox-active nor transformed in phase I to a reactive metabolite nor an inducer of lipid peroxidation *per se*[533]. Also, the lack of GSSG accumulation after phorone treatment confirms that this compound depletes GSH without inducing any signs of oxidative stress (figure 5 C). Since the block of apoptotic signaling by GSH depletion was only reversible with a cell-permeable GSH precursor and none of the other antioxidants used had this quality, we conclude that free intracellular GSH is the pivotal factor that enables DR signaling in murine liver. Therefore, an inhibition by superoxide or hydrogen peroxide of CD95-mediated apoptosis as observed in various cell lines[235,581,651] seems unlikely for the *in vivo* situation investigated here. The findings of this thesis might have important physiological and pathophysiological bearings (6.5), because liver GSH levels are not only subject to exogenous variables such as nutritional supply of amino acids, exposure to toxicants, inhalation of NO_x or O_3, radiation or UV light, but also to conditions such as ischemia, inflammation, the hormonal status and others[498].

6.2 Mechanistic interactions of cell death and glutathione depletion

6.2.1 Direct inhibition of death receptor-dependent apoptosis

Examining various murine models of cytokine-mediated liver injury, this study demonstrates that the destruction of liver tissue does not take place when glutathione is not available in sufficient amounts. This holds true for models involving both apoptosis or necrosis as the primary hepatocyte insult (table 4). In each of these models, the activation of DRs, i.e. TNF-R1 or CD95, is necessary for the induction of liver damage (1.3.1). In order to allow a concise discussion of the mechanistic implications of the presented data, a summary of possible interactions is presented in figure 18.

Within the signaling cascade of DRs, the most important interaction of GSH with apoptotic signaling is suggested to occur at the level of caspase-8 activation and before downstream signals including impairment of mitochondrial function. The following arguments support this hypothesis: (i) GSH depletion completely prevented caspase-3 processing, hepatocyte death, and allowed long-term survival in three models of lethal, apoptotic liver injury (figures 5, 7 and 17); (ii) GSH depletion or treatment of mice with the caspase-inhibitor z-VAD-fmk interrupted apoptosis signaling even after CD95 engagement, but only before caspase-3-like proteases have become active (figure 8); (iii) for CD95, two molecular experimental approaches have revealed that the activation of caspase-8 is indispensable for induction of cell death[104,187]; (iv) in this line, investigations on type I CD95 apoptosis in SKW6.4 cells demonstrated that the DISC formation still takes place

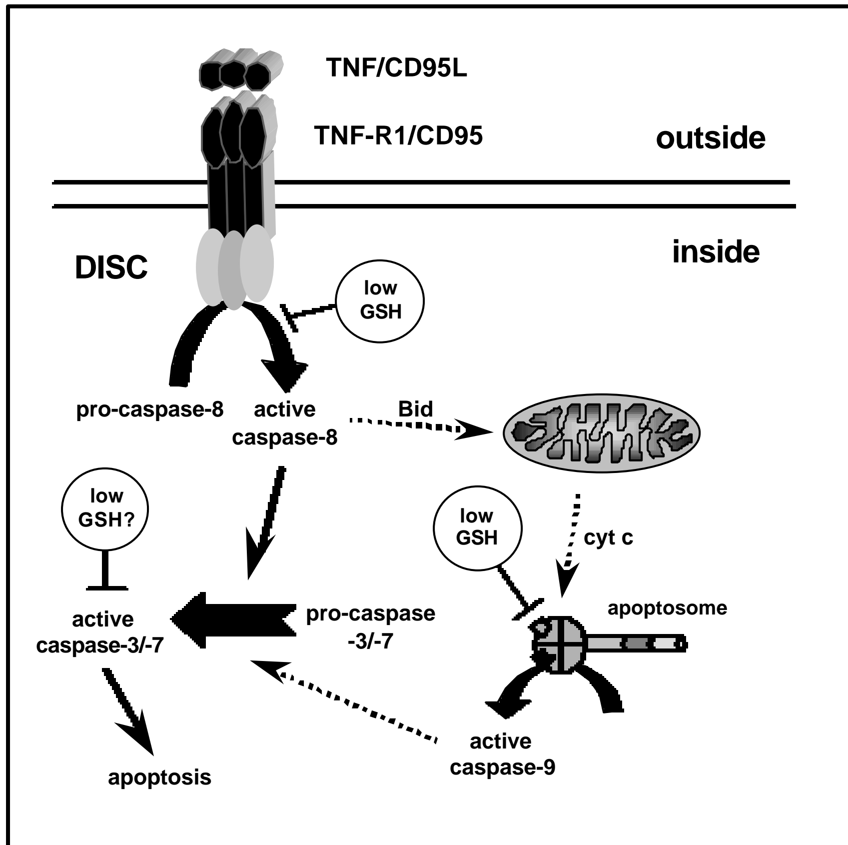


Figure 18: Proposed interactions between DR signaling and GSH deficiency.

under conditions of low GSH (figure 12), while caspase-8 processing, caspase-3-like activity and apoptosis were inhibited (figures 11 B/C and 12); (v) mitochondrial dysfunction serves as an amplifier and thus marks the point of no return in several cell death paradigms, including TNF-mediated apoptosis[312,470], and consistently, TNF-mediated hepatocyte demise was aggravated by lowering mitochondrial GSH *in vitro*, i.e. by downstream potentiation of the death signal[565]. Thus, the main mechanistic interference of low GSH with signaling of caspase-dependent models appears to be the inhibition of caspase-8 processing in caspase-dependent models (figure 18).

Furthermore, it was shown under cell-free conditions *in vitro* that the presence of a reducing agent such as GSH is required for the activity of recombinant caspase-3, and that GSSG inhibits its activity (figure 8 A). In line with these data, it was published recently that GSSG directly inhibits caspase-3-like and -6-like activities with an IC_{50} of 2.8 and 0.8 mM, respectively[236]. However, it is not possible to determine whether this effect contributes to apoptosis inhibition *in vivo*, since e.g. a possible glutathionylation of caspase-3 was not determined in the course of the present study. Apoptosome-mediated activation of caspases in liver cytosols was also investigated, and it was seen that the cyt c/dATP-initiated activation reaction was suppressed, but not blocked, when cytosols derived from phorone-treated mice were used (figure 9). Again, the actual contribution to the hepatoprotective effect of phorone cannot be estimated. However, it seems unlikely that this modulation represents a major effect, since (i) impairment of caspase-8 activation is upstream of the apoptosome pathway and suggested to mediate hepatoprotection (see above), (ii) the

suppression of cyt c/dATP-triggered caspase activation by about 50% (figure 9 F) is not sufficient to explain apoptosis complete prevention (figure 6); (iii) CD95-mediated apoptosis in the murine liver appears to represent an example for type I paradigm (figure 1/1.2.4) and is thus not dependent on the mitochondrial branch; and (iv) when only the apoptosome pathway is inhibited in DR-mediated models such as CD95 type II (1.2.4) or TNF-R1-triggered apoptosis, the mode of cell death was reported to switch from apoptosis to necrosis[9,10,287,289,657], which did not occur in any of the models investigated in the present study (figure 11, table 4).

In contrast to SKW6.4 cells, CD95-triggered apoptosis of primary cultured murine hepatocytes *in vitro* was not completely prevented when the cells were pretreated with the GSH depletors phorone, DEM or CDNB. The maximal inhibitory effect achieved with DEM was 20%, but an enhancement of CD95- or TNF-R1-mediated apoptosis after GSH depletion with DEM or phorone was never observed (without figure). How can this discrepancy between the *in vivo* and the *in vitro* situation be explained? Firstly, it has to be considered that in 40% oxygen cultured hepatocytes are more exposed to redox stress compared to hepatocytes *in vivo*, and accordingly, e.g. a GSH depletion with the compound CDNB was in contrast to a previous study[658] cytotoxic in our cell culture. Also, the metabolic situation of cultured hepatocytes is different from the *in vivo* situation with respect to e.g. cell-cell interactions, microenviroment and bile production/excretion, which is important *in vivo* for the excretion of GSH conjugates (1.4). Two further aspects are to mention: (i) *in vivo*, a significant contribution of SEC damage to the overall liver injury observed is evident in the CD95 model[411] and in the Con A model (figure 14). The observation that in endothelial cells GSH is decreased by diethyl maleate[659] demonstrates that these cells contain active GSH S-transferases, i.e. it is very likely that phorone will also deplete endothelial GSH and thus may prevent SEC apoptosis or necrosis *in vivo*; (ii) hepatoprotection by GSH depletion was also observed in models that do not involve caspase activation (Act D, α-amanitin, Con A, LPS shock, 1.3.1/table 4), suggesting that in addition to the above-mentioned mechanisms (figure 18), alternative implementations are suggested. It can be hypothesized that GSH depletion interferes with the internalization of the TNF-R1, since this important event for apoptosis induction[97,101,102,660] is linked to an intact microtubule network, which in turn requires an intact glutathione status[661,662]. In line with this assumption, microtubule-disrupting agents such as colchicine previously prevented hepatotoxicity in both the GalN/TNF[483] and the CD95 model[484]. Thus, it would be worth to study the influence of GSH depletion on TNF-R1 and CD95 internalization comparatively in hepatocytes *in vitro* and *in vivo*.

6.2.2 Discussion regarding conflicting literature

Published in the course of this thesis, a study by Jones *et al.*[430] describes similar findings as shown table 4, i.e. a protection from GalN/LPS-induced liver damage by GSH depletion, but an intermediate LPS shock - GalN/LPS model with a higher LPS dose (200 µg/kg, see 1.3.1) was used in this study. It was proposed that hepatoprotection is mediated by preventing the upregulation of adhesion molecules (VCAM-1, ICAM-1) on hepatic endothelial cells, which in turn would suppress neutrophil invasion and organ damage. This is in contrast to the conclusions above (6.2.1), and the following facts argue against it: (i) it is evident that ICAM-1 knock-out mice are susceptible towards GalN/LPS-mediated liver injury[663]; (ii) neutrophils do not play a significant role in the GalN/LPS model, as demonstrated in a study of Tiegs *et al.*[432]; (iii) in line with these arguments, the authors show that ICAM-1 protein levels are upregulated only 6 hours after LPS treatment onwards, at a time when liver damage has already been occurred. This finding further rules out a cause-relationship between ICAM expression, neutrophil invasion and liver damage; (iv) DEM is mainly used in this study to deplete GSH. In contrast to phorone, DEM impairs protein synthesis (1.4.2), possibly explaining the finding that TNF production was markedly suppressed by DEM treatment.

A recent study of Xu *et al.* is in clear contrast to the present thesis, as it was concluded that an increase in TNF cytotoxicity and hepatotoxicity would result from hepatocyte GSH depletion[664]. However, the data and the methodical informations given are not sufficient to draw this conclusion: (i) for *in vitro* studies, mainly a T antigen-transformed rat hepatocyte cell line (RALA255-10G) was used, and these cells died upon Act D/TNF treatment. However, the mode of cell death, i.e. apoptosis or necrosis, has not been elaborated, and the contradictory data given show that only 13% of RALA cells underwent nuclear apoptotic changes 8 hours after Act D/TNF treatment, but 29% of the cells died within 24 h; (ii) the increase in cell death exerted by the GSH depletor DEM was 12% in this experimental setting. Besides the problematic tool DEM (1.4.2), this effect is not very convincing; (iii) 11% of primary rat hepatocytes died upon 24 h Act D treatment alone (control data are lacking), and 31% after Act D/TNF treatment; cell death was increased only to 43% by again DEM; (iv) the authors did not address pro-apoptotic TNF-R1-related signaling events such as caspase activation, but they did not find any effects regarding GSH or H_2O_2 levels, APO-1 or NF-κB activation in Act D/TNF-mediated hepatocyte demise; (v) eventually, *in vivo* data are presented in this study (lethality in the GalN/LPS model, see also figure 17). Given the lack of BSO controls, hepatic glutathione determinations, quantitation of liver injury or data regarding the time of animal death, the absolute 2-fold rise in mortality upon BSO treatment alone does not permit any conclusion. In conclusion, the study of Xu *et al.*[664] is in apparent conflict with other studies[412,430,541].

6.2.3 Multiple mechanistic interference sites in Con A-mediated liver injury.

The Con A model differs from the other experimental models used with regard to TNF receptor subtype redundancy[665], post-receptor triggering of cell death programs[95,379,646,666], the role of endothelial cells[420] and the involvement of further cytokines in addition to TNF[415,416], i.e. IL-4[419] and IFN-γ[417,418](1.3.1), which all amplify the overactivation of immune response and causally contribute to liver injury (figure 19).

To demonstrate a protection independent of the compound phorone, glutathione was additionally depleted by other means in this model previously[412,643]: (i) the aromatic compound 1-cis-chloro-2,4-dinitrobenzene (CDNB, 100 mg/kg, i.p.)[509] depleted hepatic glutathione to 20 ± 9% of control (n = 3/group) within 30 minutes and reduced Con A-mediated liver injury (25 mg/kg, i.v.) from 4,200 ± 490 U/l to 100 ± 20 U/l ALT 8 hours after challenge; (ii) the selective, irreversible inhibitor of GSH synthesis, BSO (890 mg/kg, i.p.)[496], reduced the glutathione content of the liver to 43 ± 10% 9 hours after BSO treatment compared to untreated controls, and also conferred protection in this experiment (100 ± 60 U/l ALT 8 hours after Con A) when given 9 hours before Con A. Thus, the observed protection is unlikely to be restricted to phorone, but is due to a decrease of hepatic glutathione levels as such.

As T cell function[497,644,645], cytokine release[667], and NF-κB activation[610] are modulated by GSH, it was probable that an insufficient availability of GSH would prevent liver damage following Con A injection. A diminished T cell responsiveness, i.e. partial suppression or delayed release of circulating TNF, IFN-γ or IL-4, and a delayed induction of NF-κB (figure 14 A-C), alone might already explain protection. In order to examine whether IFN-γ suppression could be the reason for prevention of Con A hepatotoxicity[417,418], phorone/Con A-treated mice were supplemented with rmu-IFN-γ (50 µg/kg, i.v., t = -15 min before Con A). Even though this treatment restored the level of systemic IFN-γ comparable to that in control Con A-treated mice (7,330 ± 900 pg/ml at 30 minutes after IFN-γ treatment), it failed to restore sensitivity to Con A (not shown). This implies that T cell suppression alone is unlikely to be a sufficient explanation for the protective action of GSH depletors against Con A hepatotoxicity (table 4). Although the observed suppression of early pro-inflammatory cytokine release after depletion of GSH (figure 14 A/C) is likely to contribute to desensitization towards Con A, it also seems feasible that post-TNF-R-signaling events leading to hepatocyte death are disrupted under this condition. In particular, two signal transduction events known to be crucial in the Con A model, i.e. transactivation of NF-κB and activation of caspases[95,646,668], are known to be controlled by thiols[610,651](1.5.3). Therefore, a further and independent effect of GSH depletion could be located in the insufficient activation of these two factors at the hepatocyte level as depicted in figure 19.

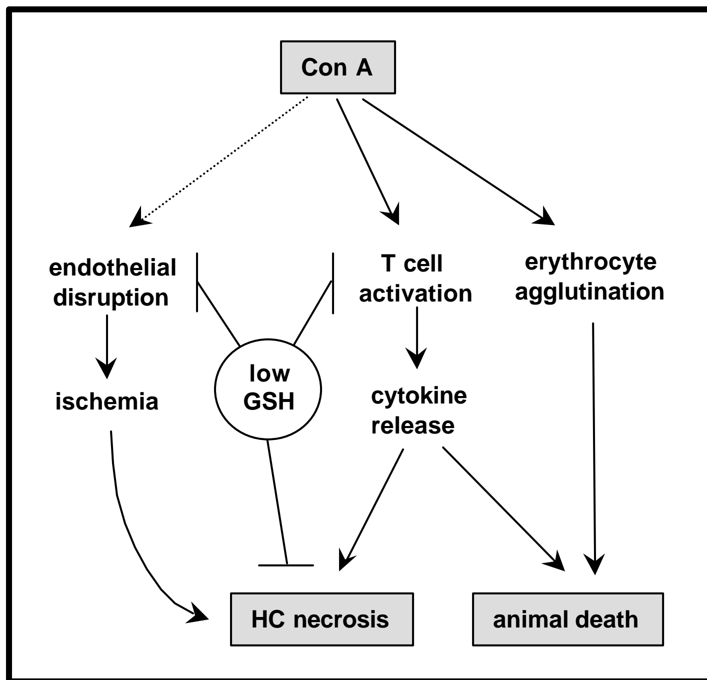


Figure 19: Hypothetical interaction sites of GSH with liver injury mechanisms initiated by Con A.

Endothelial damage has been poorly investigated in Con A hepatotoxicity. FITC-labeled Con A given *in vivo* was immediately located in hepatic sinusoids[416], and a selective damage of sinusoidal endothelial cells (SEC) was reported to be an early event in liver destruction[420]. In contrast, endothelial damage in other organs has not been reported. By intravital microscopy, it was demonstrated that after Con A injection, T cells primarily adhere to the endothelium in the periportal area[669], where liver damage was most pronounced after 8 hours (figure 13 C). In extension of these observations, electron microscopy provided evidence for a prominent SEC cell destruction after Con A injection, whereas no typical signs of hepatocyte apoptosis were visible (figure 15 B). Without prior knowledge of the experimental model, the lesions would have been characterized as being similar to those seen after an ischemic insult (figure 13 C).

Although phorone treatment prevented liver damage in the Con A model, it failed to rescue mice from a lethal Con A challenge (figure 17 B), indicating that Con A causes animal death independent of hepatic damage. This situation is interpretated as a generalized, fatal condition comparable to LPS- or superantigen-induced lethal shock[670]. The presented data allow to identify two different events as possible causal lethal conditions after Con A injection, which were both insensitive towards phorone treatment (figure 19): (i) severe hemorrhage, as evidenced by histology (figure 13 C/D; figure 15 B-D) and by a dramatic increase in spleen weight 8 hours after Con A treatment (untreated control: 90 ± 10 mg; Con A (25 mg/kg): 230 ± 20 mg; 250 mg/kg phorone + Con A (25 mg/kg): 210 ± 20 mg; n = 3/group), and (ii) exceedingly high levels of pro-inflammatory circulating cytokines from 3 hours onwards (figure 14 A/C).

6.2.4 The mode of hepatocyte demise in Con A-mediated liver injury

Initially, the actual mode of hepatocyte cell demise in the Con A model has been characterized as apoptotic[416], and this notion was repeatedly confirmed by the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) assay[668,671]. Predominantly necrotic hepatocyte death was also reported[672](this study). To appreciate the significance of these previous findings, three relevant methodological aspects must be discussed: (i) the detection of oligonucleosomal DNA fragmentation and DNA laddering in crude liver homogenate fails to discriminate between apoptosis of hepatocytes and other hepatic cell populations[399]; (ii) the TUNEL assay does not unequivocally discriminate between apoptotic and necrotic cells[673], and *in situ* monitoring of hepatic caspases have demonstrated that the TUNEL assay gives false positive results[674]; (iii) none of the previous studies investigated the morphology by electron microscopy.

Based on these three independent methodological reservations, it is concluded from the absence of hyperchromatic hepatocyte nuclei in many liver sections from Con A experiments that apoptotic *hepatocyte* death is of minor importance in this model. This conclusion is supported by the recent findings from our laboratory that Con A induced liver injury is neither associated with hepatic caspase activation, nor sensitive towards inhibition by the potent broad-spectrum caspase inhibitor z-VAD-fmk[95]. Considering the observation of early SEC damage, it is proposed that hepatocyte demise in the Con A model occurs in a necrotic way and as a result of endothelial disruption and ischemic conditions, as outlined in figure 19. Obviously, the mode of SEC death upon Con A injection and its modulation by GSH requires detailed further experiments.

6.3 GSH and NO interactions: relevance for hepatocyte apoptosis?

Besides GSH, two further endogenous mediators have been shown to control hepatocyte apoptosis: (i) the progression of apoptosis requires energy, and the energy charge of the ATP system determines whether or not the hepatocyte will undergo TNF-R1-triggered apoptosis (1.3.2 C); (ii) nitric oxide (NO) is a hepatoprotective mediator in TNF-mediated hepatocyte apoptosis and organ damage (1.3.2 D). Furthermore, S-nitrosation of caspases by NO has been shown to occur *in vitro* and was considered responsible for apoptosis inhibition in various models (1.3.2 D, 1.5.3 A). Physiological determinants of GSH/NO regulation may be additionally complicated by interactions between NO and glutathione: NO reacts with extracellular[675] and intracellular GSH[676] giving rise to S-nitroso glutathione (GSNO), which in turn can be cleaved within the cell by the antioxidant thioredoxin[677]; correspondingly, it was reported that NO can be either cytoprotective or cytotoxic for a given cell type depending on the intracellular thiol level[678-680].

Due to the above-mentioned interactions between NO and GSH, it was examined whether the observed hepatoprotective effect of phorone could be explained by an increased impact of endogenously produced NO due to decreased intracellular GSH levels: (i) the unspecific NO synthase inhibitor aminoguanidine[681] was used (3 x 15 mg/kg in saline i.p., t = -30 min, +3 h, +6 h) in order to block endogenous NO production. However, treatment of mice with aminoguanidine did not reverse the hepatoprotective effect of phorone (without figure); (ii) it was investigated whether the NO donor sodium nitropusside (SNP) would prevent liver damage in the GalN/TNF model in a synergistic manner with the GSH depletor phorone, but only an additive protective effect and no synergism was found; (iii) SNP is not hepatoprotective in the CD95 model, where GSH depletion by phorone prevented hepatic damage (see next paragraph). Thus, it can be ruled out that NO mediates hepatoprotection as a secondary effect of GSH depletion.

In the course of this thesis, the effect of NO donors on caspases in various systems was further elaborated: (i) SNP (2 mg/kg) inhibited TNF-induced liver injury and group-II caspase activation by >90%. However, liver damage and caspase activity elicited by αCD95 was enhanced by SNP by about 50% (without figure); (ii) in cultured murine hepatocytes, the NO donors S-nitroso-penicillamine and S-nitroso-glutathione did not prevent cytotoxicity, but decreased TNF- and αCD95-induced activation of group-II caspases (without figure). In respective samples, reactivation of caspases with the reductant dithiotreitol (DTT) failed, whereas DTT removed NO from *in vitro*-nitrosated hepatic caspases under cell-free conditions (EC_{50}: 59 µM); (iii) to characterize intracellular redox conditions, the denitrosating capability of the physiologic reductants glutathione (EC_{50}: 151 µM) and thioredoxin (EC_{50}: 0,12 µM) was estimated; (iv) using cytochrome c/dATP, hepatic pro-caspases were activated *ex vivo* in liver cytosols from control and also from SNP-treated mice (figure 9 F), arguing against an inhibition of pro-caspases by NO as proposed by Mannick *et al.*[247]. These data indicate that pro-caspase and caspase S-nitrosation is neither likely to occur in hepatocytes nor to contribute to NO-mediated inhibition of hepatocyte apoptosis.

6.4 Modulation of liver injury by caspase inhibition, GSH, ATP and NO

In this section, results of the present study, unpublished data of our group and data derived from the present literature are compiled. Thereby, an overview about the sensitivity of the seven liver injury models presented in table 3 (1.3.1) towards pharmacological inhibition of caspases, and the influence of hepatic GSH or ATP depletion and the impact of NO donors is given.

It is apparent that pharmacological **inhibition of caspases** (1.2.2 F) has a hepatoprotective potential only in models where caspase activity could be detected (table 3, 1.3.1), i.e. in CD95-, GalN/TNF- and GalN/LPS-mediated liver injury, but not in the Act D and α-amanitin models. In T cell-dependent Con A hepatotoxicity, it was shown by Künstle *et al.* that the caspase inhibitor z-VAD-fmk even when given in doses as high as 50 mg/kg has no hepatoprotective effect and does not block cytokine

model	caspase inh.	GSH ¯	ATP ¯	NO donors	liver damage - lethality
αCD95	- 479-481	- 412,589	- 453	- u.d.	+ 405,589
GalN/TNF	- 480, 481	- 412,430,561	- 453	- 241, 244	+ 412
GalN/LPS	- 401, 684	- 412,430,561	- 453	- 685	+ 412
Act D, α-amanitin	- 412	- 412	n.d.	¯ u.d.	n.d.
Con A	- 95 - 422,423	- 412	¯ u.d.	- u.d. ¯ 423	- 412
LPS shock	- 401	- 412	¯ u.d.	- 462	- 412

Table 5: Overview of pharmacological and metabolic modulation in murine cytokine-mediated liver injury models. *It is indicated whether caspase inhibitors, depletion of GSH/ATP or NO donors inhibit (¯) or potentiate (-) liver damage in the liver injury models, or whether these variables do not influence liver damage in the respective model (-). Additionally, it is shown whether liver damage is directly linked to animal lethality in these models (+) or not (-). References are given for each property of the respective model; "u.d.", unpublished data from our laboratory, "n.d.", not determined.*

production[95]. In contrast, the group of Fiorucci reported that the same inhibitor would block hepatotoxicity in this model due to an inhibition of IFN-γ release[422,423]. In the endotoxic shock model, caspase inhibitors did not reduce the extent of liver injury[401], but improved the animal survival, which may be attributed to cytokine release alterations[682,683].

Whereas **GSH depletion** exerted hepatoprotection in all animal models used in the study to hand [412,589], depletion of hepatic **ATP** by the carbohydrate fructose inversely regulated hepatic apoptosis upon CD95 and TNF-R1 triggering[453]. The mechanisms behind this phenomenon remain to be elucidated (1.3.2 C), and hepatoprotection after treatment of mice with fructose was also observed in the Con A and LPS shock models (M. Latta, personal communication).

The hepatoprotective potential of **NO donors** on DR-triggered liver injury remain controversial with respect to the underlying mechanisms, since liver damage in caspase-dependent models was both inhibited (GalN/TNF, GalN/LPS) and enhanced (CD95) by SNP, arguing against a direct effect of NO on caspases (6.3). Because ATP and NO donors have similar effects on these three models (table 5) and since it is known that NO donors may also deplete ATP by blocking mitochondrial ATP generation[9,657], it was checked whether SNP would deplete hepatic ATP; however, this was not the case (without figure). Furthermore, hepatoprotection was observed in the caspase-independent Act D hepatotoxicity model (Act D: 16940 ± 4710 U/L ALT activity; Act D + 4 mg/kg SNP: 3890

± 4860 U/L ALT activity; 20 h), whereas SNP (4 mg/kg) greatly enhanced cytokine release and was lethal in the Con A model (without figure). Again, different results have been published by Fiorucci *et al.*[423], since the authors clearly demonstrated that the cytokine response induced by Con A is mediated by IL-18, requires caspase activation and is inhibited by NCX-4016, a NO-releasing Aspirin derivate; it was proposed that this effect is attributed to the S-nitrosation and inhibition of caspases involved in cytokine production[423].

At last, it appears that a fulminant and selective liver destruction is directly linked to **animal lethality** in the three caspase-dependent models, because (i) the Jo-2 model is regarded as liver-specific[405], (ii) GalN inhibits transcription only in hepatocytes[433], and (iii) phorone furthermore preferentially depletes hepatic glutathione (figure 5 A, 1.4.2). In contrast, mice died in the complete absence of hepatic damage when treated with phorone in the Con A and LPS shock models (table 4, figure 17), a phenomenon that has to be considered in the interpretation of liver injury when compared with survival data in these models.

6.5 Impaired apoptosis under low GSH: implications for human liver disease

Receptor-mediated hepatocyte death in an autocrine or paracrine fashion has been recognized as an important event in toxic liver injury, and damaged or toxin-stressed hepatocytes rapidly up-regulate their death receptors TNF-R1 or CD95[374,386,387,686](1.3). When the total initial tissue lesion is below the threshold that causes organ failure, apoptotic hepatocytes are usually removed by professional phagocytes or by neighboring parenchymal cells[5,377,427]. Hence, any dysregulation of this active cell death program may have negative consequences for the organ. In the context with low hepatic GSH, three examples for this situation are discussed:

(i) The hepatic metabolism of the analgesic drug **acetaminophen** consumes and depletes liver GSH *via* phase II conjugation[527]. When overdosed, the hepatocyte is damaged by formation of reactive toxic metabolites. These include the organic iminoquinone radical NAPQI[648] and reactive oxygen species, that in turn peroxidize proteins and membrane lipids[687]. These events and the concomitant drastic GSH depletion, that may blocks DR-triggered hepatocyte apoptosis *in vivo* according to the findings presented in this study, eventually cause necrotic hepatocyte death (see below).

(ii) Secondly, **Wilson´s disease** (WD) is known as a necrotic liver destruction process due to genetically determined copper overload of the organ[688]. Recently, the CD95 system was proposed to contribute to the etiology of this disease, since an upregulation of CD95 in the liver of Wilson´s disease patients and in hepatocytes *in vitro* after copper treatment was found[387]. Also, hepatic GSH levels in affected patients were reported to be dramatically decreased at <15% of healthy controls[530]. Combining both findings, it is proposed that paracrine receptor-mediated suicide of copper-damaged hepatocytes is blocked due to low

GSH levels under this condition, thus resulting in hepatocellular necrosis as a result of copper overload and oxidative stress[688,689]. Notably, Barrow *et al.* reported in 1987 that acute galactosamine-induced liver injury, which is mediated *via* apoptosis as to our current knowledge[374,413], was potently inhibited by copper treatment of rats[690]. *Vice versa*, copper administration to rats induced a pathophysiological condition which roughly resembled acute WD in man[537], possibly explaining the inhibition of apoptotic liver damage under this condition.

(iii) Finally, **chronic alcohol consumption** eventually can lead to fibrosis due to replacement of dead hepatocytes by increased proliferation of connective tissue, and furthermore promotes hepatic GSH depletion[531,532]. Besides circumstantial evidence for a role of the CD95 system[691], a recent study provides a link to receptor-mediated apoptosis, since it was shown that transgenic mice lacking TNF-R1 are resistant to liver damage elicited by acute ethanol treatment[397].

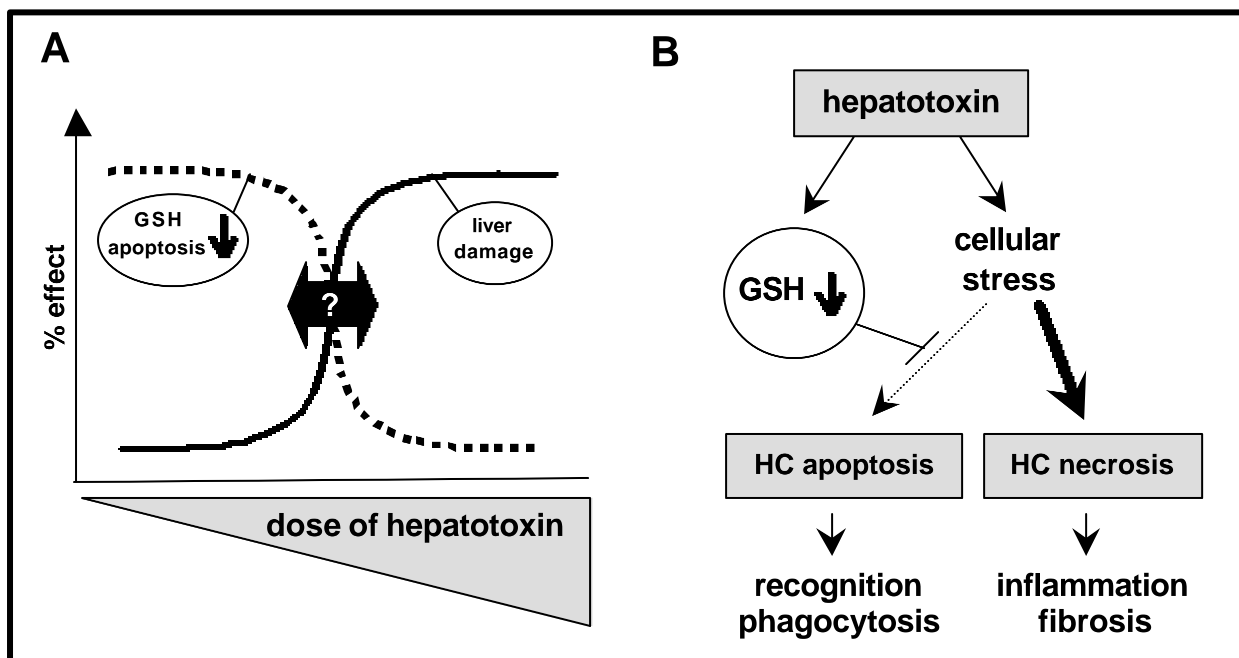


Figure 20: Differential effects of hepatotoxins on the initiation of hepatocyte apoptosis or necrosis with respect to their GSH-depleting capacity. ***(A)*** *Dose dependency of GSH depletion/anti-apoptotic action and toxicity, and* ***(B)*** *hypothetical scheme illustrating the role of GSH depletion in the decision whether HC apoptosis or necrosis occurs as a result of toxic liver injury. For details, see text.*

Considering these examples and the data provided by the study to hand, a concept emerges as to the role GSH might have in toxic liver injury (summarized in figure 20). A number of hepatotoxins consumes GSH and *at the same time* damages cellular structures. As to our current knowledge, hepatocyte undergo auto- or paracrine DR-dependent apoptotic cell death when stressed (1.3); depending on the dose of the actual hepatotoxin and thus on the relative impact of the two mechanisms *anti-apoptosis* and *hepatotoxicity*

(figure 20 A), a given hepatocyte might still be able to die by DR-induced apoptosis; alternatively, this death program may be blocked due to low GSH resulting in hepatocyte necrosis and inflammation (figure 20 B). In a certain dose window, also an intermediary appearance of cell death may occur (indicated by "?" in figure 20 A). In line with this concept, a number of studies identified apoptotic processes in the liver upon e.g. acetaminophen treatment in addition to necrosis in certain time frames and dosage regimens, as assessed by DNA fragmentation[692] and apoptotic morphology of hepatocytes[693]. Under these experimental conditions, caspase inhibitors failed to reduce hepatic damage[692], which might be explained now in the light of this study by the insufficient availability of GSH after acetaminophen metabolism. Also supporting the above-mentioned hypothesis, CD95-triggered apoptotic events such as caspase-3-like activity were directly prevented by acetaminophen[692], but liver damage still took place. In contrast, an *experimentally* induced GSH deficiency by a non-toxic tool such as phorone entirely prevented liver damage and thus was *hepatoprotective*, as described in the study to hand.

It is well established that when the ability to activate intracellular death pathways is severely confined an insult or other metabolic circumstances, the modus of cell death may switch from apoptosis to necrosis[7,377,591]. A sustained GSH deficiency caused by a hepatotoxin may impair the physiological balance between apoptosis and necrosis by blocking DR-dependent cell death programs (figure 20 B). Collectively, these considerations implicate that when the capacity of hepatocyte apoptosis is pathologically downregulated due to insufficient intracellular GSH levels, the dysregulation of receptor-mediated cell death may be a crucial factor for the clinical progression of liver disease, e.g. for the development of inflammation and fibrosis.

7 Summary

The activation of the death receptors TNF-receptor-1 (TNF-R1) or CD95, respectively, is a hallmark of inflammatory or viral liver disease. The present thesis investigated the regulation of death receptor-triggered apoptosis by glutathione alterations using different murine *in vivo* models and an *in vitro* model of CD95-mediated apoptosis. The findings provide evidence that (i) a depletion of hepatic GSH levels precludes the execution of death receptor-mediated cell death; and that (ii) the molecular target affected by GSH depletion is the activation of caspase-8 at the CD95 DISC (death-inducing signaling complex) and possibly the activation of caspases *via* the apoptosome. The data suggest that a dysregulation of cell demise at chronically decreased hepatic GSH affects the pathological processes and thus possibly the outcome of liver disease.

1. Intracellular glutathione levels were not affected in the course of CD95-triggered hepatic apoptosis. Glutathione levels were experimentally decreased in a controlled manner without inducing redox stress by the GSH-S transferase substrate phorone.

2. When GSH was depleted, CD95-initiated hepatic caspase-3-like activity and DNA-fragmentation were completely blocked, and animals were protected from liver injury as assessed by histological examination and determination of liver enzymes in plasma. Conversely, repletion of hepatic glutathione by treatment with a cell-permeable glutathione ester restored susceptibility of GSH-depleted mice towards CD95-mediated liver injury. In contrast, a number of antioxidants failed to do so.

3. The thiol sensitivity of recombinant caspase-3 was investigated *in vitro*, and it was found that enzymatic activity was dependent on the presence of a reducing agent such as GSH, while GSSG attenuated activity.

4. Using cytochrome c/dATP, hepatic pro-caspases were activated *ex vivo* in liver cytosols from control and also from phorone-treated mice. Glutathione-depleted cytosols displayed a significantly suppressed activation rate, suggesting a prerequisite of an intact intracellular glutathione status for activation of caspases *via* the apoptosome pathway.

5. In CD95 type I apoptosis in a B lymphoma cell line (SKW6.4), a depletion of intracellular glutathione by different GSH S-transferase substrates prevented apoptosis, but did not interfere with the formation of the CD95 DISC. It was further elaborated that apoptosis inhibition occured at the level of caspase-8 proenzyme processing.

6. Under GSH depletion, also caspase-independent, but TNF-R1-mediated injury (high-dose actinomycin D or α-amanitin), as well as necrotic hepatotoxicity (high-dose LPS) were entirely blocked.

7. In the T cell-dependent Concanavalin A (Con A) hepatotoxicity model, GSH depletion resulted in a suppression of IFN-γ release, delay of systemic TNF-release and hepatic NF-κB activation, an abrogation of sinusoidal endothelial cell detachment, and a prevention of hepatocyte necrosis as assessed by electron microscopy. Animals treated once with phorone survived for more than three months after an otherwise lethal challenge in selective liver injury models, i.e. liver damage elicited by CD95 activation, and by endotoxin (LPS) or TNF treatment of galactosamine- (GalN)-sensitized mice. In contrast, liver injury was not directly linked to animal lethality in the Con A model and in an endotoxin (LPS) shock model.

Zusammenfassung

Die Stimulierung der Todesrezeptoren CD95 und TNF-R1 (Tumor-Nekrose Faktor Rezeptor-1) in der murinen Leber initiiert die Aktivierung apoptosevermittelnder, zytosplasmatischer Cysteinproteasen (Caspasen) und führt letztlich zur akuten Leberschädigung („sekundäre Nekrose“). Dieser Mechanismus des Zelltodes spielt vermutlich eine wichtige Rolle in der Pathogenese verschiedener humaner Lebererkrankungen. Glutathion (GSH) gilt als primäres, intrazelluläres Antioxidans und hat in zahlreichen toxischen Leberschädigungsmodellen eine potente hepatoprotektive Wirkung. In dieser Arbeit wurde die GSH-Abhängigkeit der hepatischen rezeptor-vermittelten Apoptose in verschiedenen Mausmodellen sowie in einem *in vitro* Modell für CD95-vermittelter Apoptose in der Zell-Line SKW6.4 untersucht. Zusammenfassend konnte gezeigt werden, dass in Todesrezeptor-abhängigen apoptotischen und nekrotischen Leberschädigungsmodellen eine Verminderung des hepatischen GSH-Spiegels generell eine hepatoprotektive Wirkung hat. Vor allem einer Verhinderung der Apoptose unter chronischem GSH-Mangel könnte große Bedeutung für die Progression verschiedener Lebererkrankungen zukommen.

1. Die intrazelluläre Konzentration von Glutathion veränderte sich nicht während der CD95-vermittelten Leberschädigung. Es wurde das Glutathion-S-Transferasesubstrat Phoron verwendet, um den Einfluss einer Glutathiondepletion auf hepatische Apoptosemodelle untersuchen zu können. Phoron depletierte das Gesamtglutathion in der Leber zeit- und dosisabhängig, ohne Toxizität oder Redoxstress zu induzieren.

2. Eine Phoronvorbehandlung der Mäuse verhinderte die CD95- beziehungsweise die TNF-R1-vermittelte Aktivierung hepatischer Caspasen und DNA-Fragmentierung. Weiterhin kam es nach GSH-Depletion nicht mehr zur Leberschädigung, bestimmt anhand der Freisetzung leberspezifischer Transaminasen sowie durch histologische Untersuchungen. Andererseits führte eine Repletion von GSH *in vivo* durch Behandlung der Tiere mit einem zellgängigen GSH-Ester zur Aufhebung der Protektion. Verschiedene Antioxidantien zeigten diesen Effekt nicht.

3. Die Aktivität von rekombinanter humaner Caspase-3 *in vitro* war sowohl von der Anwesenheit von GSH als auch vom relativen GSH/GSSG abhängig.

4. Es wurde weiterhin die Cytochrom c/dATP-vermittelte Aktivierung hepatischer Pro-Caspasen *ex vivo* untersucht. Diese Aktivierungsreaktion war signifikant reduziert, wenn Leberzytosol phoronbehandelter im Vergleich zu unbehandelten Mäusen verwendet wurde. Dies weist auf die Notwendigkeit eines intakten Glutathionstatus für die Caspasenaktivierung über den Apoptosomweg hin.
5. Für die CD95-vermittelte Typ I Apoptose in der B-Zell-Lymphoma Linie SKW6.4 konnte ebenfalls eine Inhibition des Zelltodes nach GSH-Depletion festgestellt werden. Die GSH-Depletion beeinflusste nicht die Bildung des intrazellulären CD95-Signalkomplexes DISC (death-inducing signaling complex), verhinderte jedoch den ersten Aktivierungsschritt der Caspase-8 am DISC.
6. In sämtlichen untersuchten TNF-R1-abhängigen Modellen wurde durch die Depletion von hepatischem GSH eine signifikante Reduktion der Leberschädigung um >90 % auf der Basis der Plasmaspiegel von Transaminasen festgestellt. In Caspase-abhängigen, apoptotisch verlaufenden Modellen (GalN [Galaktosamin]/LPS [Endotoxin, Lipopolysaccharide], GalN/TNF) wurde zudem eine Inhibition von Caspase-3-ähnlichen Proteasen beobachtet. Unter GSH-Depletion war auch die Leberschädigung Caspase-unabhängig verlaufender Modelle (Actinomycin D und α-Amanitin) als auch die im LPS-Schock auftretende nekrotische Leberschädigung vollständig inhibiert.
7. Im Con A-Modell konnte festgestellt werden dass nach GSH-Depletion die systemische Freisetzung von IFN-γ unterdrückt wurde, die TNF-Freisetzung und die hepatische NF-κB Aktivierung verzögert waren. Durch Kurativschutz-Versuche wurde allerdings ausgeschlossen dass die Protektion auf einer Veränderung der Immunantwort beruht. Durch elektronenmikroskopische Studien wurde ausserdem gezeigt dass hepatische Endothelzellen nach GSH-Depletion im Con A Modell nicht mehr geschädigt werden, sowie dass Hepatozyten nicht mehr nekrotisch zugrunde gehen. Phoronvorbehandelte Mäuse überlebten einen Versuchszeitraum von 3 Monaten nach einer sonst letalen Behandlung mit αCD95 (aktivierender anti-CD95 Antikörper), GalN/TNF oder GalN/LPS. Demgegenüber verstarben Tiere nach GSH-Depletion im Con A- und LPS-Schock-Modell in Abwesenheit einer Leberschädigung, was darauf hinweist dass in den beiden letztgenannten Modellen die auftretende Leberschädigung nicht alleine zur Letalität führt.

8 References

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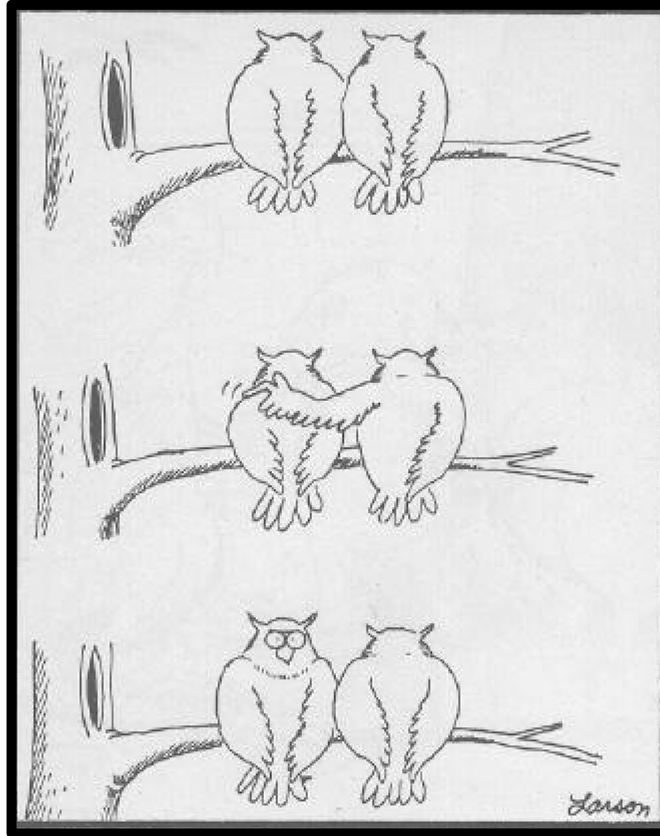

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