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Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death ☆☆☆



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ABSTRACT

Background: In chronic liver disease, hepatic stellate cells (HSCs) are activated, highly proliferative and produce excessive amounts of extracellular matrix, leading to liver fibrosis. Elevated levels of toxic reactive oxygen species (ROS) produced during chronic liver injury have been implicated in this activation process. Therefore, activated hepatic stellate cells need to harbor highly effective anti-oxidants to protect against the toxic effects of ROS.

Aim: To investigate the protective mechanisms of activated HSCs against ROS-induced toxicity.

Methods: Culture-activated rat HSCs were exposed to hydrogen peroxide. Necrosis and apoptosis were determined by Sytox Green or acridine orange staining, respectively. The hydrogen peroxide detoxifying enzymes catalase and glutathione-peroxidase (GPx) were inhibited using 3-amino-1,2,4-triazole and mercaptosuccinic acid, respectively. The anti-oxidant glutathione was depleted by L-buthionine-sulfoximine and replenished with the GSH-analogue GSH-monoethylester (GSH-MEE).

Results: Upon activation, HSCs increase their cellular glutathione content and GPx expression, while MnSOD (both at mRNA and protein level) and catalase (at the protein level, but not at the mRNA level) decreased. Hydrogen peroxide did not induce cell death in activated HSCs. Glutathione depletion increased the sensitivity of HSCs to hydrogen peroxide, resulting in 35% and 75% necrotic cells at 0.2 and 1 mmol/L hydrogen peroxide, respectively. The sensitizing effect was abolished by GSH-MEE. Inhibition of catalase or GPx significantly increased hydrogen peroxide-induced apoptosis, which was not reversed by GSH-MEE.

Conclusion: Activated HSCs have increased ROS-detoxifying capacity compared to quiescent HSCs. Glutathione levels increase during HSC activation and protect against ROS-induced necrosis, whereas hydrogen peroxide-detoxifying enzymes protect against apoptotic cell death.

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Abbreviations: 3AT, 3-amino-1,2,4-triazole; BSO, L-buthionine-sulfoximine; dNTP, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GCL, glutamate cysteine ligase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSH-MEE, GSH-monoethylester; GSSG, oxidized glutathione; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HO-1, heme-oxygenase-1; HSC, hepatic stellate cell; MS, mercaptosuccinic acid; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulphonylfluoride; PPAR- γ , peroxisome proliferator-activated receptor gamma; RNA, ribonucleic acid; ROS, reactive oxygen species; α -SMA, α -smooth muscle actin; SOD, super oxide dismutase; TGF- β , transforming growth factor beta

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1. Introduction

Oxidative stress is defined as the imbalance between pro-oxidants and anti-oxidants [1,2]. Under normal conditions, reactive oxygen species are detoxified by various enzymatic and non-enzymatic antioxidants. When pro-oxidants exceed the antioxidant capacity of the cell, oxidative stress is the result [1,2]. Prolonged oxidative stress in the liver is associated with liver fibrosis and cirrhosis [3–7]. Liver fibrosis is characterized by the loss of hepatocytes and the activation of hepatic stellate cells (HSCs) [3–5]. During the activation process quiescent HSCs transform into proliferating myofibroblast-like cells. Unlike quiescent HSCs, these activated cells lack retinoid-storing capacity, produce excessive amounts of connective tissue and proliferate [4,5].

Although generation of reactive oxygen species has been implicated in the activation of stellate cells and liver fibrosis [6–16], little is known about the role of the different antioxidant systems in activated HSCs. Several enzymes are able to generate hydrogen peroxide, e.g. NADPH-

oxidases and xanthine oxidase [1,2]. In addition, hydrogen peroxide is generated in the detoxification of superoxide anions by superoxide dismutases like the cytosolic CuZn-SOD (SOD1) and the mitochondrial Mn-SOD (SOD2) [1,2]. Pathophysiological conditions often lead to increased hydrogen peroxide levels produced by inflammatory cells, e.g. neutrophils [1]. Hydrogen peroxide is detoxified by catalase that resides in peroxisomes or by cytosolic glutathione peroxidase. Glutathione peroxidase converts reduced glutathione (GSH) into oxidized glutathione (GSSG) [1,2]. To control the hydrogen peroxide level within the cell, the cell has to balance the activity of catalase and glutathione peroxidases relative to SODs. The aim of this study was to investigate the role of antioxidant systems in the resistance of stellate cells to hydrogen peroxide-induced toxicity.

2. Material and methods

2.1. Animals

Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

2.2. Hepatic stellate cell isolation and culture

Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500–600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, the Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (12% w/v) centrifugation as described previously [17]. Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 50 µg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 µg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, the Netherlands) in a humidified atmosphere containing 5% CO₂ at 37 °C. For studying activation of hepatic stellate cells, cells were seeded, grown to confluency and harvested at the indicated time points. Primary HSC cultures were passaged via trypsinization, and then cultured in Iscove's medium with supplements as described above, except Nystatin. Prior to experiments, HSCs were serum-starved for 24 h, unless indicated otherwise.

2.3. Experimental design

HSCs were culture-activated on tissue culture plastic for at least 7 days. The activated rat HSCs were exposed to oxidative stress induced by 0.2 or 1 mM hydrogen peroxide (Merck) or 20 or 50 µM menadione (Sigma-Aldrich, the Netherlands). The glutathione depleting compound L-buthionine-sulfoximine (BSO, Sigma-Aldrich, the Netherlands) was used at 200 µM. The cell permeable glutathione donor GSH-monoethylester (GSH-MEE, Calbiochem, VWR, The Netherlands) was used at 5 mM. The glutathione peroxidase inhibitor mercaptosuccinic acid (MS, Sigma-Aldrich) was used at 10 mM and the catalase inhibitor 3-amino-1,2,4-triazole (3AT, Sigma-Aldrich) was used at 20 mM. The caspase-3 inhibitor (Z-DEVD-FMK004R & D Systems, Abingdon UK) was used at 0.05 µM. Inhibitors were added 30 min prior to exposure to hydrogen peroxide, with the exception of BSO, which was added 17–20 h prior to exposure to hydrogen peroxide.

2.4. Glutathione assay

Glutathione and glutathione disulfide content were determined using a spectrophotometry-based assay as described previously [18,19]. HSCs were harvested in a lysis buffer composed of 25 mM HEPES, 5 mM MgCl₂, 5 mM EDTA, 2 mM PMSF, 10 µg/µL pepstatin and 10 µg/µL leupeptin and then lysed by 3 cycles of snap-freezing in liquid nitrogen and thawing. Values were corrected for protein concentration, determined by the BioRad DC protein assay (Veenendaal, the Netherlands) according to the manufacturer's instructions.

2.5. RNA isolation

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using random nonamers (Sigma-Aldrich) in a final volume of 50 µL. Reverse transcription was performed in three steps: 10 min at 25 °C, 1 h at 37 °C and 5 min at 95 °C.

2.6. Quantitative real-time PCR

Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems, the Netherlands) initialized by 10 min at 95 °C, followed by 40 cycles (15 s at 95 °C, and 1 min at 60 °C). Each sample was analyzed in duplicate. mRNA levels of 18S were used as an internal control. Reaction mixture contained qPCR mastermix plus-dTTP (Eurogentec, Maastricht, The Netherlands) supplemented with 900 nM sense and anti-sense primer and 200 nM labeled probe. The primers (Invitrogen) and probe (Eurogentec) used are listed in Supplementary Table S1. Relative gene expressions were calculated using the $\Delta\Delta C_t$ method.

2.7. Western blot analysis

Western blot analysis was performed as described previously [19]. Equal amounts of protein were loaded on SDS-PAGE gels. Proteins were transferred using semi-dry electrophoretic transfer. Specific proteins were detected using primary antibodies: mouse anti-GAPDH (1:10,000, Calbiochem, VWR, the Netherlands CB1001), mouse anti- α -smooth muscle actin (1:2,000, Sigma Aldrich), rabbit polyclonal anti-Mn-superoxide dismutase (1:1000, Stressgen, Enzo life Sciences, Antwerpen, Belgium, SOD-111), polyclonal rabbit anti-catalase (1:2000, Calbiochem 219010), polyclonal rabbit anti-Pex-14 (1:2000, generous gift of Dr. M. Fransen, Leuven, Belgium), and polyclonal rabbit anti- β -actin (1:2000, Sigma-Aldrich A2066). Protein bands were detected using a Chemidoc XRS system (Bio-Rad).

2.8. Apoptosis and necrosis determination by acridine orange and Sytox green/Hoechst 33342 nuclear staining

Cells were seeded in 12-well plates and treated as indicated. Apoptosis was determined by assessment of nuclear condensation using Acridine orange staining (Sigma-Aldrich) at 2.5 µg/mL. After 6 h, the percentage of apoptotic cells was determined by dividing the number of condensed nuclei by the total number of nuclei per field, amplified with 100. Percentages are the mean of two randomly chosen fields per condition (magnification 200 \times ; 15 nuclei per field).

To determine necrosis, HSCs were incubated with Sytox green nucleic acid staining (Invitrogen, Breda, The Netherlands) at 125 nM in combination with Hoechst 33342 (Roche, Almere, the Netherlands) at 5 µg/mL. Sytox green penetrates cells with leaky plasma membranes, a hallmark of necrotic cells, but does not cross the plasma membranes of viable or apoptotic cells and has been validated before [19]. Hoechst 33342 crosses the plasma membrane of all cells. After 3 h, the percentage of necrotic cells was determined by dividing the number of Sytox green positive nuclei by the number of Hoechst 33342 positive nuclei of the same field, amplified by 100. Two randomly-chosen fields were

used to determine the average per condition (magnification 200x; 15 nuclei per field). Cells were monitored using an Olympus CKX41 microscope at 450–490 nm.

2.9. Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde, labeled and analyzed as described previously [20]. Rabbit polyclonal antibodies against catalase (dilution 1:200; Calbiochem) or MnSOD (dilution 1:100, Stressgen) were used as primary antibodies, followed by secondary antibodies labeled with Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen), respectively. Images were captured with a Leica TCS SP2-AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany).

2.10. Proliferation assay

Proliferation of HSCs was determined using the Cell Proliferation ELISA kit (Roche, Almere, the Netherlands), a chemiluminescent ELISA-based detection of BrdU incorporation, according to the manufacturer's instructions.

2.11. Statistical analysis

Statistical analyses of data were performed using SPSS 14. Data are presented as mean \pm standard deviation, unless otherwise indicated. Statistical differences between groups were calculated using the non-parametric Kruskal–Wallis test, followed by a Mann–Whitney–U-test. P-values below 0.05 were considered significant.

3. Results

3.1. Glutathione peroxidase 1 expression is induced during activation of hepatic stellate cells

Marked changes in gene expression occur when quiescent HSCs transform into activated HSCs. Therefore, we first investigated the expression of various genes involved in the detoxification of reactive oxygen species during the activation process. As expected, alpha-smooth muscle actin (α -SMA), a marker for HSC activation, was strongly induced upon activation (Fig. 1A). mRNA levels of catalase were significantly induced after two days of culture, but decreased upon complete activation of stellate cells (Fig. 1B). Manganese superoxide dismutase (Mn-SOD) mRNA levels progressively declined upon activation (Fig. 1C), while the copper-zinc superoxide dismutase (CuZn-SOD) expression remained unaltered (Fig. 1D). Interestingly, mRNA expression of glutathione peroxidase 1 (GPx1) increased during activation of HSCs (Fig. 1E), while glutamate–cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis, was not altered upon HSC activation (Fig. 1F). HO-1 expression in HSCs was very low and although a trend towards reduced heme oxygenase-1 (HO-1) expression was observed, this did not reach statistical significance (Fig. 1G).

The mRNA data were confirmed by analyzing the levels of the corresponding proteins by Western blotting (Fig. 1H). Alpha-smooth muscle actin, β -actin and Gapdh protein expression all increased upon HSC activation. In contrast, Mn-SOD protein levels sharply decreased during activation of HSC. Similarly, also catalase protein levels dropped immediately after day 1, which was not observed for the catalase mRNA levels. Despite the low protein levels of catalase and MnSOD as detected by Western blotting, both proteins remained detectable using immunocytochemistry and revealed a typical peroxisomal and mitochondrial location, respectively (Fig. 2). Given the large and unexpected difference in β -actin and Gapdh levels in quiescent versus activated HSC, we searched for alternative proteins to use as loading control for Western blot analysis. Pex14p, a protein involved in translocating proteins into peroxisomes, showed a stable signal relative to 18S mRNA level and total protein loaded for Western blot analysis (Fig. 1H, bottom panel).

3.2. Activated hepatic stellate cells have a higher glutathione content than quiescent hepatic stellate cells

Total glutathione levels were determined in quiescent (1 day after isolation) HSCs and in fully activated (>7 days after isolation) HSCs. The total cellular glutathione content was increased 5.6-times upon activation of HSC from 0.18 μ mol/ μ g protein in quiescent HSC to 1.0 μ mol/ μ g protein in activated HSC (Fig. 3), despite unchanged GCL mRNA and protein levels, the rate-limiting enzyme in the synthesis of glutathione (Fig. 1). Subsequent analysis revealed that 88% of the total glutathione content was in the reduced (GSH) form (mean of 3 different isolates of HSCs).

3.3. Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells

Pre-incubation of activated, serum-starved HSCs with BSO reduced total glutathione levels by 87% (Fig. 4A). No increase was observed in either necrotic or apoptotic cell death (data not shown) as well as no gross changes in cellular morphology was observed in BSO-treated HSC. Likewise, BSO treatment also reduced glutathione content by 88% in activated HSCs cultured in medium containing 20% FCS (data not shown), without visible morphological changes.

To investigate whether glutathione depletion leads to increased oxidative stress in HSCs, we determined the mRNA level of the oxidative stress-responsive gene heme-oxygenase-1 (HO-1). Depletion of glutathione increased HO-1 mRNA levels 2.3-fold (Fig. 4B), which is only minor when compared to the induction of HO-1 by menadione or hydrogen peroxide (5–50 fold) [6,19]. In addition, mRNA expression of GCL, the rate-limiting enzyme in glutathione synthesis, was increased only 1.6-fold (Fig. 4B). In contrast, glutathione depletion had no effect on the expression of the hydrogen peroxide-detoxifying enzymes GPx1 and catalase and the superoxide dismutases Mn-SOD, and CuZn-SOD (Fig. 4B, C). These data indicate that glutathione depletion alone only minimally induces markers of oxidative stress. Furthermore, glutathione depletion did not change the expression of the known markers of stellate cell activation α -smooth muscle actin (α -SMA), collagen type 1, and TGF- β (Fig. 4D). Finally, glutathione depletion or glutathione supplementation, using GSH-MEE did not alter stellate cell proliferation (data not shown). Next, we analyzed whether glutathione depletion sensitizes HSCs for oxidative stress.

3.4. Depletion of glutathione increases sensitivity to hydrogen peroxide-induced necrosis

After depletion of glutathione with BSO, 35% and 75% of the cells became necrotic after a 3 h exposure to 0.2 or 1 mM hydrogen peroxide, respectively (Fig. 5). This indicates that glutathione depletion greatly enhanced sensitivity to hydrogen peroxide-induced necrosis. Restoration of glutathione content, using GSH-MEE, almost completely reversed hydrogen peroxide-induced necrosis in BSO-treated HSCs (Fig. 5).

3.5. Glutathione peroxidase and catalase protect against oxidative stress-induced apoptosis

The importance of the antioxidant enzyme GPx in the protection of activated HSCs against oxidative stress was investigated using the GPx inhibitor mercaptosuccinic acid (MS). Inhibition of GPx using mercaptosuccinic acid increased apoptosis in the absence of hydrogen peroxide and in the presence of 0.2 mM and 1 mM hydrogen peroxide (Fig. 6A). No effects on necrosis were observed under these conditions (data not shown).

The importance of the antioxidant enzyme catalase in the protection of activated HSCs against oxidative stress was investigated using the catalase inhibitor 3-amino-1,2,4-triazole (3-AT). This inhibitor significantly

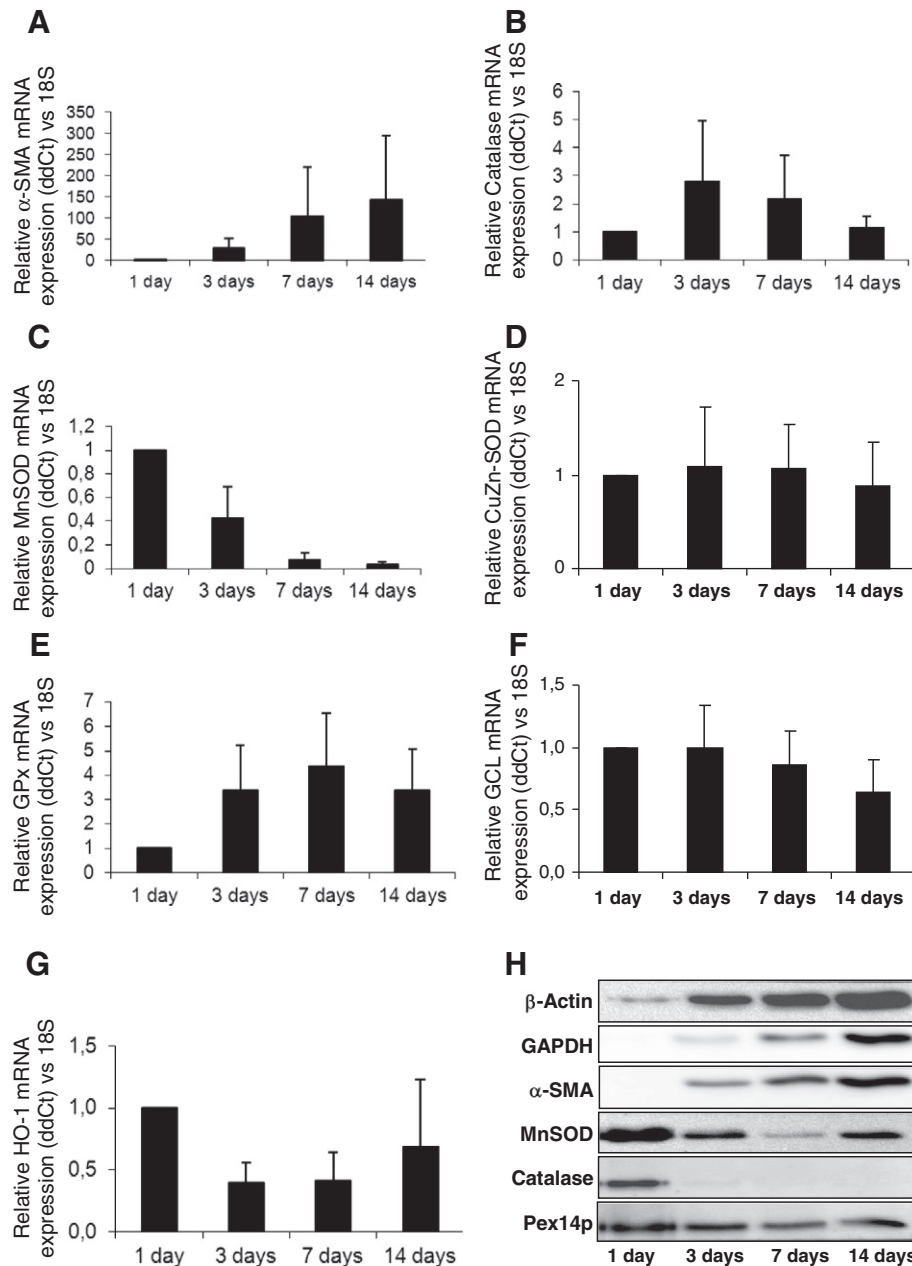


Fig. 1. Expression of ROS-detoxifying enzymes during hepatic stellate cell activation. Primary rat HSC were culture-activated for 1, 3, 7 and 14 days and mRNA (A–G) and protein (H) levels of activation markers and anti-oxidant enzymes were quantified by RT-Q-PCR and western blotting, respectively. Alpha-SMA mRNA (A) and protein (H) levels progressively increased during HSC activation. Catalase mRNA levels (B) transiently increased, while the corresponding protein rapidly declined in total cell extracts after day 1 (H). Expression of Mn-SOD progressively decreased during HSC activation, both at mRNA (C) and protein (H) level, while CuZn-SOD levels did not change (D). The expression of GPx1 increased (E), while the expression of GCL did not change (F, H). The expression of HO-1 was low and not significantly changed during HSC activation (G). The expression of Pex14p was used as loading control for Western blot analysis, since β -actin and Gapdh protein levels strongly increased upon HSC activation (H). The Western blots are representative of four independent experiments. mRNA data was corrected for 18S and is presented as means \pm SD. *Significant difference compared to quiescent HSCs at day 1, $p < 0.05$.

induced apoptotic cell death (18%) in HSCs after 6 h, even in the absence of exogenous hydrogen peroxide (Fig. 6A). Cells treated with 0.2 or 1 mM hydrogen peroxide in the presence of the inhibitor catalase showed even higher levels of apoptosis, 22% and 44% respectively (Fig. 6A). No effects on necrosis were detected at this time point.

To investigate the importance of the hydrogen peroxide detoxifying enzymes catalase and GPx in the protection against superoxide anions that are converted into hydrogen peroxide by superoxide dismutases, we exposed activated stellate cells to the superoxide anion generator menadione. As described previously, menadione at 20 μ M induced predominantly apoptotic cell death, which was reduced by the glutathione donor GSH-MEE [19]. Inhibition of catalase in the presence of menadione had no effect on apoptotic cell death, but slightly increased necrotic

cell death, whereas inhibition of GPx did neither modulate apoptotic nor necrotic cell death (Fig. 6C). Prior reduction of cellular glutathione levels by BSO did not aggravate apoptotic cell death induced by 20 μ M menadione. Menadione at 50 μ M resulted in detachment of cells and massive necrotic cell death.

3.6. Combined inhibition of glutathione peroxidase and catalase elevates apoptosis of HSCs

Inhibiting both GPx and catalase by cotreatment with MS and 3AT resulted in 22% apoptotic cells after 6 h even in the absence of exogenous hydrogen peroxide (Fig. 6A). Exposing these cells to hydrogen peroxide induced apoptosis even further: to 49% in co-treatment

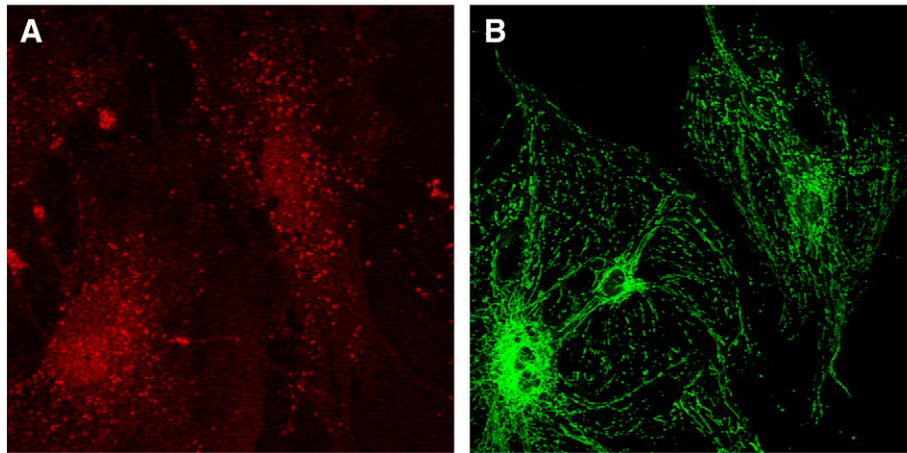


Fig. 2. Catalase and Mn-SOD protein is still detectable in activated HSC. Fully activated HSC were analyzed for the presence and subcellular location of catalase (A) and MnSOD (B) using immunofluorescence microscopy. A punctate staining typical for a peroxisomal location was observed for catalase (red stain), while MnSOD staining (green) was restricted to mitochondria.

with 0.2 mM hydrogen peroxide and to 62% in co-treatment with 1 mM hydrogen peroxide (Fig. 6A). Increasing the glutathione content using the glutathione donor GSH-MEE did not change the cell viability under these conditions (Fig. 6B). Inhibition of caspase-3 partially decreased apoptosis of HSCs (Fig. 6B). Necrotic cell death was not significantly enhanced in the described conditions (data not shown).

4. Discussion

Chronic liver injury is almost invariably accompanied by increased oxidative stress, activation of stellate cells and fibrogenesis [3–7]. Activated HSCs must be well protected against oxidative stress, since they survive and proliferate in the chronically injured liver. The oxidative stress in chronically injured liver is composed of several reactive oxygen species, including hydrogen peroxide and superoxide anions. In this study, we have investigated the resistance of hepatic stellate cells to hydrogen peroxide-induced injury. We demonstrate that this resistance is to a large extent due to a high intracellular glutathione content and increased expression of glutathione peroxidase in activated stellate cells. Although an increased glutathione content in activated stellate cells has been reported before [21], the implications have never been investigated in the context of oxidative stress-induced cell death.

Maher et al. showed, in addition to increased glutathione levels upon activation, an increase in the activity and mRNA level of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis [21]. We did not observe an induction of GCL mRNA upon stellate cell activation in our experiments. The increased cellular glutathione content is most likely due to a higher activity of GCL, especially since GCL activity is known to be regulated by the glutathione content [22]. It is unlikely that the increased glutathione content is due to reduced activity of the GSSG export pump Mrp1, since we have previously shown that the

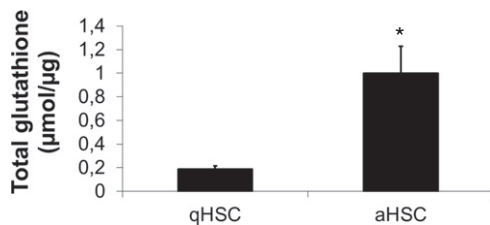


Fig. 3. Total glutathione content is increased upon hepatic stellate cell activation. Total glutathione levels were quantified in quiescent (qHSC, 1 day in culture) and fully activated (aHSC, >7 days in culture) HSC. Activated HSCs have approximately 5.6-fold higher glutathione levels than quiescent HSCs. Results are shown as mean \pm S.E.M of four independent experiments, * significantly different from quiescent stellate cells, $p < 0.05$.

expression of this transporter is increased in activated stellate cells and contributes significantly to the survival of activated stellate cells [23].

Upon HSC activation, the expression of the hydrogen peroxide detoxifying enzyme glutathione peroxidase 1 (GPx1) is increased. This increase may be an adaptive response to oxidative stress. Indeed, mice over-expressing GPx1 are better protected against oxidative stress and they survive concentrations of the oxidant paraquat that are lethal in wild type mice and even more harmful in GPx1 knockout mice [24,25]. Mice that overexpress GPx1 are also more resistant to oxidative stress due to myocardial ischemia-reperfusion injury [26]. It should be noted that glutathione is essential for the activity of GPx, since GPx converts reduced glutathione into oxidized glutathione. This might explain the coordinated increase in cellular glutathione content and GPx expression during the activation process of hepatic stellate cells. Therefore, our data suggest that activated hepatic stellate cells may be more resistant against oxidative stress than quiescent stellate cells. Interestingly, upon activation the mRNA expression level of the mitochondrial superoxide anion converting enzyme Mn-SOD is reduced, both at the mRNA and at the protein level. This finding is partially at variance with previous reports that revealed an initial increase in MnSOD mRNA expression, followed by a steady decrease of MnSOD mRNA expression [27]. Disruption of the Mn-SOD gene, a known tumor suppressor gene, is lethal in mice, which is a direct result of mitochondrial dysfunction, leading to metabolic acidosis, ketosis and accumulation of lipids in the liver and skeletal muscle [28,29]. Characterization of the heterozygous Mn-SOD knockout mice revealed no compensatory increase in other ROS-detoxifying enzymes, like glutathione peroxidase, CuZn-SOD or catalase [30]. Since Mn-SOD is restricted to the mitochondria, changes in its activity may not affect other components of the antioxidant defense system in other cellular compartment like the cytoplasm [30]. At present it is not known how the activated stellate cells detoxify the reactive oxygen species generated in mitochondria. One possibility is that the residual MnSOD protein content, as determined by Western-blotting and immunofluorescence, is sufficient to detoxify ROS generated in mitochondria. The reduction in Mn-SOD mRNA expression during activation could be due to the strong reduction of the transcription factor peroxisome proliferator-activated receptor- γ (PPAR- γ) that occurs during stellate cell activation (data not shown and [31]). MnSOD expression is controlled by PPAR- γ : in PPAR- γ knockout mice, the expression of Mn-SOD is also reduced [32] and activation of PPAR- γ with the agonists rosiglitazone or rosuvastatin enhanced MnSOD activity and expression [33,34].

The catalase mRNA levels transiently increased during the activation process of HSC, which is largely in line with observations made by De Bleser et al. who analyzed catalase regulation during HSC activation by

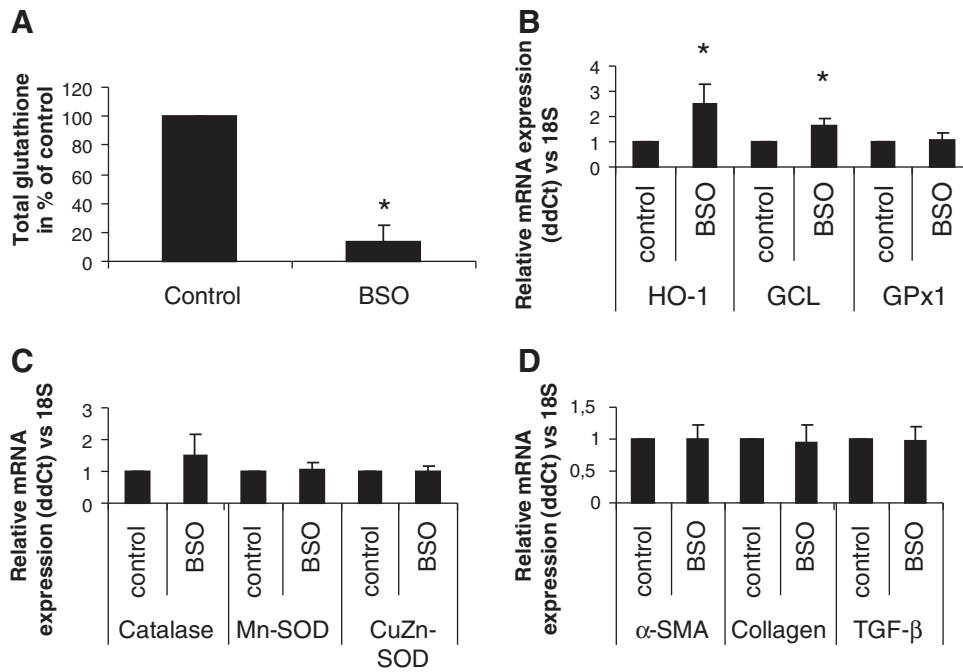


Fig. 4. Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells. Fully-activated HSC were treated with BSO for 20 h and analyzed for total glutathione content (A) and mRNA levels of anti-oxidant enzymes (B,C) and activation markers (D). Pre-treating HSCs with BSO depleted cellular glutathione content by 87% in serum-starved HSCs (A). BSO treatment moderately induced HO-1 and GCL and had no effect on GPx1 (B) nor on the expression of the anti-oxidant enzymes catalase, Mn-SOD, and CuZn-SOD (C) or the expression of the HSC activation markers, α -SMA, collagen type 1 and TGF- β (D). Results are shown as mean \pm st. dev. of at least four independent experiments, * significantly different from controls; $p < 0.05$.

Northern blot analysis [27]. However, in contrast to the mRNA levels, the cellular catalase protein content sharply dropped 1 day after plating HSC. A typical peroxisomal staining of catalase remained detectable in fully activated HSC, indicating that some residual catalase protein was present in these cells. However, clearly, the catalase protein level was not an accurate reflection of the catalase mRNA content. At present it is unclear why the catalase protein disappears from activated stellate cells, but this may be a result of a rapid and selective degradation of this antioxidant protein. The low catalase levels did not sensitize activated HSCs to acute H_2O_2 -induced necrosis, indicating the presence

of an alternative H_2O_2 scavenging mechanism(s) in activated HSCs. However, blocking catalase activity by 3-AT made HSCs highly sensitive for apoptotic cell death, which was further enhanced by exposure to 0.2 or 1.0 mM H_2O_2 . A similar effect was observed after blocking GPx activity, indicating that these 2 proteins play a key role in regulating apoptotic cell death in HSCs.

HSCs increase their glutathione levels upon activation. However, glutathione depletion has no direct effect on stellate cell morphology [35] and in this study, we show that glutathione depletion also has no effect on the expression of markers of stellate cell activation, such as collagen type I, α -SMA and TGF- β or anti-oxidant genes like catalase, Mn-SOD, CuZn-SOD, and GPx1. Additionally, glutathione depletion did not affect the proliferation of hepatic stellate cells. The glutathione levels per se do not seem to be a key mechanism in developing the activated phenotype.

Although increased mRNA levels of GCL and HO-1 were observed after glutathione depletion, these increases were modest, indicating that depletion of glutathione alone does not exert significant oxidative stress on stellate cells. Induction of GCL mRNA levels after glutathione depletion has also been shown in lung epithelial cells [36], endothelial cells [37], and in rat liver *in vivo* [38]. Although HO-1 has been reported to inhibit HSC proliferation via p38 activation [39], we did not find an alteration in the proliferation rate of stellate cells after HO-1 induction due to glutathione depletion. Possibly, the induction of HO-1 by glutathione depletion in our study is too modest to have an effect on p38 phosphorylation and subsequent HSC proliferation. The induction of HO-1 by glutathione depletion was only 2.3-fold, whereas Li et al showed at least a 10-fold induction of HO-1 expression using 15-deoxy-delta-12,14-prostaglandin J2 [39,40].

Although glutathione depletion per se had no effect on stellate cell viability, glutathione depletion increased the sensitivity of the cells to hydrogen peroxide-induced necrosis. Replenishing glutathione reduced necrotic cell death, without a shift towards apoptosis. Inhibition of the hydrogen peroxide-detoxifying activity by inhibitors of glutathione peroxidase or catalase induced HSC apoptosis, both in the absence and in the presence of exogenous hydrogen peroxide. Apoptotic cell death as

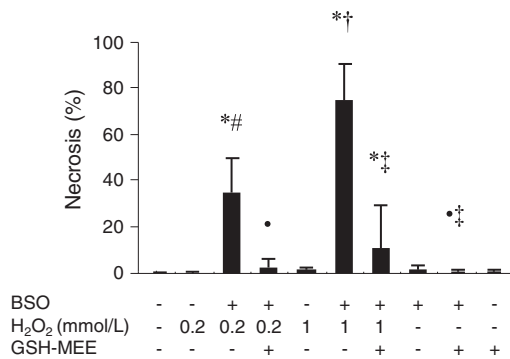


Fig. 5. Glutathione depletion increases sensitivity of hepatic stellate cells to hydrogen peroxide-induced necrosis. Fully-activated HSCs were exposed for 3 h to H_2O_2 in the absence or presence of BSO and/or GSH-MEE and analyzed for necrotic cell death by Sytox green nuclear staining. BSO treatment followed by exposure to 0.2 mM or 1 mM hydrogen peroxide (H_2O_2) induced necrosis in 35% and 75% of the cells, respectively. Co-treatment with GSH-MEE almost completely prevented the induction of HSC necrosis by BSO + H_2O_2 . Results are shown as mean \pm st. dev. of four independent experiments. *Significant difference compared to control, $p < 0.05$; # Significant difference compared to 0.2 mM hydrogen peroxide, $p < 0.05$; †Significant difference compared to 0.2 mM hydrogen peroxide + BSO, $p < 0.05$; ‡Significant difference compared to 1 mM hydrogen peroxide + BSO, $p < 0.05$.

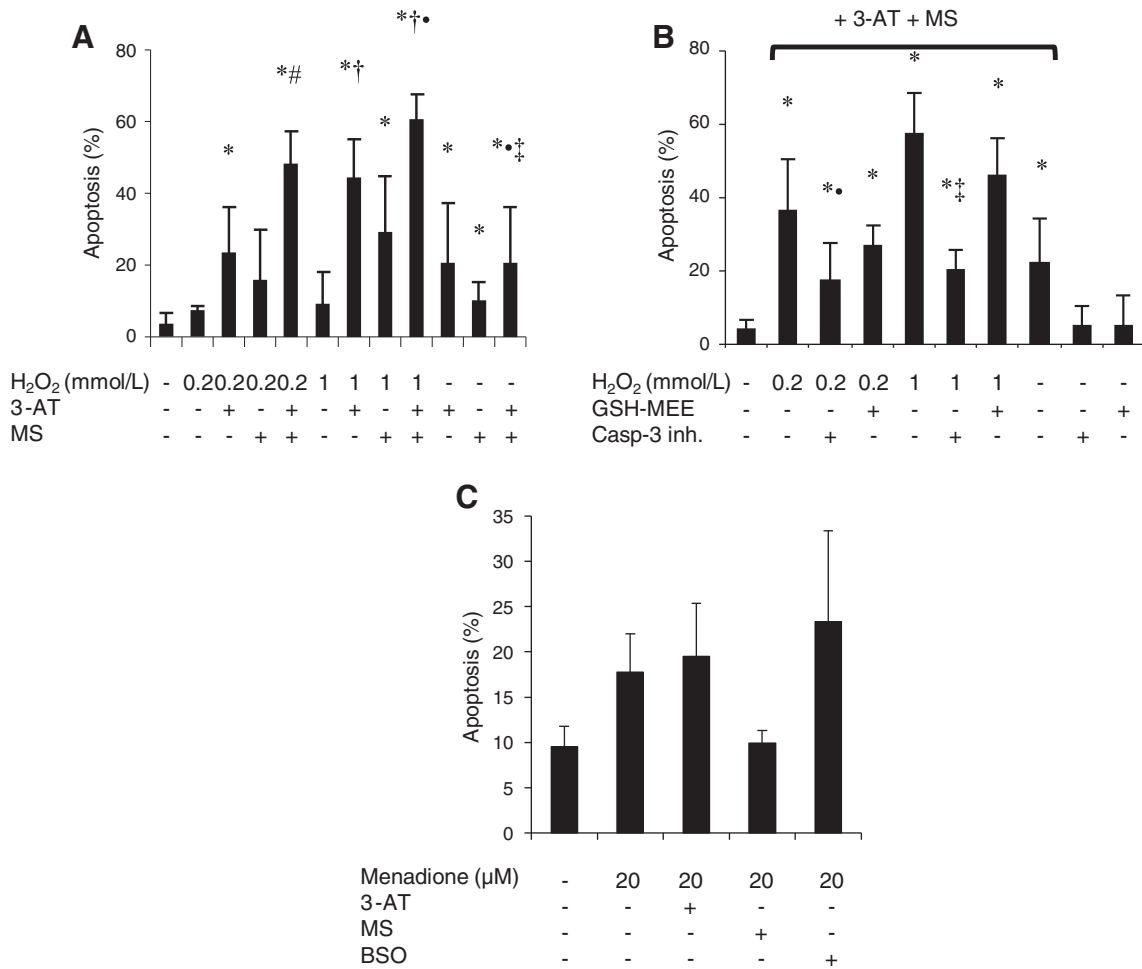


Fig. 6. Inhibition of glutathione peroxidase and/or catalase induces apoptosis in activated hepatic stellate cells. Fully-activated HSCs were exposed for 6 h to H₂O₂ in the presence or absence of the catalase inhibitor 3-AT and/or GPx inhibitor MS (A), as well as in the presence or absence of GSH-MEE or the caspase-3 inhibitor Z-DEVD-FMK (B) and analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. 3-AT and MS treatment alone significantly increased the number of apoptotic HSC, which was further enhanced by cotreatment with H₂O₂ (A). Apoptosis induced by treatment with H₂O₂ in the presence of inhibitors of GPx and catalase is inhibited by a blocker of caspase-3 activity, but not by supplementing glutathione through GSH-MEE (B). Results are shown as mean \pm stdev of at least four independent experiments. *Significant difference compared to control, $p < 0.05$; ^Significant difference compared to MS, $p < 0.05$; §Significant difference compared to 3AT, $p < 0.05$; †Significant difference compared to 1 mM hydrogen peroxide, $p < 0.05$; *Significant difference compared to MS + 3AT, $p < 0.05$; #Significant difference compared to 0.2 mM hydrogen peroxide, $p < 0.05$; *Significant difference compared to 0.2 mM hydrogen peroxide + 3-AT + MS, $p < 0.05$; ‡Significant difference from 0.2 mmol/L hydrogen peroxide + 3-AT + MS, $p < 0.05$; *Significant difference from 1 mmol/L hydrogen peroxide + 3-AT + MS, $p < 0.05$. (C). Fully-activated HSCs were exposed for 9 h to 20 μ M menadione in the presence or absence of the catalase inhibitor 3-AT, the GPx inhibitor MS, as well as the glutathione depleting agent BSO analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. Menadione induced apoptotic cell death at 20 μ M. 3-AT and MS did not significantly modulate apoptotic cell death by menadione. Moreover, depletion of glutathione using BSO did not aggravate menadione-induced apoptotic cell death. Results are shown as mean \pm st. dev. of at least four independent experiments.

a result of combined inhibition of glutathione peroxidase and catalase was shown to be independent of glutathione content, but partially dependent on caspase-3 activity. The superoxide anion donor menadione dose-dependently induces apoptotic cell death in activated HSCs [19]. Inhibition of hydrogen peroxide detoxifying enzymes did not significantly modulate menadione-induced cell death, indicating that increased superoxide generation did not lead to a massive, superoxide dismutase-mediated, increase in hydrogen peroxide.

The observed difference in mode of cell death, necrosis after glutathione depletion and apoptosis after inhibition of hydrogen peroxide detoxifying enzymes, may be explained by the cellular redox state. Glutathione is the most important regulator of the cellular redox state [41,42]. Changes in the glutathione content will affect redox status and is known to influence activation of MAP-kinases, transcription factors and caspases [41–43]. In the presence of glutathione, caspases that require reduced cysteine-sulphydryl groups in their catalytic site, can still be activated when the enzymes GPx and catalase are inhibited, because this inhibition is not likely to change the redox state of the cell.

In contrast, in the absence of glutathione, e.g. after glutathione depletion, cells exposed to hydrogen peroxide cannot activate caspases and the apoptotic program and cell death is shifted towards necrosis. Such a shift from apoptotic to necrotic cell death has been reported before in hepatocytes exposed to superoxide anions [44].

In summary, our study reveals important changes in the defense against oxidative stress of hepatic stellate cells during activation. These changes are characterized by increased cellular glutathione content and GPx1 mRNA expression. Furthermore, we demonstrate that both glutathione and the hydrogen peroxide-converting enzymes GPx and catalase are important in the resistance against hydrogen peroxide-induced cell death. Our data suggests that activated hepatic stellate cells *in vivo* may acquire increased resistance to necrotic cell death, while remaining sensitive to apoptosis, providing an explanation for their survival in the fibrotic liver and their apoptotic clearance during reversal of fibrosis.

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References

- [1] V.B. Djordjevic, Free radicals in cell biology, *Int. Rev. Cytol.* 237 (2004) 57–89.
- [2] M. Inoue, E.F. Sato, M. Nishikawa, A.M. Park, Y. Kira, I. Imada, K. Utsumi, Mitochondrial generation of reactive oxygen species and its role in aerobic life, *Curr. Med. Chem.* 10 (2003) 2495–2505.
- [3] D. Wu, A.I. Cederbaum, Alcohol, oxidative stress, and free radical damage, *Alcohol Res. Health.* 27 (2003) 277–284.
- [4] S.L. Friedman, Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver, *Physiol. Rev.* 88 (2008) 125–172.
- [5] A.Y. Hui, S.L. Friedman, Molecular basis of hepatic fibrosis, *Expert Rev. Mol. Med.* 5 (2003) 1–23.
- [6] H. Tsukamoto, R. Rippe, O. Niemela, M. Lin, Roles of oxidative stress in activation of Kupffer and Ito cells in liver fibrogenesis, *J. Gastroenterol. Hepatol.* 10 (Suppl 1) (1995) S50–S53.
- [7] K.S. Lee, S.J. Lee, H.J. Park, J.P. Chung, K.H. Han, C.Y. Chon, S.I. Lee, Y.M. Moon, Oxidative stress effect on the activation of hepatic stellate cells, *Yonsei Med. J.* 42 (2001) 1–8.
- [8] K.S. Lee, M. Buck, K. Houghlum, M. Chojkier, Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myc expression, *J. Clin. Invest.* 96 (1995) 2461–2468.
- [9] G. Svegliati Baroni, L. D'Ambrosio, G. Ferretti, A. Casini, A. Di Sario, R. Salzano, F. Ridolfi, S. Saccomanno, A.M. Jezequel, A. Benedetti, Fibrogenic effect of oxidative stress on rat hepatic stellate cells, *Hepatology* 27 (1998) 720–726.
- [10] N. Nieto, S.L. Friedman, A.I. Cederbaum, Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species, *Hepatology* 35 (2002) 62–73.
- [11] E. Zamara, E. Novo, F. Marra, A. Gentilini, R.G. Romanelli, A. Caligiuri, G. Robino, E. Tamagno, M. Aragno, O. Danni, R. Autelli, S. Colombatto, M.U. Dianzani, M. Pinzani, M. Parola, 4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells, *J. Hepatol.* 40 (2004) 60–68.
- [12] A. Galli, G. Svegliati-Baroni, E. Ceni, S. Milani, F. Ridolfi, R. Salzano, M. Tarocchi, C. Grappone, G. Pellegrini, A. Benedetti, C. Surrenti, A. Casini, Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism, *Hepatology* 41 (2005) 1074–1084.
- [13] E.L.M. Guimarães, M.F.S. Franceschi, I. Grivicich, F. Dal-Pizzol, J.C.F. Moreira, R.M. Guaragna, R. Borojevic, R. Margis, F.C. Guma, Relationship between oxidative stress levels and activation state on a hepatic stellate cell line, *Liver International.* 26 (2006) 477–485.
- [14] E. Novo, F. Marra, E. Zamara, L. Valfre di Bonzo, A. Caligiuri, S. Cannito, C. Antonaci, S. Colombatto, M. Pinzani, M. Parola, Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells, *Gut.* 55 (2006) 90–97.
- [15] K.Y. Kim, I. Choi, S.S. Kim, Progression of hepatic stellate cell activation is associated with the level of oxidative stress rather than cytokines during CCl4-induced fibrogenesis, *Mol. Cells.* 10 (2000) 289–300.
- [16] P. Greenwel, J.A. Dominguez-Rosales, G. Mavi, A.M. Rivas-Estilla, M. Rojkind, Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells, *Hepatology* 31 (2000) 109–116.
- [17] H. Moshage, A. Casini, C.S. Lieber, Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes, *Hepatology* 12 (1990) 511–518.
- [18] O.W. Griffith, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine, *Anal. Biochem.* 106 (1980) 207–212.
- [19] S. Dunning, R.A. Hannivoort, J.F. de Boer, M. Buist-Homan, K.N. Faber, H. Moshage, Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death, *Liver Int.* 29 (2009) 922–932.
- [20] A. Pellicoro, F.A. van den Heuvel, M. Geuken, H. Moshage, P.L. Jansen, K.N. Faber, Human and rat bile acid-CoA:amino acid N-acyltransferase are liver-specific peroxisomal enzymes: implications for intracellular bile salt transport, *Hepatology* 45 (2007) 340–348.
- [21] J.J. Maher, J.M. Saito, B.A. Neuschwander-Tetri, Glutathione regulation in rat hepatic stellate cells - Comparative studies in primary culture and in liver injury in vivo, *Biochem. Pharmacol.* 53 (1997) 637–641.
- [22] G.F. Seelig, R.P. Simonsen, A. Meister, Reversible dissociation of gamma-glutamylcysteine synthetase into two subunits, *J. Biol. Chem.* 259 (1984) 9345–9347.
- [23] R.A. Hannivoort, S. Dunning, S. Vander Borgh, B. Schroyen, J. Woudenberg, F. Oakley, M. Buist-Homan, F.A. van den Heuvel, M. Geuken, A. Geerts, T. Roskams, K.N. Faber, H. Moshage, Multidrug resistance-associated proteins are crucial for the viability of activated rat hepatic stellate cells, *Hepatology* 48 (2008) 624–634.
- [24] H. Jaeschke, Y.S. Ho, M.A. Fisher, J.A. Lawson, A. Farhood, Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress, *Hepatology* 29 (1999) 443–450.
- [25] X.G. Lei, Glutathione peroxidase-1 gene knockout on body antioxidant defense in mice, *Biofactors* 14 (2001) 93–99.
- [26] T. Yoshida, M. Watanabe, D.T. Engelman, R.M. Engelman, J.A. Schley, N. Maulik, Y.S. Ho, T.D. Oberley, D.K. Das, Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury, *J. Mol. Cell. Cardiol.* 28 (1996) 1759–1767.
- [27] P.J. De Bleser, G.X. Xu, K. Rombouts, V. Rogiers, A. Geerts, Glutathione levels discriminate between oxidative stress and transforming growth factor-beta signaling in activated rat hepatic stellate cells, *J. Biol. Chem.* 274 (1999) 33881–33887.
- [28] Y. Li, T.T. Huang, E.J. Carlson, S. Melov, P.C. Ursell, J.L. Olson, L.J. Noble, M.P. Yoshimura, C. Berger, P.H. Chan, D.L. Wallace, C.J. Epstein, Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase, *Nat. Genet.* 11 (1995) 376–381.
- [29] R.M. Lebovitz, H. Zhang, H. Vogel, J. Cartwright Jr, L. Dionne, N. Lu, S. Huang, M.M. Matzuk, Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice, *Proc. Natl. Acad. Sci. U.S.A.* 93 (1996) 9782–9787.
- [30] H. Van Remmen, C. Salvador, H. Yang, T.T. Huang, C.J. Epstein, A. Richardson, Characterization of the antioxidant status of the heterozygous manganese superoxide dismutase knockout mouse, *Arch. Biochem. Biophys.* 363 (1999) 91–97.
- [31] H. She, S. Xiong, S. Hazra, H. Tsukamoto, Adipogenic transcriptional regulation of hepatic stellate cells, *J. Biol. Chem.* 280 (2005) 4959–4967.
- [32] G. Ding, M. Fu, Q. Qin, W. Lewis, H.W. Kim, T. Fukai, M. Bacanamwo, Y.E. Chen, M.D. Schneider, D.J. Mangelsdorf, R.M. Evans, Q. Yang, Cardiac peroxisome proliferator-activated receptor gamma is essential in protecting cardiomyocytes from oxidative damage, *Cardiovasc. Res.* 76 (2007) 269–279.
- [33] X. Yu, X.G. Shao, H. Sun, Y.N. Li, J. Yang, Y.C. Deng, Y.G. Huang, Activation of cerebral peroxisome proliferator-activated receptors gamma exerts neuroprotection by inhibiting oxidative stress following pilocarpine-induced status epilepticus, *Brain Res.* 1200 (2008) 146–158.
- [34] W. Verreth, D. De Keyser, P.C. Davey, B. Geeraert, A. Mertens, M.C. Herregods, G. Smith, F. Desjardins, J.L. Balligand, P. Holvoet, Rosuvastatin restores superoxide dismutase expression and inhibits accumulation of oxidized LDL in the aortic arch of obese dyslipidemic mice, *Br. J. Pharmacol.* 151 (2007) 347–355.
- [35] J.J. Maher, B.A. Neuschwander-Tetri, Manipulation of glutathione stores in rat hepatic stellate cells does not alter collagen synthesis, *Hepatology* 26 (1997) 618–623.
- [36] L. Tian, M.M. Shi, H.J. Forman, Increased Transcription of the Regulatory Subunit of gamma-Glutamylcysteine Synthetase in Rat Lung Epithelial L2 Cells Exposed to Oxidative Stress or Glutathione Depletion, *Arch. Biochem. Biophys.* 342 (1997) 126–133.
- [37] Y. Urata, H. Yamamoto, S. Goto, H. Tsushima, S. Akazawa, S. Yamashita, S. Nagataki, T. Kondo, Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1beta and tumor necrosis factor-alpha in mouse endothelial cells, *J. Biol. Chem.* 271 (1996) 15146–15152.
- [38] N. Kiyosawa, K. Ito, K. Sakuma, N. Niino, M. Kanbori, T. Yamoto, S. Manabe, N. Matsunuma, Evaluation of glutathione deficiency in rat livers by microarray analysis, *Biochem. Pharmacol.* 68 (2004) 1465–1475.
- [39] L. Li, B. Julien, P. Grenard, F. Teixeira-Clerc, A. Mallat, S. Lotersztajn, Molecular mechanisms regulating the antifibrogenic protein heme-oxygenase-1 in human hepatic myofibroblasts, *J. Hepatol.* 41 (2004) 407–413.
- [40] L. Li, P. Grenard, J.T. Nhieu, B. Julien, A. Mallat, A. Habib, S. Lotersztajn, Heme oxygenase-1 is an antifibrogenic protein in human hepatic myofibroblasts, *Gastroenterology* 125 (2003) 460–469.
- [41] D. Han, N. Hanawa, B. Saberi, N. Kaplowitz, Mechanisms of liver injury. III. Role of glutathione redox status in liver injury, *Am. J. Physiol. Gastrointest. Liver Physiol.* 291 (2006) G1–G7.
- [42] L. Cesaratto, C. Vascotto, S. Calligaris, G. Tell, The importance of redox state in liver damage, *Ann. Hepatol.* 3 (2004) 86–92.
- [43] C. Garcia-Ruiz, J.C. Fernandez-Checa, Redox regulation of hepatocyte apoptosis, *J. Gastroenterol. Hepatol.* 22 (Suppl 1) (2007) S38–S42.
- [44] L. Conde de la Rosa, M.H. Schoemaker, T.E. Vrenken, M. Buist-Homan, R. Havinga, P.L. Jansen, H. Moshage, Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases, *J. Hepatol.* 44 (2006) 918–929.