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Protective role of HO-1 and carbon monoxide in ethanol-induced cell death in hepatocytes and liver injury in mice

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Abstract

Background and Aims—Alcoholic liver disease is associated with inflammation and cell death. Heme oxygenase-1 (HO-1) is a stress-inducible enzyme with anti-apoptotic and anti-inflammatory properties. Here we tested the hypothesis that induction of HO-1 or treatment with a carbon monoxide releasing molecule (CORM) during chronic ethanol exposure protects and/or reverses ethanol-induced liver injury.

Methods—Female C57BL/6J mice were allowed free access to a complete liquid diet containing ethanol or pair-fed control diets for 25 days. Mice were treated with cobalt protoporphyrin (CoPP) to induce HO-1 expression during ethanol feeding or once injury had been established. Mice were also treated with CORM-A1, a CO-releasing molecule (CORM), after ethanol-induced liver injury was established. The impact of HO-1 induction on ethanol-induced cell death was investigated in primary cultures of hepatocytes.

Results—Induction of HO-1 during or after ethanol feeding, as well as treatment with CORM-A1, ameliorated ethanol-induced increases in AST and expression of mRNA for inflammatory cytokines. Treatment with CoPP or CORM-A1 also reduced hepatocyte cell death, indicated by decreased accumulation of CK18 cleavage products and reduced RIP3 expression in hepatocytes. Exposure of primary hepatocyte cultures to ethanol increased their sensitivity to TNF α -induced cell death; this response was attenuated by necrostatin-1, an inhibitor of necroptosis, but not by caspase inhibitors. Induction of HO-1 with CoPP or CORM-3 treatment normalized the sensitivity of hepatocytes to TNF α -induced cell death after ethanol exposure.

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Conclusions—Therapeutic strategies to increase HO-1 and/or modulate CO availability ameliorated chronic ethanol-induced liver injury in mice, at least in part by decreasing hepatocellular death.

Alcoholic liver disease (ALD) develops in approximately 20% of all alcoholics [1]. The development of fibrosis and cirrhosis is a complex process involving both parenchymal and non-parenchymal cells resident in the liver, as well as the recruitment of other cell types to the liver in response to damage and inflammation [2]. Alcohol consumption results in hepatocyte injury and death, both via apoptotic and necroptotic pathways [1,3]. Hepatocellular injury and death occurs, at least in part, in response to ethanol metabolism and oxidative stress [1], as well as to the pro-inflammatory milieu in the liver resulting from activation of innate immune responses [2]. There is a recent appreciation that rationally-designed treatments for ALD need to be both hepato-protective and able to normalize the innate immune response.

Heme oxygenase-1 (HO-1) activity is a possible candidate to initiate both hepato-protective and anti-inflammatory mechanisms. HO-1 plays an important anti-inflammatory role in chronic inflammatory diseases and also has anti-apoptotic and anti-proliferative properties [4]. Heme oxygenases catalyze the initial and rate limiting step in the oxidative degradation of heme, yielding equimolar amounts of biliverdin IX α (BV), carbon monoxide (CO), and free iron [4]. HO-2 and HO-3 are constitutive forms, while HO-1 (also known as heat shock protein 32) is an inducible isozyme that is up-regulated by a broad spectrum of stressors [4]. Surprisingly, chronic ethanol exposure induces HO-1 expression in some, but not all, pre-clinical models [5], in part dependent on the animal's age [6,7]. However, pharmacological induction of HO-1 prevents ethanol-induced inflammation in intestine [8], as well as oxidative damage and apoptosis in cultured hepatocytes [9,10]. Further, HO-1-mediated pathways can be activated in Kupffer cells after chronic ethanol feeding to suppress TLR4-dependent signaling in primary cultures of Kupffer cells, as well as an *in vivo* mouse model of LPS challenge [5,11].

Given the protective effects of HO-1 in both hepatocytes and its anti-inflammatory impact on LPS-stimulated cytokine expression after chronic ethanol exposure, here we have tested the hypothesis that induction of HO-1 or treatment with carbon monoxide releasing molecules (CORMs) will both prevent and treat ethanol-induced liver injury in a pre-clinical mouse model. Indeed, the HO-1/CO axis was both protective and therapeutic for the early phases of ethanol-induced liver injury in mice. Importantly, HO-1/CO was effective at protecting hepatocytes from chronic ethanol-induced cell death via both apoptotic and necroptotic pathways.

Materials and Methods

Materials and biochemical methods

See Supplemental Methods for sources of materials and methodological details.

Chronic ethanol feeding to mice and treatment with CoPP and CORM-A1

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Eight to ten week old female mice were allowed free access to a Lieber-DeCarli liquid diet (no.710260) (Dyets, Bethlehem, PA) containing ethanol or isocalorically-substituted maltose dextrins, as previously described [12]. After adjusting to the control liquid diet for two days, the ethanol-fed group was given a liquid diet containing 1% v:v ethanol for two days (data reported as “d2”), 2% for two days (d4), 4% for 1 week (d11), 5% for 1 week (d18) and finally 6% (32% total kcal) for 1 week (d25).

Mice were placed on the following protocols. 1) *Prevention*: Mice were treated with 5 mg/kg CoPP or vehicle (saline) three times at 7 day intervals on d5, d12 and d19 of the ethanol feeding protocol. 2) *Treatment*: a) On d25, mice were treated with 5 mg/kg CoPP or vehicle (saline) via intraperitoneal injection and euthanized 24 h later or b) Mice were treated with 3mg/kg CORM-A1, the CO releasing molecule sodium boranocarbonate ($\text{Na}_2[\text{BH}_3\text{CO}_2]$) [13], or inactivated CORM-A1 (iCORM-A1) via intraperitoneal injection five times at 24 h intervals on days 21–25. CORM-A1 was prepared in sterile saline each day; each mouse received ~0.1 ml of the diluted drug, based on body weight or an equivalent volume of sterile saline. While CoPP has been associated with decreased food intake and body weight via the hypothalamus [14], treatment with CoPP or CORM-A1 during ethanol feeding had no effect on food intake or body weights (Table 1). Ethanol-induced increases in CYP2E1 expression were also not affected by treatments (Table 2).

Hepatocyte cultures and cytotoxicity assays

Hepatocytes were isolated from male Wistar rats [15]. Cytotoxicity was assessed in primary hepatocytes and AML12 cells by MTT assay and flow cytometric analysis, as detailed in Supplemental Materials.

Statistical analysis

All values are reported as means $\pm$ standard error of the mean (SEM), n=4 pair-fed and n=6 for ethanol-fed mice. Data were analyzed by general linear models procedure (SAS; Carey, NC). Data were log transformed, if needed, to obtain a normal distribution. Follow-up comparisons were made by least square means testing.

Results

Chronic ethanol feeding to mice as part of a complete liquid diet results in the development of early stages of steatosis and inflammatory cytokine expression. After 25 days on the ethanol diet, mice exhibit increased concentration of ALT (Table 1) and AST in the circulation (Figure 1A) and increased hepatic triglycerides (Figure 1B). These markers of injury are associated with increased expression of mRNA for TLR4, as well as cytokines (TNF α and IL-6) and chemokines (CXCL10) (Figure 1C). Accumulation of 4-hydroxynonenal adducts (Figure 1D), an indication of oxidative stress in the liver, as well as an increase in TUNEL staining (Figure 1E) are also evident in ethanol-fed mice compared to their pair-fed controls.

HO-1 is an anti-inflammatory and anti-apoptotic enzyme that is induced in response to a variety of stresses. However, expression of HO-1 in livers was not increased after chronic ethanol feeding either by immunohistochemistry (Figure 1F) or Western blotting (data not shown). Therefore, we hypothesized that treatment of mice with CoPP, an inducer of HO-1, might exert protective effects in liver of mice during ethanol feeding. HO-1 was induced in livers of both pair-fed and ethanol-fed mice after weekly challenge with CoPP (Figure 1F). While expression of HO-1 was dominant in non-parenchymal cells in response to CoPP, expression was also elevated in hepatocytes (Figure 1F). Importantly, induction of HO-1 during chronic ethanol feeding reduced ethanol-induced increases in circulating ALT (Table 1) and AST (Figure 1A), mRNA associated with inflammation (Figure 1C), reduced accumulation of 4-HNE (Figure 1D) and ameliorated induction of apoptosis in response to chronic ethanol (Figure 1E). The only markers of injury not protected by weekly CoPP was the ethanol-induced increase in hepatic triglycerides (Figure 1B) and induction of CYP2E1 (Table 2).

Since induction of HO-1 prevented the development of ethanol-induced liver injury, we next asked if CoPP-mediated induction of HO-1 could reverse already established liver injury. To test this hypothesis, mice were given a single injection with CoPP on d24 of ethanol feeding to induce HO-1 expression. CoPP rapidly induced HO-1 expression in liver of both ethanol- and pair-fed mice (Figure 2F) in both parenchymal and non-parenchymal cells (Figure 1F). Induction of HO-1 was associated with a decrease in chronic ethanol-induced AST, hepatic triglycerides and mRNA associated with inflammatory cytokine and chemokine expression (Figure 2 A, B, C), but did not decrease ALT (Table 1) or reduce expression of CYP2E1 (Table 2).

Accumulation of M30, a cleavage product of CK18, specifically identifies apoptotic hepatocytes. M30 accumulation was increased by chronic ethanol feeding and reduced by 24 h after CoPP treatment (Figure 2D). Chronic ethanol feeding also induced expression of RIP3, a mediator of necroptosis, compared to pair-fed mice (Figure 2E); a single treatment with CoPP rapidly ameliorated this response to ethanol (Figure 2E).

Both the enzymatic products of heme degradation by HO-1, biliverdin and CO, have potential protective effects in the liver against injury. Here we have investigated the effect of CO in reducing chronic ethanol-induced liver injury by treating mice with CORM-A1 during the last 5 days of chronic ethanol feeding. Treatment with CORM-A1 reduced the effects of ethanol on plasma ALT (Table 1) and AST (Figure 3A), but not triglycerides (Figure 3B) and had no effect on the induction of CYP2E1 by chronic ethanol (Table 2). These data are consistent with a recent report that treatment of mice with CORM-2 prevented release of liver enzymes in response to acute exposure to ethanol [16]. Treatment of mice with iCORM-A1 did not ameliorate the effects of ethanol feeding on markers of liver injury (Table 3).

Interestingly, CORM-A1 had differential effects on regulation of mRNA for inflammatory mediators, decreasing TNF α , IL6 and CXCL10 expression, but not TLR-4 (Figure 3C). Chronic ethanol feeding increased Ly6C⁺ cells, an indicator of infiltrating immune cells, in liver compared to pair-fed controls; treatment with CORM-A1 reduced this effect of ethanol

(Figure 3D). CORM-A1 also decreased the number of inflammatory foci seen on Hematoxylin and Eosin stained sections (Supplemental Figure 1). Accumulation of γ -ketoaldehydes-adducts, such as iso-levuglandins (iso[4]LGE₂)-adducts, is another indicator of oxidative stress [17]. γ -Ketoaldehydes can form protein adducts orders of magnitude faster than the well-known lipid peroxidation products, 4-hydroxynonenal or malondialdehyde, and thus provide a very sensitive “dosimeter” of oxidative stress. Chronic ethanol feeding increased the accumulation of iso[4]LGE₂ protein adducts; this response was ameliorated in mice treated with CORM-A1 (Figure 3E). CORM-A1 treatment also reduced M30 accumulation (Figure 3F) and attenuated the induction of RIP3 (Figure 3G).

Induction of HO-1 or treatment with CORM-A1 decreased hepatocellular death, reducing markers of both apoptosis and necroptosis. This protective effect could be a direct cytoprotective effect on hepatocytes and/or an indirect consequence of decreased inflammatory mediators in the liver [11]. In order to understand whether HO-1/CO could directly modulate hepatocyte cell death pathways in the context of ethanol, primary rat hepatocytes were cultured with 50 mM ethanol. Cytotoxicity, assessed by MTT assay, was increased and cells were sensitized to TNF α -induced cell death (Figure 4A) [18]. While treatment with the pan-caspase inhibitor, Z-VAD-FMK, protected control hepatocytes from TNF α -induced cytotoxicity, inhibition of caspases did not protect, and even enhanced, the sensitivity of primary hepatocytes to TNF α -induced cytotoxicity after ethanol exposure (Figure 4A). FACS analysis of propidium iodine and annexin V revealed that hepatocytes cultured in ethanol exhibited both necrotic and apoptotic characteristics (Figure 4B). These data suggest that ethanol sensitized hepatocytes to caspase-independent, TNF α -mediated programmed necrotic cell death. Consistent with this hypothesis, pre-treatment of hepatocytes with necrostatin-1, an inhibitor of RIP1 kinase, reduced TNF α -induced cell death to control values in hepatocytes exposed to ethanol (Figure 4C). Further, pull-down of FADD from control cells brought down very little RIP3; however, after culture with ethanol, the association between FADD and RIP3 increased (Figure 4D), characteristic of formation of necroptosomes [22]. Treatment with TNF α slightly increased FADD-RIP3 association in control cells, but did not further increase the interaction of FADD with RIP3 in ethanol-treated cells (Figure 4D).

If protection from ethanol-induced hepatocyte death *in vivo* (Figure 1 and 2) in response to CoPP was due, at least in part to a direct protective effect of HO-1 and/or CO on hepatocytes, then induction of HO-1 and/or treatment with CORM in hepatocyte cultures should protect hepatocytes from TNF α -induced cell death. Pre-treatment of the primary hepatocytes with CoPP-induced the expression of HO-1 (Figure 4E), but did not decrease expression of CYP2E1 (Supplemental Figure 2). Treatment of hepatocytes with CoPP or CORM-3 protected hepatocytes from TNF α -induced cell death (Figure 4F). Finally, AML12 cells, a murine hepatocyte cell line, overexpressing HO-1 were protected from ethanol-induced sensitization to TNF α -induced cytotoxicity (Figure 4G).

Discussion

Two hallmarks of alcoholic liver injury are increased inflammation and hepatocyte injury [1,2]. The development of treatment strategies for ALD must therefore address both these

critical mechanisms of injury. HO-1 has a potent anti-inflammatory and anti-apoptotic properties in a number of pathophysiological diseases [4]. In previous work, we have shown that induction of HO-1 ameliorates the ethanol-induced sensitization of hepatic macrophages to stimulation with LPS both in primary cultures of Kupffer cells and in an *in vivo* model of chronic ethanol exposure to mice [5,11]. Here we find that induction of HO-1 during ethanol feeding protected mice from hepatocyte injury and death, as well as reduced expression of inflammatory mediators in the liver. Further, we have identified a direct protective role for HO-1 in primary hepatocytes against ethanol-induced sensitization to TNF α -induced cell death. Importantly, induction of HO-1 or treatment with its enzymatic product CO reversed established liver injury, suggesting that HO-1/CO might be important therapeutic strategies for treatment of ALD. Taken together with previous studies, these data indicate that HO-1/CO have multiple protective effects against ethanol-induced liver injury, by serving anti-inflammatory functions as well as protecting hepatocytes from cell death.

Given the anti-inflammatory effects of HO-1 and CO in Kupffer cells [5,11], these protective and therapeutic effects of HO-1/CO against ethanol-induced liver injury are likely due, at least in part, to the reduction in inflammatory cytokine expression. However, HO-1 and CO may also have direct protective effects on hepatocytes against ethanol-induced hepatocellular injury. Ethanol exposure is associated with apoptotic hepatocyte death, as evidenced by the accumulation of CK18 cleavage products in the liver [2] (Figure 2 and 3). More recent studies have also identified an important role for necroptotic cell death in ethanol-induced liver injury, associated with an increase expression of RIP3 in the liver [3]. Treatment of mice with CoPP to induce HO-1 or treatment with CORM-A1 reduced hepatocellular cell death via both apoptosis and necroptosis, evidenced by decreased accumulation of CK18 cleavage product and reduced expression of RIP3 in the liver of ethanol-fed mice.

Here we have used primary cultures of hepatocytes to investigate the direct hepatocellular protective effects of HO-1 in response to challenge with ethanol. Exposure of primary hepatocytes to ethanol during culture sensitizes them to TNF α -induced cell death that is independent of caspase-8 [18,20] or pan-caspase inhibitors (Figure 4A). TNF α -induced cytotoxicity in the presence of pan-caspase inhibitors (Figure 4A) is typically defined as necroptotic cell death [23,24]. While there are no direct measures of necroptosis available, necrostatin-1, an inhibitor of RIP1 kinase activity that blocks necroptotic cell death, effectively prevented the sensitization of hepatocytes to cell death after ethanol exposure (Figure 4). Further, the association of RIP3 with FADD is another hallmark of the necroptotic pathway [22]. In control hepatocytes, very little RIP3 was associated with FADD, even after challenge with TNF α ; in contrast, RIP3 protein associated with FADD in hepatocytes after culture with ethanol (Figure 4). Induction of HO-1 or provision of CO protected primary hepatocytes from the ethanol-induced sensitization to TNF α -induced cell death.

Interestingly, pan-caspase inhibitors actually increased TNF α -induced cytotoxicity after ethanol exposure. This phenomenon has been observed in a number of cell lines [19,21]. While not completely understood, this sensitization to necroptotic death may be related to

the more damaging impact of necroptosis vs apoptosis; i.e. necroptotic cells release damaging molecules into the local environment that can actually enhance cell death [19].

Taken together, these data are consistent with the hypothesis that HO-1 protects from ethanol-induced liver injury by both reducing inflammatory cytokine expression and protecting hepatocytes from cell death. A similar dual protective function for HO-1 on hepatocytes has also been proposed in response to ischemia/reperfusion injury [25]. This protection is due, at least in part, to a suppression of TNF α -induced apoptotic signaling, evidenced by a reduction in the formation of death-inducing signaling complex (DISC) in hepatocytes [26], as well as a decrease in the expression of inflammatory cytokines by resident Kupffer cells [27]. The exact mechanisms for HO-1-mediated protection of hepatocytes from death are not known. HO-1 is also critical for hepatocyte proliferation; mice treated with CO exhibit a more robust proliferative response after partial hepatectomy [28]. HO-1 mediates the effects of several agents, including globular adiponectin and quercetin, in protecting HepG2 cells and rat hepatocytes from ethanol-induced cell death [9,29].

While both induction of HO-1 with CoPP or treatment of mice with CORM-A1 ameliorated the ethanol-induced increase in expression of inflammatory mediators, CoPP had a broader effect, acting to decrease TLR4 and MCP-1 expression, genes which were not affected by CORM-A1. The broader effects of CoPP are likely due to the generation of bilirubin/biliverdin during heme degradation. Indeed, biliverdin is a known regulator of TLR4 transcription [30].

Systemic provision of pharmaceutical agents makes it difficult to determine the precise site of therapeutic action. Making use of primary cultures, induction of HO-1 or the provision of CORM-A1 ameliorated the impact of ethanol in both Kupffer cells [5] [11] and hepatocytes (Figure 4), suggesting that these two cell types are key targets for the protective effects of HO-1/CO. However, it is also possible that additional cell types/organs contribute to the protective effects of HO-1/CO *in vivo*. The progression of ethanol-induced liver injury involves interactions between different cell types in the liver, as well as cross-talk with a number of other organs, including adipose tissue and the intestine. Treatment of mice with CoPP increases expression of adiponectin by adipocytes in obese diabetic mice [36]. Since adiponectin protects mice from ethanol-induced liver injury [37], the protective effects of CoPP may also be due, at least in part, to increased availability of adiponectin. Finally, since CO and HO-1 promote bacterial clearance in the colon [38], HO-1/CO may dampen the contribution of the gut-liver axis in the progression of ethanol-induced liver injury. Taken together, the multiple sites of protection afforded by the induction of HO-1 or provision of CO suggest that it is an important therapeutic target for the treatment of ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALD	Alcoholic liver disease
AST	aspartate amino transferase
CK18	cytokeratin 18
CO	carbon monoxide
CoPP	Cobalt protoporphoryin
CORM	CO-releasing molecule
CYP2E1	cytochrome P450 2E1
EtOH	ethanol
FADD	Fas-associated protein with death domain
HO-1	hemeoxygenase-1
iCORM-A1	inactive CORM-A1
IL-6	interleukin 6
iso[4]LGE₂	iso-levuglandins
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
PAGE	polyacrylamide electrophoresis
RIP1	Receptor interacting kinase 1
RIP3	Receptor interacting kinase 3
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor α

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Prevention - CoPP treatment during ethanol feeding

Figure 1. Induction of hemeoxygenase-1 (HO-1) with cobalt protoporphyrin (CoPP) treatment during chronic ethanol feeding to mice prevents the development of liver injury
C57BL/6 mice were allowed free access to ethanol containing diets or pair-fed control diets for 25d. Mice were treated with 5 mg/kg CoPP or vehicle (saline) three times at 7 day intervals on d5, d12 and d19 of the ethanol feeding protocol. (A) Plasma AST activity and (B) liver triglycerides were measured by biochemical assay. (C) Expression of mRNA for TLR-4, TNF α , IL6 and CXCL10 was measured by qRT-PCR. Values are expressed as the percent increase over pair-fed controls. (D) Immunoreactive 4-HNE adducts, (E) TUNEL positive nuclei and (F) HO-1 expression were visualized and semi-quantified in paraffin-embedded liver sections. Images were acquired using a 10 $\times$ (4HNE and HO-1) or 20 $\times$ (TUNEL) objective. For panels A, B, D, E, F, values with different superscripts are significantly different from each other ($P < 0.05$). For panel C, * $P < 0.05$ compared to pair-fed mice.

Therapy - CoPP treatment after ethanol feeding

Figure 2. Induction of hemeoxygenase-1 (HO-1) with cobalt protoporphyrin (CoPP) treatment after the development of chronic ethanol feeding to mice reverses liver injury

C57BL/6 mice were allowed free access to ethanol containing diets or pair-fed control diets for 25d. On d25 of the ethanol feeding protocol, mice were treated with 5 mg/kg CoPP or vehicle (saline) via intraperitoneal injection and euthanized 24h later. (A) Plasma AST activity and (B) liver triglycerides were measured by biochemical assay. (C) Expression of mRNA for TLR-4, TNF α , IL6 and CXCL10 was measured by qRT-PCR. Values are expressed as the percent increase over pair-fed controls. (D) M30, a caspase-cleavage product of CK18, was visualized in paraffin-embedded livers. M30-positive cells were

counted and expressed as total number of cells per 20X frame. (E/F) Paraffin-embedded livers were immunostained for (E) RIP3 and (F) HO-1 expression. For panels A, B, D, E, F, values with different superscripts are significantly different from each other ($P < 0.05$). For panel C, $*P < 0.05$ compared to pair-fed mice.

CORM-A1 treatment during ethanol feeding

Figure 3. Treatment with the CO-releasing molecule, CORM-A1, after the development of chronic ethanol feeding to mice reverses liver injury

C57BL/6 mice were allowed free access to ethanol containing diets or pair-fed control diets for 25d. Mice were treated with 3mg/kg CORM-A1, a CO releasing molecule, via intraperitoneal injection five times at 24 h intervals on days 21–25 of the ethanol feeding protocol. (A) Plasma AST activity and (B) liver triglycerides were measured. (C) Expression of mRNA for TLR-4, TNF α , IL6 and CXCL10 was measured by qRT-PCR. Values are expressed as the percent increase over pair-fed controls. (D) Ly6C⁺ cells were visualized by immunostaining OCT-embedded frozen liver sections and positive cells were counted per

40X frame. (E) Paraffin-embedded liver sections were immunostained for isolevuglandin-2 (iso[4]LGE₂). (F) M30, a caspase-cleavage product of CK18, was visualized in paraffin-embedded livers. M30-positive cells were counted and expressed as total number of cells per 20X frame. (G) Paraffin-embedded livers were immunostained for RIP3. For panel C, *P<0.05 compared to pair-fed mice. For all other panels, values with different superscripts are significantly different from each other (P < 0.05).

Figure 4. Sensitization of primary hepatocytes to TNF α -induced cell death after exposure to ethanol

Primary hepatocytes were cultured in the presence or absence of 50 mM ethanol for 24h, then treated with or without inhibitors (20 μ M Z-VAD-FMK, 20 μ M necrostatin-1, 4 μ M CoPP, 10 μ M CORM-3) for 4 h prior to challenge with 30 ng/ml TNF α for a further 24 h. (A, C, F) TNF α -induced cytotoxicity was assessed by MTT assay. (B) FACS analysis of propidium iodide and annexin V. Histograms are representative of 5 independent experiments. (D/E) Hepatocyte lysates were used to assess (D) RIP3-FADD interaction in a

FADD-pull down assay (normal IgG was used as a negative control) or (E) probed for HO-1 expression in response to CoPP (HSC70 was used as a loading control). (F) AML12 cells were transfected with an HO-1 expression vector or empty vector, cultured with ethanol and TNF α -induced cytotoxicity assessed by MTT assays. Values represent means $\pm$ SEM, n=4–6, except for panel F, where n=2. *p<0.05 compared to cells not exposed to ethanol, †p<0.05 compared to cells not treated with TNF- α and **p<0.05 compared to cells treated with TNF- α within an ethanol/no ethanol treatment group.

Table I

Characteristics of ethanol- and pair-fed mice treated with CoPP or CORM-A1

Prevention with CoPP				
	saline		CoPP	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Initial body weight(g)	17.6 ± 0.4	17.6 ± 0.3	17.8 ± 0.6	18.2 ± 0.5
Final body weight (g)	20.8 ± 0.8	18.4 ± 0.5	21.3 ± 0.8	19.2 ± 0.7
Food intake last 5 days (mls)	paired	19.4 ± 0.2	paired	20 ± 0.2
ALT (U/ml)	14.6 ± 1.7 ^a	52.7 ± 4.1 ^b	16.2 ± 2 ^a	30.8 ± 3.3 ^c
Treatment with CoPP				
	saline		CoPP	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Initial body weight(g)	18.0 ± 0.5	18.4 ± 0.3	17.8 ± 0.5	18.4 ± 0.4
Final body weight (g)	21.2 ± 0.9	20.7 ± 0.4	21.2 ± 0.4	21.1 ± 0.6
Food intake last 24h (mls)	paired	19.8 ± 0.1	paired	20 ± 0.3
ALT (U/ml)	15.5 ± 1.5 ^a	55.4 ± 1.1 ^b	15.8 ± 1.2 ^a	53.8 ± 3.1 ^b
Treatment with CORM-A1				
	saline		CORM-A1	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Initial body weight(g)	16.8 ± 0.7	17.7 ± 0.5	16.8 ± 0.4	17.3 ± 0.4
Final body weight (g)	19.4 ± 0.7	19.0 ± 0.3	19.9 ± 0.5	19.9 ± 0.4
Food intake last 24h (mls)	paired	18.9 ± 1.0	paired	19.1 ± 0.4
ALT (U/ml)	24.1 ± 2.1 ^a	118.4 ± 28.3 ^b	23.4 ± 4.8 ^a	69.4 ± 6.6 ^c

(n=4 for pair-fed and n=6 for ethanol-fed).

Table 2

Immunoreactive CYP2E1 in mouse livers normalized to HSC70

	Pair-fed	EtOH-fed
No treatment	0.99 ± 0.14	$1.54 \pm 0.18^*$
Prevention with CoPP	1.18 ± 0.14	$1.60 \pm 0.25^*$
Treatment with CoPP	1.02 ± 0.10	$1.97 \pm 0.22^*$
Treatment with CORM-A1	0.95 ± 0.10	$1.64 \pm 0.12^*$

(*p<0.05 compared to pair-fed)

Table 3

ALT and AST activity in ethanol- and pair-fed mice treated with or without inactive CORM-A1 (iCORMA1)

	saline		iCORM-A1	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
ALT (U/ml)	16.2 ± 1.3 ^a	58.9 ± 9.4 ^b	14.2 ± 1.5 ^a	70.5 ± 6.5 ^b
AST (U/ml)	34.1 ± 1.2 ^a	67.4 ± 6.8 ^b	33.4 ± 1.8 ^a	79.2 ± 5.2 ^b

(n=4 for pair-fed and n=6 for ethanol-fed).