Digestive Diseases

Dig Dis 2010;28:792–798
DOI: 10.1159/000324287

Protective Role of HO-1 for Alcohol-Dependent Liver Damage

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Key Words
HO-1 · Hepatocytes · Quercetin · Oxidative stress

Abstract
Background/Aims: Alcoholic liver disease is continuously increasing in developed countries being a leading cause of death worldwide. Chronic ethanol consumption induces oxidative stress by accumulation of reactive oxygen intermediates (ROI) while reducing the cellular antioxidant defense. Induction of heme oxygenase-1 (HO-1) may protect primary human hepatocytes (hHeps) from such damage. Thus, the aim of this study was to investigate the potential of polyphenols to protect hHeps from ethanol-dependent oxidative damage. ***Methods:*** hHeps were isolated by collagenase perfusion. ROI and cellular glutathione (GSH) were measured by fluorescent-based assays. Cellular damage was determined by lactate dehydrogenase (LDH) leakage and staining for apoptosis and necrosis. Nuclear translocation of Nrf2 and HO-1 expression were analyzed by Western blot. ***Results:*** Ethanol and TGF-β rapidly increase ROI and reduce GSH in hHeps, causing apoptosis with a release of approximately 40% total LDH after 72 h. Similar to incubation with hemin preincubation and co-incubation of cells with nifedipine, verapamil and quercetin significantly reduce oxidative stress and resulting cellular damage, in a dose-dependent manner, by initiating nuclear translocation of Nrf2 which in turn induces HO-1 under the control of p38 and ERK. Blocking of HO-1 activity with ZNPP9 reverses the protective effect of all three substances. ***Conclusion:*** Our results suggest that increasing HO-1 activity in hHeps protects them from oxidative stress-dependent damage. As polyphenols have great potential to induce HO-1 expression, they may play an important role for future therapeutic strategies to protect liver from oxidative stress-dependent damage observed during chronic alcohol consumption.

Introduction

The liver is probably the organ with the best regenerative capacity in the human body. However, repeated damage, e.g. from alcohol abuse, causes liver cells to produce excessive extracellular matrix (ECM) which eventually prevents regeneration. Alcoholic liver disease (ALD) progresses from steatohepatitis to clinical manifestations like fibrosis, cirrhosis and even hepatocellular carcinoma. Acute and chronic ethanol consumption increases the production of reactive oxygen intermediates (ROI),

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e.g. hydroxyl ethyl free radical, hydroxyl radical and superoxide anion. The produced ROI can react with and in turn damage complex cellular molecules, e.g. lipids, proteins or DNA. ROI are produced during metabolism of ethanol by alcohol dehydrogenase (ADH1), catalase and more selectively through the microsomal ethanol oxidative system (MEOS) with its main component cytochrome P450 2E1 (CYP2E1) [1, 2].

It is generally accepted that lipid peroxidation, altered immune responses and antioxidant defenses play an important role in the pathogenesis of ethanol-induced cellular injury, as patients suffering from ALD show enhanced lipid peroxidation, protein carbonyl formation, production of the reactive products acetaldehyde [3] and 1-hydroxyl ethyl radical as well as lipid radicals. This goes along with mobilization of iron [4], induction of CYP2E1 [5] and reduction of antioxidant enzymes and chemicals, particularly mitochondrial and cytosolic glutathione (GSH) [6], representing a dysbalance between the cells' ROI production and its own cellular defense mechanisms, consisting of various enzymatic and non-enzymatic mechanisms. The elevated 'oxidative stress' in combination with changes in redox state and hypoxia then further damages mitochondria and cell membranes [7]. Furthermore, ethanol affects the immune system by altering production of cytokines [8]. This is of particular importance as human hepatocytes (hHeps) exposed to ethanol are more sensitive towards damage from the pro-inflammatory cytokine TGF-β [9]. The resulting signal transduction in liver cells is critically required for progression of liver disease. TGF-β further enhances the 'oxidative stress' on hHeps by upregulating NADPH oxidases [10] while lowering GSH.

We expect antioxidant supplementation to be a promising strategy to prevent alcohol-dependant redox disequilibrium and oxidative damage. Polyphenolic substances have strong free radical scavenging properties due to their structure. Quercetin for example, one of the most common naturally occurring polyphenols present not only in vegetables and fruit but also to a great extent in red wine, is even proposed to lower the mortality of coronary heart disease due to regular consumption of red wine ('French paradox'), although the detailed mechanisms are not yet fully understood [11–13]. The protective effect of quercetin cannot be explained by only direct hydrogen-donating properties to quench ROI. Just recently we focused on the influence of flavonoids on signaling pathways and its interaction with the endogenous antioxidative defense system [14–16]. In this system, heme oxygenase-1 (HO-1) is of particular interest, as it is finely upregulated under 'oxidative stress' and helps to protect the liver against damage from several chemical compounds such as acetaminophen, carbon tetrachloride and heavy metals [17]. There is increasing evidence that HO-1 induction represents an adaptive response or enhanced resistance against various oxidative stresses. Recently we have shown that HO-1 induction by cobalt protoporphyrin (CoPP) protects hHeps from ethanol-induced cytotoxicity [18]. However, most inducers of HO-1, including CoPP and hemin, show toxic side effects. Polyphenols have been reported to upregulate HO-1 expression by activating nuclear factor erythroid-2-related factor (Nrf2) to bind with the antioxidant response element (ARE) in the *ho-1* gene promoter region.

As quercetin has been shown to positively influence heart diseases, we wanted to investigate the effect of the two structurally related antihypertensives, nifedipine and verapamil, on ethanol- or TGF-β-induced damage of hHeps. More specifically, the aim of this study was to investigate whether nifedipine and verapamil can, similarly to quercetin, actively induce HO-1 expression via Nrf2 activation and thus reduce oxidative stress and its resulting cellular damage in ethanol- or TGF-β-treated hHeps.

Materials and Methods

Human recombinant active TGF-β_1 was from PeproTech (London, UK), cell culture medium and supplements were from PAA Laboratories (Cölbe, Germany), and chemicals were obtained from Sigma (Munich, Germany).

Isolation of Primary Human Hepatocytes

Human liver tissue was obtained from liver resections of tumor patients with primary or secondary liver tumors according to the institutional guidelines (Technische Universität München, MRI, Munich, Germany). Written informed consent was obtained from all patients. hHeps were isolated by a two-step collagenase perfusion technique followed by Percoll gradient centrifugation [19]. Hepatocyte viability was consistently above 90% as assessed by trypan blue exclusion method. hHeps were cultured on collagen-coated culture plates in Williams' medium E (10% FCS, 1 mM insulin, 15 mM Hepes, 0.8 μg/ml hydrocortisone, 100 U/ml penicillin, 100 μg/ml streptomycin). Prior to all experiments, cells were serum starved ON.

Measurement of ROI and GSH

Cells stimulated according to the experimental setup were washed three times with PBS. Cells were incubated for 30 min at 37°C with 10 μM DCFH-DA (ROI) or 10 μM monochlorobimane (GSH) in serum-free medium. Afterwards, cells are washed three times with PBS. Immediately afterwards, cells were lysed with 0.1% Triton X-100 (in PBS) and fluorescence was determined at ex/em = 485/527 nm and ex/em = 355/360 nm, respectively.

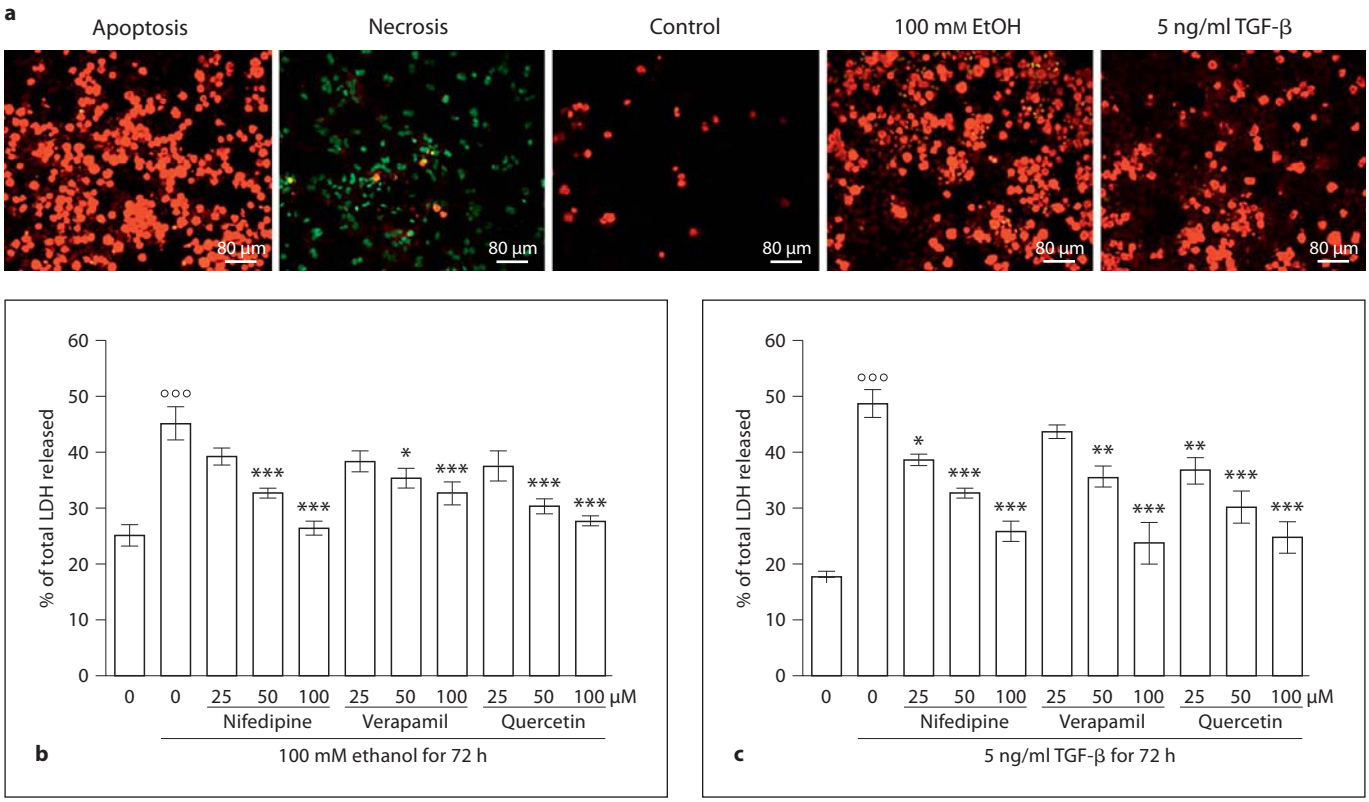


Fig. 1. Polyphenols reduce ethanol- and TGF-β-dependent LDH release in hHeps. **a** hHeps stained with Annexin-V-Cy3 (red/cell membranes) as indicator for apoptosis and Sytox Green (green/nucleus) as indicator for necrosis. Nuclear counterstain with Hoechst 33342 (blue). Ethanol- and TGF-β-dependent cellular damage is mainly caused by apoptosis. **b** hHeps treated for 72 h with 100 mM ethanol release about 45% of total LDH into the culture supernatant. **c** hHeps treated for 72 h with 5 ng/ml TGF-β release almost 50% of total LDH into the culture medium. Nifedipine, verapamil and quercetin reduce both ethanol and TGF-β-dependent LDH leakage in a dose-dependent manner. °°° $p < 0.001$ compared to untreated cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared to ethanol or TGF-β-treated cells, respectively.

Toxicity Assay

Damage of cells was determined by measuring lactate dehydrogenase (LDH) release into the culture supernatant. LDH activity was determined using the LDH test kit (HiTaDo Diagnostic Systems, Möhnesee, Germany) according to the manufacturer's instructions.

Apoptosis and Necrosis Staining

Cells were fixed to culture plastic with 4% paraformaldehyde (in PBS, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$). Cells were covered with PBS (2 mM $CaCl_2$) containing 5 nM Sytox Green and 0.25 µg/ml Annexin-V-Cy3. After washing, cells were analyzed by fluorescence microscopy.

Western Blot

Extraction of cytosolic and nuclear protein fractions was based on published protocols [20]. 25 µg of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Hybridization and detection were performed as reported [16, 18].

Statistics

Results are expressed as whiskers graphs showing median, 25- and 75-percentile and extreme values. Datasets were compared by Kruskal-Wallis followed by Dunn's multiple comparison test. $p < 0.05$ was taken as minimum level of significance.

Results

Ethanol and TGF-β Damage hHeps by Rapidly Inducing Oxidative Stress

Treatment of hHeps with ethanol or TGF-β for 72 h or longer causes a significant release of LDH into the culture supernatant, representing cellular damage. This cellular damage increases with an increasing concentration of substances. For example, 100 mM ethanol or 5 ng/ml TGF-β cause approximately 35–45% of total LDH to be released into the culture medium. Annexin-V staining

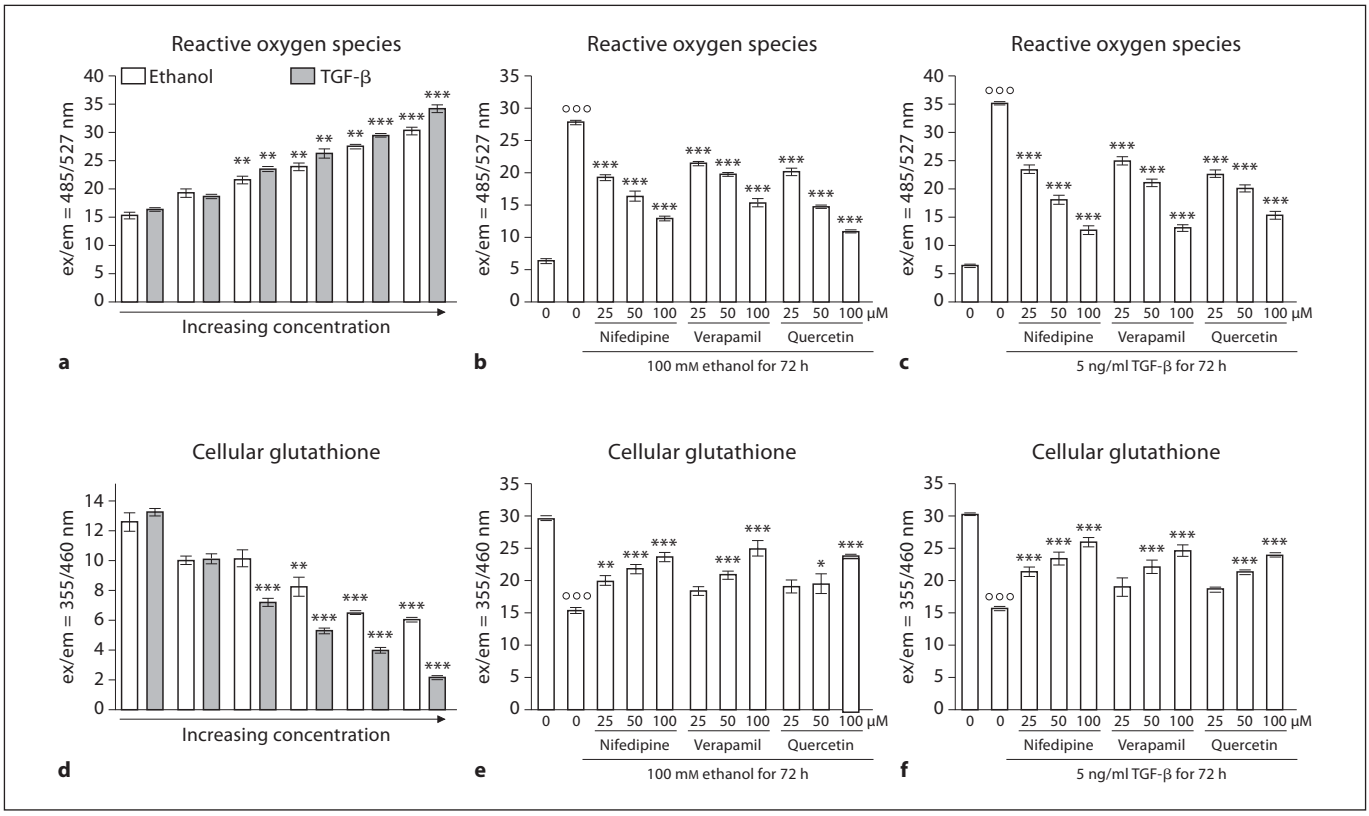
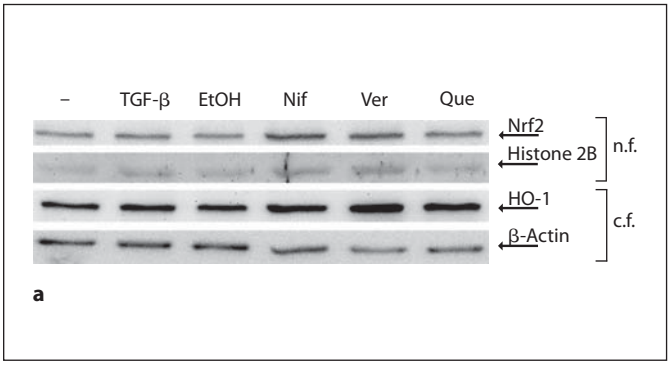


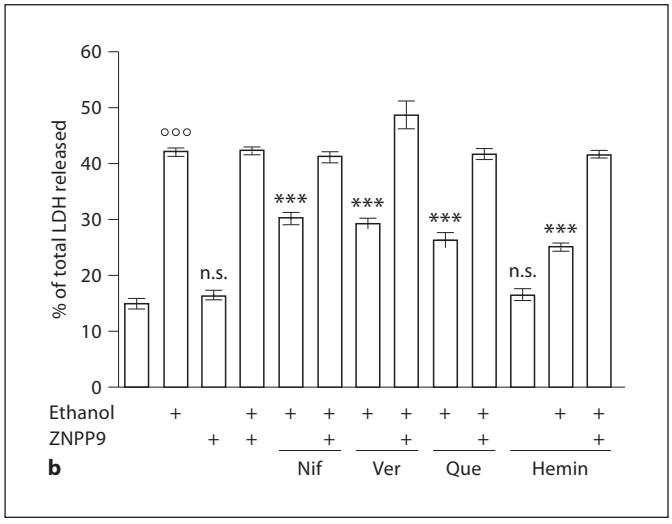
Fig. 2. Ethanol- and TGF-β-induced oxidative stress can be buffered by polyphenols. hHeps treated for 4 h with ethanol (white bars; 0, 12.5, 25, 50, 100, 200 mM) or TGF-β (shaded bars; 0, 0.1, 0.5, 1, 5, 10 ng/ml) were assessed for ROI production (**a**) and cellular GSH (**b**). Both substances increase ROI while decreasing GSH levels dose-dependently. The polyphenols nifedipine, verapamil and quercetin decrease ROI accumulation (**b**, **c**) while increasing cellular GSH (**e**, **f**) in cells treated for 4 h with 100 mM ethanol (**b**, **e**) or 5 ng/ml TGF-β (**c**, **f**). °°° $p < 0.001$ compared to untreated cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared to ethanol or TGF-β-treated cells.

identified the majority of the cells affected to be apoptotic (fig. 1a). Co-incubation with the polyphenols nifedipine (EC_{50} ~1.29 mM), verapamil (EC_{50} ~335.8 µM) or quercetin (EC_{50} ~238.5 µM) significantly reduced cellular damage from ethanol and TGF-β in a dose-dependent manner (fig. 1b, c). Ethanol and TGF-β have in common that they rapidly induce oxidative stress in hHeps by increasing production of ROI (fig. 2a) and reducing cellular GSH (fig. 2d) dose-dependently. The increase in ROI as well as the drop in cellular GSH peaks between 4 and 8 h, and recovers to normality after 24 h (data not shown), thus we repeated stimulation of the cells with ethanol and TGF-β every 24 h.

Polyphenols Buffer Ethanol and TGF-β-Induced Oxidative Stress in hHeps by Upregulating HO-1

Polyphenols significantly reduce oxidative stress in hHeps dose-dependently (fig. 2b, c). Reversely, cellular GSH levels are less reduced in the presence of the polyphenols (fig. 2e, f). This effect is even more pronounced when hHeps were preincubated with these polyphenols before application of ethanol or TGF-β (data not shown). This indicates that these substances not only have direct free radical scavenging properties but also regulate the endogenous antioxidative defense system. Thus, we investigated translocation of Nrf2 into the nucleus and expression of HO-1 in hHeps treated with ethanol, TGF-β, nifedipine, verapamil or quercetin for 24 h. HO-1 mRNA levels were slightly increased in hHeps treated with 100 mM ethanol or 5 ng/ml TGF-β. HO-1 levels further increased when hHeps were treated with nifedipine, verapamil or quercetin. In those samples the amount of Nrf2 in the nuclear fraction was also increased (fig. 3a). To investigate if the protective effect of our polyphenols is caused by increased HO-1 levels, we blocked HO-1 activity with ZNPP9. Induction of HO-1 with hemin had





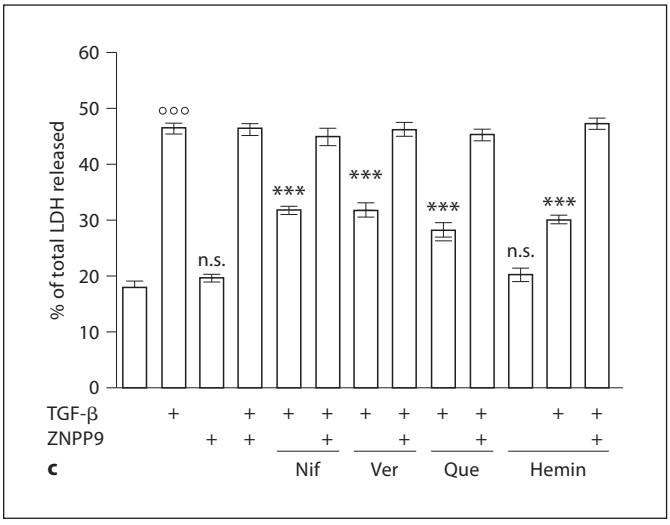


Fig. 3. Polyphenols protect hHeps from ethanol and TGF-β-induced damage by upregulating HO-1. **a** Western blot for nuclear accumulation of Nrf2 (histone 2B) in the nuclear fraction (n.f.) of untreated hHeps as well as hHeps treated with 100 mM ethanol, 5 ng/ml TGF-β, 100 μM nifedipine, 100 μM verapamil or 100 μM quercetin for 24 h. The expression of HO-1 and β-actin was investigated in the cytoplasmic fraction. Polyphenols clearly induce nuclear translocation of Nrf2 and subsequently induce HO-1 expression. **b**, **c** Blocking HO-1 activity by ZNPP9 reversed the protective effects of all three polyphenols on LDH release induced by treating hHeps with 100 mM ethanol (**b**) or 5 ng/ml TGF-β (**c**) for 72 h. °°° $p < 0.001$, n.s. = $p > 0.05$ compared to untreated cells. *** $p < 0.001$ compared to ethanol- or TGF-β-treated cells.

comparable effects as the observed polyphenols. However, inhibiting HO-1 activity by ZNPP9 supplementation completely reversed the protective effect of our three polyphenols (fig. 3b, c). Similar results were observed when inhibiting p38 (SB203580) and ERK (PD98059) phosphorylation/signaling (data not shown).

Discussion

As patients suffering from ALD show enhanced levels of lipid peroxidation, protein carbonyl formation and production of reactive products [3], it is widely accepted that oxidative stress and altered immune responses play an important role in the pathogenesis of ethanol-induced cellular injury. Oxidative stress is represented by a dysbalance between production of ROI and cellular antioxidant defenses.

Ethanol consumption rapidly increases production of ROI during its metabolism by ADH1, catalase and CYP2E1 [1, 2]. Ethanol results in downregulation of ADH1 expression which in turn leads to excessive accumulation of lipids in hHeps [9]. CYP2E1 expression is increased by ethanol producing further reactive products in the MEOS [5]. Increased formation of ROI in combination with reduction of antioxidant enzymes and chemicals, particularly cellular GSH [6], causes damage to the hepatocytes. At the same time, ethanol affects the immune system by altering production of cytokines [8]. Thus, infiltrating macrophages secrete pro-inflammatory cytokines, e.g. TGF-β_1 whose signal transduction in liver cells is critically required for progression of liver disease. TGF-β_1 activates the resident hepatic stellate cells and Kupffer cells. Besides an excessive ECM production, those cells produce even more TGF-β_1 resulting in a vicious cycle of autostimulation. In hepatocytes, TGF-β_1 stimulates ROI production by upregulating NADPH oxidases (NOX) [10]. It also reduces expression of ADH1 which favors accumulation of lipids. Interestingly, hHeps exposed to ethanol are more sensitive towards damage

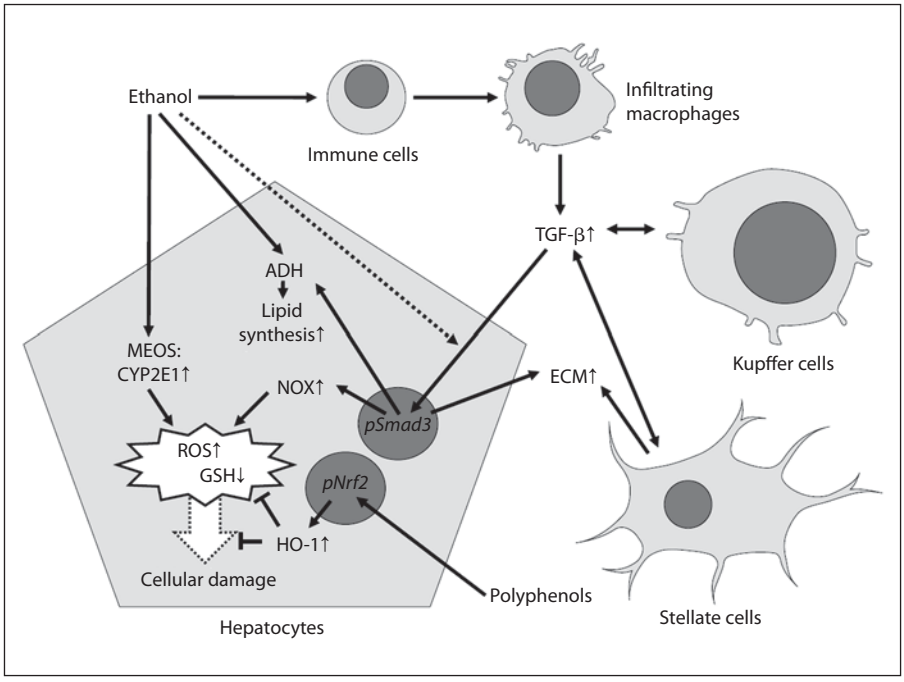


Fig. 4. Schematic overview on cellular responses on ethanol in the liver. Ethanol induces expression of CYP2E1. This leads to accumulation of ROI and reduction of GSH, inducing cellular damage. At the same time, ethanol reduces ADH1 expression which in turn favors accumulation of neutral lipids. The resulting changes in the liver cause immune cells to change their cytokine expression favoring inflammation, e.g. increased expression of TGF-β_1. TGF-β_1 then activates hepatic stellate cells and Kupffer cells to produce ECM and even more TGF-β. In the hepatocytes, TGF-β_1 further increases oxidative stress by upregulating NOX further increasing cellular damage. Interestingly, ethanol enhances all TGF-β-dependent effects in hepatocytes.

from the pro-inflammatory cytokine TGF-β_1 [9] (fig. 4). We tested if medium supplementation with polyphenols nifedipine, verapamil and quercetin can protect hHeps from oxidative damage induced by stimulation with ethanol and TGF-β_1. All three substances were able to reduce cellular damage induced by ethanol and TGF-β_1 in a dose-dependent manner. The protective effect observed when polyphenols were added to the cells at the same time as ethanol and TGF-β_1 can be explained partly by the fact that polyphenolic substances have strong free radical scavenging properties due to their structure. However, cells pretreated with nifedipine, verapamil and quercetin showed even better outcome, suggesting that polyphenols can stimulate the cells' own antioxidant defense mechanisms. In this system, HO-1 is of particular interest as it is upregulated during 'oxidative stress' and helps to protect the liver against damage from several chemical compounds such as acetaminophen, carbon tetrachloride and heavy metals [17]. Just recently we have shown that HO-1 induction by CoPP protects hHeps from ethanol-induced cytotoxicity [18]. However, most inducers of HO-1, including CoPP and hemin, show toxic side effects in animals and human. Therefore, we investigated the influence of naturally occurring polyphenols (flavonoids), e.g. curcurmin and quercetin, on signaling pathways and its interaction with the endogenous antioxidative defense system [14–16]. We could show that upregulation of HO-1 expression is strongly dependent on phosphorylation and nuclear translocation of Nrf2 to bind with the ARE in the *ho-1* gene promoter region [18]. Our latest results even suggest p38 and ERK being involved in the regulation of Nrf2 activation (data not shown). However, these flavonoids can often not be taken up in sufficient amounts needed to protect the liver from ethanol-induced oxidative damage. Thus, substances should have a good bioavailability, be non-toxic and either reduce ROI formation by inhibiting CYP2E1 and NOX4 activity and/or increase the cellular oxidative defense mechanisms to allow future therapeutic strategies. For example, the naturally occurring CYP2E1 inhibitor diallyl sulfoxide (DAS from garlic) was reported to also induce HO-1 expression, however, concentrations needed to inhibit CYP2E1 activity are often toxic, especially in vivo [21]. As inhibiting CYP2E1 activity also affects the metabolism of other xenobiotics, focusing on reduction of oxidative stress in alcohol-induced tissue injury seems to be the most promising strategy. As basic information continues to emerge regarding the role of oxidative stress in disease development and the mechanisms underlying ROI-related cellular toxicity, these findings will lead to more rational antioxidant therapeutic approaches. Polyphenolic substances often used in the clinic include antihypertensive drugs. Thus, we investigated the effect of nifedipine and verapamil on ethanol- and TGF-β_1-in-

duced cellular damage. Both drugs were able to reduce oxidative stress and resulting cellular damage in hHeps effectively. The mechanism is mediated by increased expression of HO-1 which is Nrf2-dependent and regulated by MAP kinases p38 and ERK.

Summarizing, our results suggest that increasing HO-1 activity in liver cells protects them from ROI-dependent damage by increasing cellular GSH. Under these conditions, GSH favors formation of non-toxic products from ROI. Therefore, food supplementation with antioxidants, such as the polyphenol quercetin, supports liver function by interfering with increased ROI production observed during chronic alcohol consumption. As the antihypertensives nifedipine and verapamil showed similar effects, adjustment of medication might also favor the outcome of ALD of many patients, but for this purpose many drugs have been left to test for future therapeutic strategies.

Acknowledgement

This project was partially supported by the BMBF (01GG0732).

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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