Hydrophobic Bile Salts Induce Hepatocyte Shrinkage via NADPH Oxidase Activation

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Key Words

Oxidative stress • Bile acids • CD95 • Fas • CD95
Ligand • p47^{phox}

Abstract

Hydrophobic bile salts activate NADPH oxidase through a ceramide and protein kinase C ζ -dependent pathway as an important upstream event of bile salt-induced hepatocyte apoptosis. As shown in the present study, hydrophobic bile salts such as glycochenodeoxycholate, taurochenodeoxycholate or tauroolithocholylsulfate (TLCS) also induce within 30min hepatocyte shrinkage in perfused rat liver. TLCS-induced hepatocyte shrinkage was strongly blunted in presence of desipramine, apocynin, bafilomycin and DIDS, *i.e.* manoeuvres previously shown to inhibit TLCS-induced NADPH oxidase activation and the subsequent oxidative stress response. The antioxidant N-acetylcysteine inhibited TLCS-induced hepatocyte shrinkage. N-acetylcysteine by itself increased hepatocyte hydration, suggesting that a basal production of reactive oxygen intermediates

is involved in the regulation of liver cell hydration. TLCS failed to induce shrinkage of hepatocytes from p47^{phox} knock-out, but not control mice. Likewise, hepatocytes from p47^{phox} knock-out mice were resistant towards TLCS-induced apoptosis and failed to activate the CD95 system. No cell shrinkage was observed in response to taurocholate and tauroursodesoxycholate, *i.e.* bile salts which do not induce an oxidative stress signal and apoptosis. NADPH oxidase activation also counteracts volume recovery in response to hyperosmotic hepatocyte shrinkage. The findings indicate that hydrophobic, proapoptotic bile salts induce hepatocyte shrinkage largely through NADPH oxidase-derived oxidative stress. Because cell shrinkage in turn activates NADPH oxidase, which blunts cell volume recovery, a vicious cycle ensues between oxidative stress and cell shrinkage, which propagates CD95 activation and may finally lead to apoptosis. In addition, cell shrinkage induced by proapoptotic bile salts may augment apoptosis by increasing protein breakdown and induction of cholestasis.

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Introduction

Cell shrinkage is one hallmark of apoptotic cell death [1-3]. In Jurkat cells CD95 (CD95 receptor, Apo-1/Fas) activation inhibits Na^+/H^+ antiport [4], the Na^+/K^+ -ATPase [5] and stimulates efflux of taurine [6], which results in an "apoptotic cell volume decrease" (AVD). Blockade of AVD by inhibition of volume-sensitive Cl^- or K^+ channels [7] or by elevation of extracellular K^+ [8-10] counteract apoptosis, whereas inhibition of the Na^+/H^+ exchange accelerates apoptosis [4]. On the other hand, hyperosmotic hepatocyte shrinkage can induce a ligand-independent activation of CD95, which may result in apoptosis induction [11-13]. Thus, a mutual interaction between cell shrinkage and apoptosis induction can be anticipated, however, the underlying mechanisms are unclear.

Also hydrophobic bile salts induce hepatocyte apoptosis *in vivo* and *in vitro* [14-17] through a ligand-independent activation of the CD95 (CD95 receptor, Apo-1/Fas) death receptor [15, 18]. CD95 activation by hydrophobic bile salts is initiated by a NADPH oxidase-derived oxidative stress signal [19], which triggers activation of c-Jun-N-terminal kinases (JNK) [20, 21] and of the Src kinase family member Yes [22]. Activated Yes rapidly activates the epidermal growth factor receptor (EGFR) [22], which then associates in a JNK-dependent way with CD95 and catalyzes CD95 tyrosine phosphorylation. The latter is required for CD95 membrane trafficking, formation of the death-inducing signalling complex (DISC) and induction of apoptosis [21]. These complex signal transduction events towards CD95 activation are not only activated by hydrophobic bile salts, but also by hyperosmotic hepatocyte shrinkage [12]. Bile salt- and hyperosmolarity-induced activation of NADPH oxidases involves a rapid acidification of endosomes with subsequent activation of acidic sphingomyelinase (ASM) and a ceramide-dependent activation of protein kinase C (PKC) ζ , which triggers an activating serine-phosphorylation of p47^{phox} [19], a regulatory subunit of the NADPH oxidase complex [23]. Here we report that bile salt-induced NADPH oxidase activation not only triggers CD95 activation, but also hepatocyte shrinkage. Because hepatocyte shrinkage in turn activates NADPH oxidase, a self-amplifying cycle ensues between cell shrinkage and NADPH oxidase-derived oxidative stress, which perpetuates CD95 activation and finally will lead to apoptosis.

Materials and Methods

Materials

The materials used were purchased as follows: collagenases from Boehringer (Mannheim, Germany); William's E medium, tauroolithocholate-3-sulfate (TLCS) taurochenodeoxycholate (TCDC), taurocholate (TC), glycochenodeoxycholate (GCDC) and desipramine from Sigma-Aldrich (Deisenhofen, Germany); penicillin/streptomycin from Biochrom (Berlin, Germany); fetal calf serum from Gibco Life Technologies (Gaithersburg, MD, USA); apocynin, bafilomycin A1 and DIDS from Calbiochem (Darmstadt, Germany); diphenyleneiodonium chloride (DPI) from Biomol (Hamburg, Germany); TUNEL assay from Roche Diagnostics (Mannheim, Germany); CM-H₂DCFDA from Molecular Probes (Eugene, Oregon, USA). The antibodies used were purchased as follows: rabbit anti-CD95, rabbit anti-EGFR, rabbit anti-FADD and mouse anti-caspase 8 antibodies from Santa Cruz Biotechnology (CA, USA); rabbit anti-Yes, sheep anti-EGFR and mouse anti-phosphotyrosine antibodies from Upstate Biotechnology (Lake Placid, NY); rabbit anti-phospho-Src family-Y⁴¹⁸ antibody from Cell Signalling (Beverly, MA, USA); horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Bio-Rad (Hercules, CA, USA). All other chemicals were from Merck (Darmstadt, Germany) at the highest quality available.

Cell preparation and culture

As described previously [13], hepatocytes were isolated by a collagenase perfusion technique from livers of 5-6 weeks old male wild type or p47^{phox} knock-out mice, both with the C57BL/6 background and fed *ad libitum* with a standard diet. p47^{phox} knock-out mice [24] were obtained from Taconic, Ry, Denmark. Aliquots of 1.5×10^6 hepatocytes were plated on collagen-coated 6-well culture plates (Falcon, Heidelberg, Germany) and cultured as published recently [13] for 24h, unless indicated otherwise, before the experiments were started. The viability of the hepatocytes was more than 95% as assessed by trypan blue exclusion.

Western blot analysis

At the end of the incubations, medium was removed, and cells were washed briefly with phosphate-buffered saline (PBS) and immediately lysed. Samples were transferred to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and proteins were then blotted to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia Biotech, Freiburg, Germany) as recently described [21]. Blots were blocked for 2 hours in 5% (w/v) BSA-containing 20mmol/L Tris, pH 7.5, 150mmol/L NaCl and 0.1% Tween 20 (TBS-T), and then incubated at 4°C overnight with the first antibody [antibodies used: anti-phospho-Src family-Y⁴¹⁸ and anti-Yes (1:2500), anti-EGFR, anti-CD95, anti-FADD, anti-caspase 8 and anti-phospho-tyrosine (1:10000)]. Following washing with TBS-T and incubation with horseradish peroxidase-coupled anti-

mouse, anti-sheep or anti-rabbit IgG antibody (all diluted 1:10000) at room temperature for 2h respectively, the blots were washed extensively and developed using enhanced chemiluminescent detection (Amersham Pharmacia Biotech, Upsala, Sweden). Blots were exposed to Kodak X-OMAT AR-5 film (Eastman Kodak Co., Rochester, NY, USA).

Immunoprecipitation

Hepatocytes were harvested in lysis buffer as recently published [21]. Equal protein amounts (200µg) of each sample were incubated for 2h at 4°C with a polyclonal rabbit anti-CD95, rabbit anti-EGFR or rabbit anti-Yes antibody (dilution 1:100; Santa Cruz, CA, USA) in order to immunoprecipitate CD95, EGFR or Yes. Then 10µl of protein A- and 10µl of protein-G-agarose (Santa Cruz, CA, USA) were added and incubated at 4°C overnight. Immunoprecipitates were washed 3 times as published recently [21] and then transferred to Western blot analysis as described above. The anti-phospho-Src family-Y⁴¹⁸ antibody was used in order to detect activating phosphorylation of Yes [25] and the anti-phospho-tyrosine antibody in order to detect tyrosine phosphorylation of EGFR in the respective immunoprecipitates. FADD and caspase 8 association or tyrosine phosphorylation of the immunoprecipitated CD95 samples were detected by Western blot analysis using the respective antibodies (anti-FADD, -caspase 8 and -phospho-tyrosine).

Detection of reactive oxygen species (ROS)

Hepatocytes were seeded on collagen-coated 6-well culture plates (Falcon, Heidelberg, Germany) and cultured for 24h. Cells were incubated with phosphate buffered saline (PBS) containing 5µmol/L of CM-H₂DCFDA for 30min at 37°C and 5% CO₂. In order to detect ROS generation, CM-H₂DCFDA-loaded cells were supplemented again with culture medium and then exposed to the respective bile salt for the indicated time period. Then cells were washed briefly using icecold PBS and cells were lysed in 0.1% Triton X 100 (vol/vol) dissolved in aqua bidest. Lysates were centrifuged immediately (10000g, 4°C, 1min) and fluorescence of the supernatant was measured at 515 to 565nm using a luminescence spectrometer LS-5B (Perkin-Elmer, Wellesley, MA, USA) at 488nm excitation wavelength. Fluorescence of untreated control cells was arbitrarily set to 1.

Detection of apoptosis

Terminal deoxynucleotidyl transferase-mediated X-dUTP nick-end labelling of FITC-conjugated deoxyuridine triphosphate (TUNEL) technique was performed as described recently [11]. The number of apoptotic cells was determined by counting the percentage of fluorescein-positive cells. At least 100 cells from three different cell preparations were counted for each condition. Cells were visualised on an Axioskop (Zeiss, Oberkochen, Germany).

Liver Perfusions

Livers from male Wistar rats (120–150g body wt), fed a standard chow, were perfused as described previously in a non-recirculating manner [21, 22]. The perfusion medium was

the bicarbonate-buffered Krebs-Henseleit saline plus L-lactate (2.1mmol/L) and pyruvate (0.3mmol/L), gassed with O₂/CO₂ (95/5 vol/vol). The temperature was 37°C. The average flow rate was kept constant throughout the individual perfusion experiment and was 3.5–4.5mL/min/g liver. Perfusate osmolality was 305mosmol/L. Livers from wild type and p47^{phox} knock-out mice (20–25g body weight, both with the C57BL/6 background) were perfused with a flow rate 7–8mL/min/g liver using the perfusion medium described above.

Determination of the intracellular water space in perfused liver

The intracellular water space in the intact perfused rat liver was calculated from the difference of washout profiles of simultaneously infused [¹⁴C]urea and [³H]inulin (approximately 500Bq/mL perfusate), as described previously [26]. In brief, both isotopes were infused for 5min in order to allow equilibration of both tracers within the intracellular and extracellular compartments. Then, tracer infusion was stopped and the influent perfusate was collected for another 5min, a time period allowing complete washout of both isotopes. The effluent was monitored for the total sum of released [³H] and [¹⁴C]label by dual spectrum scintillation spectrometry. Taking the [³H]/[¹⁴C] ratio under steady state conditions, a putative ¹⁴C-accessible, but not ³H-accessible space was calculated. This space, expressed as intracellular water space (µL/g liver), was seen to reflect the cellular hydration state. The cellular hydration state was measured under control conditions and again 30min after institution of the respective condition. A spontaneous decrease of the intracellular water space of 2.6% within 30min in fed rats [26], and of 2.0% within 30min in fed mice, respectively, was taken into account. In livers from fed animals, the cell water under control conditions was 572±11µL/g (n=28), as described previously [26].

Statistics

Results from at least three independent experiments are expressed as means±SEM (standard error of the mean). n refers to the number of independent experiments. Results were analyzed using the Student's t-test: p<0.05 was considered statistically significant.

Results

Hydrophobic bile salts induce hepatocyte shrinkage in perfused rat liver

The effect of proapoptotic hydrophobic bile salts on hepatocellular hydration was studied in perfused rat liver using a dual label washout technique, as described previously [26]. As shown in Figure 1, TLCS, GCDC and to a lesser extent TCDC, led within 30min to a decrease of hepatocyte hydration in perfused rat liver. No significant net K⁺ release from the liver was detectable in response to TLCS and GCDC induced only a slight net

Fig. 1. Effect of bile salts on liver cell hydration in perfused livers from rats, wild type or p47^{phox} knock-out mice. The intracellular water space was determined using a dual label washout technique in perfused rat liver (□, for details see *Methods* and ref. [26]) under control conditions and 30min after institution of the effector. An adapted perfusion technique was used for determination of intracellular water spaces in mouse livers (■). n refers to the number of different perfusion experiments. The following bile salts were perfused: 50 $\mu\text{mol/L}$ (n=4) and 20 $\mu\text{mol/L}$ TLCS (n=4), 50 $\mu\text{mol/L}$ GCDC (n=4), 50 $\mu\text{mol/L}$ TCDC (n=4) and 100 $\mu\text{mol/L}$ TC (n=7, § data from ref. [29]). The following inhibitors were used either alone [N-acetylcysteine (NAC, 10mmol/L, n=12), desipramine (10 $\mu\text{mol/L}$, n=4), apocynin (20 $\mu\text{mol/L}$, n=5), DIDS (50 $\mu\text{mol/L}$, n=6) and bafilomycin A1 (10 nmol/L, n=9)] or in combination with 20 $\mu\text{mol/L}$ TLCS (+ NAC, n=4; + desipramine, n=4; + apocynin, n=5; DIDS, n=3 or bafilomycin A1, n=4), 50 $\mu\text{mol/L}$ GCDC (+ desipramine, n=5; + apocynin, n=4) or 50 $\mu\text{mol/L}$ TCDC (+ NAC, n=4). Bile salt-induced hepatocyte shrinkage was significantly inhibited by NAC, desipramine, apocynin, DIDS and bafilomycin A1 (* $p < 0.05$). TLCS-induced hepatocyte shrinkage was further inhibited in p47^{phox} knock-out mice (n=3) compared to wild type mice (n=3) (* $p < 0.05$). Data are given as means $\pm$ SEM.

K⁺ release by $2.4 \pm 0.5 \mu\text{mol/g}$ liver (n=4). TLCS-induced hepatocyte shrinkage was no longer observed in presence of N-acetylcysteine (NAC) (Fig. 1), indicating an involvement of oxidative stress in triggering hepatocyte shrinkage in response to hydrophobic bile salts. In line with this, endogenous and exogenous hydroperoxides were shown to trigger hepatocyte shrinkage and K⁺ channel opening [27, 28]. Other bile salts such as taurocholate or taurochenodesoxycholate, which do not induce hepatocyte apoptosis, did not induce hepatocyte shrinkage [29].

Role of NADPH oxidase in triggering bile salt-induced hepatocyte shrinkage

Hydrophobic bile salts such as taurochenodesoxycholate (TLCS), glycochenodesoxycholate (GCDC) and taurochenodesoxycholate (TCDC) activate NADPH oxidase isoforms in hepatocytes [19] and the resulting oxidative stress response was identified as an important upstream event for bile salt-induced CD95 activation and apoptosis induction [19, 21]. A role of NADPH oxidase isoforms in mediating hepatocyte shrinkage in response to TLCS and GCDC is suggested by the finding that in

Fig. 2. TLCS-induced ROS generation, activation of the CD95 system and apoptosis are inhibited in hepatocytes from p47^{phox} knock-out mice. Hepatocytes were either isolated from wild type (WT; □) or p47^{phox} knock-out mice (k.o.; ■) as described in the *Methods* section and cultured for 24h. Cells were then exposed to control medium or TLCS (100μmol/L). **A**) ROS generation: Hepatocytes from either wild type or p47^{phox} knock-out mice were loaded with 5μmol/L CM-H₂DCFDA as described in the *Methods* section before TLCS was added. In all experiments, the TLCS-induced increase of DCFDA fluorescence was measured 1min after bile salt exposure. Fluorescence obtained in untreated control cells was arbitrarily set to 1. TLCS induced a rapid ROS-response compared to untreated control cells which is significantly reduced in hepatocytes from p47^{phox} knock-out mice (n=3) (* p<0.05). **B**) Activation of the CD95 system: Yes, EGFR and CD95 were immunoprecipitated as described in the *Methods* section and analyzed by Western blotting. Activating Yes-Y⁴¹⁸-phosphorylation (P-Yes-Y⁴¹⁸) and EGFR-tyrosine phosphorylation (P-EGFR-Y) were detected 1min after TLCS addition, CD95-tyrosine phosphorylation (P-CD95-Y), caspase 8/CD95 and FADD/CD95 association (i.e. DISC formation) 120min after TLCS addition. Total Yes, EGFR and CD95 served as respective loading controls. Compared to the wild type mice, in the p47^{phox} knock-out mice TLCS-induced Yes, EGFR and CD95 phosphorylation as well as TLCS-induced DISC formation were prevented (n=3). **C**) Apoptosis: Hepatocytes from either wild type or p47^{phox} knock-out mice were cultured for 24h and then exposed to control medium or TLCS (100μmol/L) for 12h in order to determine hepatocyte apoptosis using the TUNEL assay as described in the *Methods* section. Whereas the percentage of apoptotic cells was not affected under control conditions, TLCS-induced apoptosis was inhibited in hepatocytes from p47^{phox} knock-out mice compared to wild type mice (n=3) (* p<0.05).

presence of apocynin, an inhibitor of NADPH oxidase isoenzymes, cell shrinkage was no longer induced by these bile salts (Fig. 1). The upstream events leading to bile salt-induced NADPH oxidase activation were recently

characterized [19]. They involve a rapid endosomal acidification with stimulation of acidic sphingomyelinase-catalyzed ceramide formation and a subsequent protein kinase Cζ dependent activating phosphorylation of p47^{phox},

Fig. 3. Effect of hyperosmolarity on liver cell hydration in perfused livers from rats, wild type or p47^{phox} knock-out mice. The intracellular water space was determined using a dual label wash-out technique in perfused rat liver (□, for details see *Methods* and ref. [26]) under control conditions and 30min after institution of hyperosmolarity (385mosmol/L). An adapted perfusion technique was used for determination of intracellular water spaces in mouse livers (■). n refers to the number of different perfusion experiments. Hyperosmolarity-induced hepatocyte shrinkage (n=12) was significantly inhibited in presence of N-acetylcysteine (NAC, 10mmol/L, n=8), apocynin (20μmol/L, n=7) and DIDS (n=3) (*p<0.05), while desipramine (20μmol/L, n=5) and bafilomycin A1 (10nmol/L, n=5) were ineffective. Hyperosmotic induced hepatocyte shrinkage was further inhibited in p47^{phox} knock-out mice (n=5) compared to wild type mice (n=4) (*p<0.05). Data are given as means±SEM.

a regulatory subunit of NADPH oxidase [23, 30].

As shown in Figure 1, inhibitors of upstream events leading to bile salt-induced NADPH oxidase activation and the corresponding oxidative stress signal [19] largely prevented bile salt-induced hepatocyte shrinkage. This was found not only in presence of desipramine, an inhibitor of acidic sphingomyelinase (ASM), but also in presence of bafilomycin and DIDS, which were recently shown to prevent bile salt-induced endosomal acidification and subsequent ASM and NADPH oxidase activation [31]. Neither desipramine, nor apocynin or bafilomycin had by itself significant effects on liver cell hydration, indicating that a basal NADPH oxidase activity had little effect on hepatocyte hydration. However, NAC (10mmol/L) by itself induced an increase of hepatocellular hydration by about 5%, which was accompanied by a net K⁺ uptake by the liver of 6.2±1.7μmol/g liver (n=5). These findings may indicate that a basal rate of oxidative stress may exert effects on hepatocyte hydration, however, this may involve NADPH oxidase to a minor extent only.

TLCS does not induced hepatocyte shrinkage in p47^{phox} knock-out mice

The important role of TLCS-induced ROS formation via NADPH oxidase activation for triggering both, hepatocyte shrinkage and apoptosis was also confirmed in studies with hepatocytes from wild type and p47^{phox} knock-out mice. TLCS failed to induce hepatocyte shrinkage in perfused livers from p47^{phox} knock-out mice, but not in livers from wild type mice (Figure 1). In 24h-cultured hepatocytes from p47^{phox} knock-out mice, TLCS did no longer induce an oxidative stress response (Fig. 2A) and as a consequence, ROS-dependent downstream signaling towards CD95 activation [21, 22], *i.e.* Yes and EGFR activation, CD95-Tyr-phosphorylation and DISC formation was also strongly blunted (Fig. 2B). In line with this, apoptosis induction by TLCS as assessed by TUNEL staining, was significantly diminished in hepatocytes from p47^{phox} knock-out mice compared to hepatocytes from wild type mice (Fig. 2C).

NADPH oxidases and hyperosmotic hepatocyte volume recovery

Hyperosmotic exposure of perfused rat liver leads to hepatocyte shrinkage followed by a volume-regulatory increase (RVI), which involves net K^+ uptake by hepatocytes due to combined activation of Na^+/H^+ exchange, $Na-K-2Cl$ -cotransport and Cl^-/HCO_3^- exchange [32-34]. This volume-regulatory response, which is completed within 20min of hyperosmotic exposure, however, is incomplete and the hepatocyte is left in a partially shrunken state [32-34]. Because hyperosmolarity also induces NADPH oxidase activation [13], the possibility was addressed to what extent the NADPH oxidase-derived oxidative stress may contribute to the residual hepatocyte shrinkage, which is observed after completion of RVI. As shown in Figure 3, 30min after hyperosmotic exposure (385mosmol/L) and completion of volume regulatory ion fluxes, hepatocyte hydration was about 14% below the normoosmotic control level. Residual hepatocyte shrinkage after hyperosmotic exposure for 30min, however, was strongly blunted in presence of N-acetylcysteine and after inhibition of NADPH oxidase by apocynin (Figure 3). In line with a role of NADPH oxidase-derived oxidative stress in triggering sustained hyperosmotic hepatocyte shrinkage, livers from p47^{phox} knock-out, but not from wild type mice largely restored their hydration state in response to hyperosmolarity. These findings suggest that NADPH oxidase-derived oxidative stress is involved in the residual hepatocyte shrinkage 30min after hyperosmotic exposure. However, in contrast to the findings with hydrophobic bile salts, bafilomycin and desipramine had little effect on sustained hyperosmotic hepatocyte shrinkage.

Discussion

NADPH oxidase activation induces hepatocyte shrinkage

As shown in the present study, proapoptotic bile salts induce hepatocyte shrinkage, whereas the nonapoptotic bile salts TC and TUDC were previously shown to induce hepatocyte swelling [35]. The mechanisms underlying TLCS-induced hepatocyte shrinkage apparently relates to the NADPH oxidase-derived ROS response. This view is supported by the finding that TLCS induced no longer hepatocyte shrinkage in p47^{phox} knock-out animals or after inhibition of NADPH oxidase by apocynin. Furthermore, in rat liver, TLCS-induced hepatocyte shrinkage was prevented by inhibitors acting upstream of bile salt-

induced NADPH oxidase activation, such as bafilomycin, DIDS and desipramine and also N-acetylcysteine prevented TLCS-induced hepatocyte shrinkage. Exogenous and endogenous hydroperoxides are known to induce hepatocyte shrinkage due to K^+ -channel opening [27]. N-acetylcysteine by itself caused hepatocyte swelling, suggestive for a basal production rate of reactive oxygen species, which exerts control on cell hydration. In view of the multiple effects of cell hydration on liver metabolism, transport and gene expression (for review see [36]), regulation of Nox/Duox enzyme activities may be a novel site for control of liver function via cell hydration changes.

NADPH oxidase activation apparently also accounts for the incomplete volume recovery of hepatocytes following hyperosmotic exposure. In line with this, residual hepatocyte shrinkage after completion of RVI was strongly blunted in presence of N-acetylcysteine, after inhibition of NADPH oxidase by apocynin and was largely absent in livers from p47^{phox} knock-out mice. However, bafilomycin and desipramine, which were shown to blunt hyperosmotic NADPH oxidase and CD95 activation [13], had little effect in this respect. The reason for this apparent discrepancy is unknown, however, several Nox/Duox isoforms are present in rat liver and the possibility must be considered that these isoforms are differentially regulated and differentially participate in triggering CD95 activation and cell shrinkage, respectively.

Hepatocyte shrinkage and apoptosis induction

Hyperosmotic cell shrinkage was recently identified as an independent proapoptotic signal, which activates the CD95 system [12]. In addition, hepatocyte shrinkage stimulates autophagic proteolysis [36], inhibits protein synthesis [37] and diminishes the capacity of canalicular bile formation due to a rapid retrieval of canalicular transporters from the canalicular membrane [35]. It should be noted that cell shrinkage in response to hydrophobic bile salts occurs already within 30min, *i.e.* a time period at which CD95 was shown to be phosphorylated at tyrosine residues and thereby activated, but at which DISC formation at the CD95 has not yet been started [21]. Thus it is at present unclear to what extent bile salt-induced hepatocyte shrinkage contributes to their proapoptotic effect, however, apart from CD95 activation, other sequelae of cell shrinkage, such as protein wasting and cholestasis [35-37] may synergistically promote cell death.

It should be noted that cell hydration and oxidative stress are apparently closely interlinked. On the one hand

Fig. 4. Oxidative stress, hepatocellular dehydration and apoptosis. The figure summarizes our current view on the interplay between oxidative stress and hepatocellular hydration in bile salt-induced apoptosis. Proapoptotic bile salts like TLCS, TCDC or GCDC lead to a NADPH oxidase (Nox/Duox)-dependent ROS formation (oxidative stress) which triggers cellular dehydration. Cellular dehydration in turn activates NADPH oxidase and leads to the generation of ROS and thereby promotes a feed-forward amplifying system, which finally ends up in hepatocyte apoptosis. This vicious cycle may be interrupted by cosignals which either counteract hepatocyte shrinkage (such as growth factors, amino acids, organic osmolytes, PI3 kinase signals or hypoosmolarity) or cosignals which counteract the oxidative stress (such as organic osmolytes or antioxidants).

cell shrinkage induces oxidative stress [12], and on the other oxidative stress leads to cell shrinkage in hepatocytes [27]. Likewise, cell dehydration and apoptosis are related. For example, hepatocyte shrinkage causes ligand-independent CD95 activation [12], whereas CD95 activation by CD95 ligand (CD95L) was shown in other cell types to induce a so-called “apoptotic cell volume decrease” [38], possibly as a consequence of CD95L-induced ROS formation [12, 39]. Thus, this relationship between oxidative stress and cell shrinkage may represent a feed-forward amplifying system, whose activation will inevitably result in apoptotic cell death, unless this cycle is interrupted by cosignals either favouring hepatocyte swelling or counteracting the oxidative stress response (Fig. 4). Such cosignals may be growth factors, amino acids, betaine and PI3 kinase signals, all of which have been shown to induce hepatocyte swelling [36, 40] and are simultaneously known to exert antiapoptotic effects [41].

Abbreviations

ASM (acidic sphingomyelinase); CD95 (CD95 receptor, Fas, APO-1); CD95L (CD95 ligand); CM-H₂DCFDA (5-(and-6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate); DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid disodium salt); DISC (death inducing signaling complex); DPI (diphenyleneiodonium chloride); EGFR (epidermal growth factor receptor); FADD (Fas associated death domain); GCDC (glycochenodeoxycholate); JNK (c-Jun-N-terminal kinase); PBS (phosphate-buffered saline); PKC (protein kinase C); ROS (reactive oxygen species); TC (taurocholate); TCDC (taurochenodeoxycholate); TLCS (tauroolithocholate-3-sulfate); TUDC (tauroursodeoxycholate); TUNEL (terminal deoxynucleotidyl transferase-mediated X-dUTP nick-end labelling).

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