The anti-inflammatory effects of adiponectin are mediated via a heme oxygenase-1-dependent pathway in rat Kupffer cells

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Abstract

Altered expression and activity of immunomodulatory cytokines plays a major role in the pathogenesis of alcoholic liver disease. Chronic ethanol feeding increases the sensitivity of Kupffer cells, the resident hepatic macrophage, to lipopolysaccharide (LPS), leading to increased tumor necrosis factor-α (TNF-α) expression. This sensitization is normalized by treatment of primary cultures of Kupffer cells with adiponectin, an anti-inflammatory adipokine. Here we tested the hypothesis that adiponectin-mediated suppression of LPS signaling in Kupffer cells is mediated via an interleukin-10 (IL-10)/heme oxygenase-1 (HO-1) pathway after chronic ethanol feeding. Knock-down of IL-10 expression in primary cultures of Kupffer cells with siRNA prevented the inhibitory effect of globular adiponectin (gAcrp) on LPS-stimulated TNF-α expression. gAcrp increased IL-10 mRNA and protein expression, as well as expression of the IL-10 inducible gene, HO-1; expression was higher in Kupffer cells from ethanol-fed rats compared to pair-fed controls. While IL-10 receptor surface expression on Kupffer cells was not affected by ethanol feeding, IL-10-mediated phosphorylation of STAT3 and expression of HO-1 was higher in Kupffer cells after ethanol feeding. Inhibition of HO-1 activity, either by treatment with the HO-1 inhibitor, zinc protoporphyrin, or by siRNA knock-down of HO-1, prevented the inhibitory effect of gAcrp on LPS-stimulated TNF-α expression in Kupffer cells. LPS-stimulated TNF-α expression in liver was increased in mice after chronic ethanol exposure. When mice were treated with cobalt protoporphyrin to induce HO-1 expression, ethanol-induced sensitivity to LPS was ameliorated.

Conclusion—gAcrp prevents LPS-stimulated TNF-α expression in Kupffer cells via the activation of the IL-10/STAT3/HO-1 pathway. Kupffer cells from ethanol-fed rats are highly sensitive to the anti-inflammatory effects of gAcrp; this sensitivity is associated with both increased expression and sensitivity to IL-10.

Introduction

The innate and adaptive immune systems have been implicated in the progression of alcoholic liver disease (ALD). Disruption in the regulation of the innate immune response is thought to be particularly important in the early stages of ethanol-induced liver injury (1). Accumulating evidence suggests that an imbalance between the activities of pro- and anti-inflammatory mediators contributes to ethanol-induced liver injury. For example, ethanol consumption leads to elevated lipopolysaccharide (LPS)/endotoxin in the portal blood, as well as a sensitization of Kupffer cells to activation, resulting in production of a number of inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin (IL)-6 and reactive oxygen species (ROS). Among the pro-inflammatory mediators, TNF-α plays a
critical role in the pathogenesis of ALD (1); treatment with TNF-α neutralizing antibody reduces ethanol-induced liver injury in animals and TNF-α receptor 1 (TNFR-1) knock-out mice are resistant to the toxic effects of ethanol exposure (1).

Loss of anti-inflammatory mediators may also contribute to a pro-inflammatory state in the liver and facilitate injury. For example, IL-10 is an immunomodulatory cytokine with potent anti-inflammatory and immunosuppressive properties. IL-10 decreases production of pro-inflammatory cytokines, including TNF-α and IL-1β (2). While little is known about the regulation of IL-10 expression and activity in the liver in response to chronic ethanol, impaired expression of IL-10 contributes to inflammation in alcoholic cirrhotics (3) and IL-10 deficient mice are more sensitive to ethanol-induced liver injury (4). Disruption in the expression and activity of adiponectin, an abundant 30-kDa adipokine with potent anti-inflammatory properties (5), may also contribute to a pro-inflammatory imbalance during chronic ethanol exposure. Adiponectin suppresses macrophage activity via a number of mechanisms. For example, adiponectin inhibits the proliferation of myelomonocytic progenitor cells, dampens the upregulation of endothelial adhesion molecules in response to inflammatory signals, suppresses phagocytic activity, as well as reduces LPS-stimulated cytokine production in macrophages (6–8). Chronic ethanol exposure decreases adiponectin concentrations in rats and mice (9,10); treatment of mice with adiponectin during chronic ethanol exposure prevents the development of liver injury, decreasing both steatosis and TNF-α expression in the liver (10). While the mechanisms for these therapeutic effects of adiponectin are not well understood, the decrease in steatosis is most likely related to the critical role of adiponectin in regulation of glucose and lipid homeostasis. Further, we have previously reported that adiponectin treatment normalizes LPS-induced TNF-α production in primary cultures of Kupffer cells after chronic ethanol exposure (9) suggesting that adiponectin therapy may directly suppress the pro-inflammatory activity of Kupffer cells after chronic ethanol feeding.

Recent data suggest an important link between adiponectin and IL-10, two critical anti-inflammatory mediators which may contribute to ethanol-induced liver injury. For example, adiponectin induces the expression of IL-10 mRNA and protein in cultured macrophages (11,12). Expression of IL-10 is required for the anti-inflammatory effects of adiponectin in RAW 264.7 macrophages since immunoneutralization of IL-10 prevents gAcrp-mediated desensitization to LPS (11). IL-10 mediates its anti-inflammatory functions via induction of IL-10-inducible genes, including heme oxygenase-1 (HO-1) and suppressor of cytokine signaling 3 (SOCS3)(2). Induction of these genes involves the activation of STAT3 signaling pathways (2). Adiponectin and HO-1 pathways also interact. For example, increased adiponectin expression is associated with increased expression of HO-1 and enhanced cardiac protection in diabetic rats (13). Further, induction of HO-1 increases adiponectin expression in Zucker rats, leading to decreased TNF-α expression and reduced adipogenesis (14).

HO-1 has anti-apoptotic, anti-inflammatory and anti-proliferative properties (15). There is a growing appreciation that HO-1, in particular, is an important down-stream mediator of the anti-inflammatory effects of IL-10 in macrophages (15). HO-1, and its down-stream mediator carbon monoxide (CO), both inhibit LPS-induced expression of pro-inflammatory cytokines and increase LPS-induced expression of IL-10 in macrophages (15). Induction of HO-1 prevents ethanol-induced oxidative damage in cultured hepatocytes (16) and also decreases complement-mediated injury in the endothelium (17,18).

Since a failure in the ability to induce adequate anti-inflammatory responses likely contributes to chronic inflammation during long-term ethanol exposure, here we tested the hypothesis that there is a beneficial interplay between gAcrp, IL-10 and HO-1 in the
regulation of LPS-induced TNF-α expression by Kupffer cells after chronic ethanol exposure. We found that induction of both IL-10 and HO-1 expression are required for the anti-inflammatory effects of gAcrp in Kupffer cells. Importantly, the increased sensitivity of Kupffer cells from ethanol-fed rats to gAcrp was associated with increased expression of IL-10, as well as enhanced IL-10 receptor signaling, leading to the greater expression of HO-1. When HO-1 expression was increased in mice by treatment with cobalt protoporphyrin, chronic ethanol-induced sensitization of LPS-stimulated TNF-α expression in liver was normalized. These data suggest that therapeutic strategies to enhance IL-10 and/or HO-1 expression or signaling may be effective strategies for dampening the sensitivity of Kupffer cells to stimulation after chronic ethanol.

Materials and Methods

Materials

Adult male Wistar rats weighing 140–150 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Lieber-DeCarli ethanol diet (regular, no. 710260) was purchased from Dyets (Bethlehem, PA). Cell culture reagents were from Invitrogen (Grand Island, NY). Recombinant human gAcrp expressed in E. coli and full-length adiponectin expressed in HEK293 cells were purchased from Peprotech, Inc. (Rocky Hill, NJ) and BioVendor Lab Medicine (Candler, NC), respectively. Additional materials are described in Supplemental Material.

Chronic ethanol feeding and Kupffer cell isolation

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Chronic ethanol-feeding to rats and mice, as well as the isolation and culture of Kupffer cells, were performed as previously described (9,19,20) (see Supplemental Material for further details). Isolated Kupffer cells were then either plated immediately or used for nucleofection prior to plating. One to four hours after plating, nonadherent cells were removed by aspiration and fresh media with or without 1 μg/ml gAcrp added. After 18h in culture, cells were treated with or without 100 ng/ml LPS or 10 ng/ml IL-10, as indicated in the figure legends. In some experiments, inhibitors were added to the Kupffer cell culture media 30 min prior to the IL-10 treatment. The dose and time of exposure of Kupffer cells to gAcrp and LPS were based on previous studies (9,19).

Nucleofection in rat Kupffer cells

Freshly isolated Kupffer cells were transfected using the Amaxa mouse macrophage Nucleofector kit according to the instructions of the manufacturer using the Y-001 program (Lonza, Cologne, Germany), except for the following modifications. Samples were processed individually and the entire nucleofection procedure for each sample was completed in less than 5 min. For each nucleofection sample, 2 x 10⁶ Kupffer cells were centrifuged for 10 min at 300 x g. The pellet was washed with 1 ml PBS, collected at 300 x g for 5 min and then resuspended in 105 μl nucleofector solution and transferred to a 1.5 mL eppendorf tubes for a final concentration of ~2 x 10⁶ cells/100 μl. Cells were then treated or not with 2.0 μg specific or scrambled siRNA (siRNA sequences are provided in Supplemental Materials), transferred into the electroporation cuvette and placed in the Nucleofector device. After nucleofection, cells were immediately removed from the cuvette and plated in a 96-well plate (150 μl/well) at 0.5 x 10⁶ cells/well. After 4 h, the cell culture medium was replaced with fresh medium with or without 1 μg/ml gAcrp or 10 ng/ml IL-10 for 18 h and then treated with or without LPS or IL-10, as described in the figure legends.
RNA isolation and quantitative real-time PCR (qRT-PCR)
Total RNA was isolated, reverse transcribed and qRT-PCR amplification was performed as previously described (9). The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S. Details of the procedure and primer sequences are provided in Supplemental Material.

IL-10 ELISA
The quantity of secreted IL-10 protein was measured in the media from Kupffer cells after treatment with gAcrp for 18 h using a rat IL-10 ELISA kit (Biosource, Camarillo, CA).

Western blot analysis
Western blot analysis was performed using enhanced chemiluminescence for signal detection. Signal intensities were quantified by densitometry using Image J software (NIH).

Flow cytometry Analysis
After 18h culture with or without gAcrp, Kupffer cells were gently scraped and adjusted to 1 million cells per ml with culture media. Cells were greater than 90% viable as determined by Trypan blue exclusion. Expression of IL-10 receptor A subunit was then measured by flow cytometry, as described in the figure legend. Data were acquired and processed using FlowJo software (Becton Dickinson).

Statistical analysis
Because of the limited number of Kupffer cells available from each animal, data from several feeding trials are presented in this study. Values are means ± standard error of the mean (SEM). Data were analyzed by general linear models procedure (SAS; Carey, IN). 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 increases the sensitivity of Kupffer cells to LPS-stimulated TNF-α expression; LPS increased TNF-α mRNA accumulation was 2.7-fold higher in Kupffer cells from ethanol-fed rats compared to pair-fed rats (Figure 1)(9). Treatment of primary cultures of Kupffer cells with gAcrp for 18 h suppressed LPS-stimulated responses in Kupffer cells isolated from both pair-fed and ethanol-fed rats (Figure 1)(9). In other cellular model systems, adiponectin exerts its anti-inflammatory actions through induction of IL-10 (11). Therefore, we tested if knock-down of IL-10 expression with siRNA ameliorated the ability of gAcrp to suppress LPS-stimulated TNF-α expression in Kupffer cells. Transfection of Kupffer cells with siRNA against IL-10 effectively suppressed IL-10 mRNA accumulation (Supplementary Figure 1A) and prevented the suppression of LPS-stimulated TNF-α mRNA accumulation by gAcrp (Figure 1). Scrambled siRNA had no effect on IL-10 mRNA (Supplementary Figure 1A) or the response to gAcrp (Figure 1).

Primary cultures of Kupffer cells from ethanol-fed rats are more sensitive than cells from pair-fed rats to the anti-inflammatory actions of both gAcrp and full-length adiponectin to suppress LPS-dependent responses (9). Since IL-10 is required for gAcrp to suppress LPS-stimulated TNF-α mRNA accumulation in Kupffer cells, the more poten effects of adiponectin after ethanol feeding may be due to increased gAcrp-stimulated expression of IL-10 and/or increased sensitivity of the Kupffer cells to stimulation by IL-10. To test these hypotheses, isolated Kupffer cells were treated with increasing concentrations of gAcrp for 18h and IL-10 protein secreted in the media was measured by ELISA. Accumulation of
IL-10 protein was higher in Kupffer cells from ethanol-fed rats compared to cells from pair-fed controls (Figure 2A). Similarly, IL-10 mRNA expression was also higher in Kupffer cells from ethanol-fed rats compared to cells from pair-fed rats when incubated with gAcrp (Figure 2B) or full-length adiponectin (Figure 2C). These data suggested that increased gAcrp-stimulated IL-10 expression may contribute, at least in part, to the higher sensitivity of Kupffer cells from ethanol-fed rats to gAcrp. siRNA knock-down of adiponectin receptors (AdipoR) revealed that the effects of gAcrp on IL-10 mRNA were dependent on the expression of AdipoR1, but not AdipoR2 (Figure 2D).

IL-10 mediates its anti-inflammatory effects via interactions with IL-10 receptors and activation of specific signaling pathways; STAT3 activation is required for IL-10-mediated signaling (2). Surface expression of the IL-10 receptor subunit A, the ligand binding subunit of the IL-10 receptor, on Kupffer cells was not affected by either ethanol feeding or gAcrp treatment (Figure 3). Stimulation with IL-10 increased the phosphorylation of JAK1 within 30 min in Kupffer cells from ethanol-fed, but not pair-fed, rats (Figure 4). Phosphorylation of STAT3 in response to IL-10 was both more rapid (within 10 min) and more robust in Kupffer cells from ethanol-fed rats compared to pair-fed rats (Figure 4). IL-10 had no effect on phosphorylation of JAK2, p38 or ERK1/2 mitogen-activated protein kinases in Kupffer cells under these conditions (data not shown).

IL-10R activation of the STAT3 pathway increases expression of STAT3 responsive genes, such SOCS3 and HO-1 (2). Culture of Kupffer cells with gAcrp increased the expression of SOCS3 and HO-1 mRNA (Figure 5A/B). Consistent with the increased gAcrp-stimulated IL-10 expression and phosphorylation of STAT3 after chronic ethanol feeding, gAcrp treatment increased HO-1 and SOCS3 mRNA expression to a greater extent in Kupffer cells from ethanol-fed compared to pair-fed rats (Figure 5A/B). gAcrp increased HO-1 protein expression in Kupffer cells from ethanol-fed rats compared to pair-fed rats (Figure 4). IL-10 had no effect on phosphorylation of JAK2, p38 or ERK1/2 mitogen-activated protein kinases in Kupffer cells under these conditions (data not shown).

Because HO-1 is a critical mediator of the anti-inflammatory effects of IL-10 (15), we further investigated the mechanisms by which gAcrp increased HO-1 expression in Kupffer cells. To test whether gAcrp induces HO-1 expression through an IL-10 dependent pathway, Kupffer cells were transfected with siRNA against IL-10 in order to knock-down IL-10 expression. When IL-10 expression was inhibited, gAcrp-stimulated HO-1 mRNA expression was suppressed in Kupffer cells from both pair- and ethanol fed rats (Figure 6A). Scrambled siRNA administration had no effect on gAcrp-stimulated HO-1 mRNA expression (Figure 6A). The signaling pathways down-stream of gAcrp-stimulated IL-10 expression were investigated with the use of selective inhibitors. gAcrp stimulated HO-1 mRNA expression was attenuated when Kupffer cells were pre-treated with a selective inhibitor of STAT3 (JSI-124) (Figure 6B). Finally, IL-10-stimulated HO-1 mRNA expression was suppressed in Kupffer cells transfected with siRNA against STAT3; scrambled siRNA had no effect on IL-10-dependent HO-1 expression (Figure 6C). siRNA knock-down of STAT3 decreased STAT3 protein expression (Supplementary Figure 1C). Taken together, these data demonstrate that gAcrp induces HO-1 expression via an IL-10/STAT3-dependent pathway.

Since HO-1 has potent anti-oxidant and anti-inflammatory activity, we investigated the role of HO-1 in mediating the effect of gAcrp using both biochemical and siRNA knock-down strategies. First, when Kupffer cells were treated with zinc protoporphyrin (ZnPP), a competitive inhibitor of HO-1 activity, prior to culture with gAcrp, the inhibitory effect of gAcrp on LPS-stimulated TNF-α expression was ameliorated (Figure 7A). Similar results
were obtained using a siRNA strategy. When Kupffer cells were transfected with siRNA against HO-1, expression of HO-1 protein was decreased compared to Kupffer cells transfected with scrambled siRNA (Supplementary Figure 1B). Knock-down of HO-1 with siRNA prevented the inhibitory effect of gAcrp on LPS-stimulated TNF-α mRNA, whereas scrambled siRNA had no effect (Figure 7B).

Previous studies have shown that treatment of mice with supra-physiological concentrations of adiponectin during chronic ethanol exposure protects from ethanol-induced steatosis and inflammation (10). Based on our novel findings in Kupffer cells that HO-1 is a down-stream mediator of the anti-inflammatory effects of adiponectin, we designed an in vivo experiment to ascertain whether induction of HO-1 would normalize LPS-stimulated TNF-α expression in liver after chronic ethanol exposure. HO-1 mRNA and protein expression in mouse liver were not affected by chronic ethanol feeding (Figure 8A); however, treatment with cobalt protoporphyrin (CoPP) increased HO-1 expression in liver of both ethanol-and pair-fed mice (Figure 8A). After chronic ethanol feeding, LPS-stimulated TNF-α mRNA expression was increased 2-fold compared to pair-fed controls (Figure 8B). However, when mice were pre-treated with CoPP to induce HO-1 expression, LPS-stimulated TNF-α expression was reduced and did not differ between ethanol- and pair-fed mice (Figure 8B).

**Discussion**

Increased expression of TNF-α contributes to ethanol-induced liver injury (1). Treatment of mice with adiponectin, a potent adipokine with anti-inflammatory properties, prevents ethanol-induced steatosis and TNF-α expression (10). Kupffer cells isolated from rats exposed to chronic ethanol exhibit increased sensitivity to LPS-stimulated TNF-α expression and are used as a model system to understand the interaction between ethanol and LPS-mediated responses in macrophages (21). Interestingly, the anti-inflammatory actions of adiponectin are enhanced in Kupffer cells isolated from rats chronically exposed to ethanol, compared to pair-fed controls (9). Despite the efficacy of adiponectin in decreasing LPS-mediated responses, both in mouse models (10) and primary cultures of Kupffer cells (9), the development of adiponectin for therapeutic interventions in patients with alcoholic liver disease is likely of limited utility, due to the high concentration of adiponectin in the circulation, as well as the complex oligomeric structure of adiponectin. Therefore, here we made use of primary cultures of Kupffer cells to investigate the molecular mechanisms for the anti-inflammatory effects of adiponectin after chronic ethanol exposure. Understanding the mechanisms of adiponectin action, particularly in ethanol-treated macrophages, could illuminate molecular targets of adiponectin action that are more amenable to pharmacological intervention. Here we have identified an IL-10/STAT3/HO-1 dependent pathway that mediates the anti-inflammatory effects of adiponectin in Kupffer cells. The activity of this pathway is enhanced in Kupffer cells from ethanol-fed rats due to both an increased gAcrp-mediated expression of IL-10, as well as a greater IL-10 stimulated phosphorylation of STAT3 and expression of HO-1. Importantly, induction of HO-1 was also effective at normalizing LPS-stimulated TNF-α expression in an in vivo model of chronic ethanol exposure.

Adiponectin has potent anti-inflammatory properties, both in vivo and in cultured macrophages. Initially, treatment of macrophages with adiponectin increases the expression of inflammatory cytokines, such as TNF-α and IL-6 (11,22). However, upon continued exposure to gAcrp, the expression of anti-inflammatory mediators, such as IL-10 and IL-1 receptor antagonist, is increased (11,12). Increased expression of IL-10 is critical for the anti-inflammatory effects of adiponectin in macrophages; immunoneutralization of IL-10 prevents the suppression of LPS-stimulated TNF-α production by 1 μg/ml gAcrp in RAW 264.7 macrophages (11). However, in one recent report from the Libby group, IL-10 was not
critical in mediating the anti-inflammatory effects of 10 μg/ml full-length adiponectin in human macrophages (23). Here we report that knock-down of IL-10 in primary cultures of Kupffer cells prevented gAcrp-mediated suppression of LPS-stimulated TNF-α mRNA accumulation, demonstrating that IL-10 is necessary and sufficient to mediate the anti-inflammatory effects of gAcrp in primary cultures of Kupffer cells. We also demonstrated that the induction of IL-10 by gAcrp in Kupffer cells was dependent on AdipoR1, but not AdipoR2, expression. The contribution of AdipoR1, which has a higher affinity for globular adiponectin compared to full-length adiponectin (24), may explain the differences between our results indicating an essential role of IL-10 and that of the Libby group (23), using higher concentrations of full-length adiponectin, that reported the induction of multiple anti-inflammatory mediators.

Kupffer cells isolated from ethanol-fed rats are more sensitive to the long-term anti-inflammatory effects of either gAcrp or full-length adiponectin, exhibiting decreased LPS-stimulated NFκB and MAPK activation, as well as decreased TNF-α expression relative to Kupffer cells from pair-fed controls (9). Because IL-10 is essential to the anti-inflammatory role of gAcrp in Kupffer cells, we hypothesized that ethanol feeding increased the sensitivity to gAcrp via increased IL-10 expression and/or increased sensitivity to IL-10 mediated responses. Our data demonstrate that chronic ethanol feeding increased the sensitivity of Kupffer cells to gAcrp-stimulated IL-10 expression; expression of both IL-10 mRNA as well as the quantity of secreted IL-10 protein is increased in Kupffer cells from ethanol-fed rats compared to cells from control rats. Kupffer cells from ethanol-fed rats also exhibited enhanced IL-10-dependent signaling (Figure 4), independent of any effect of chronic ethanol on the cell surface expression of IL-10RA, the ligand binding subunit of the IL-10 receptor complex (Figure 3). Chronic ethanol accelerated and enhanced IL-10-stimulated phosphorylation of STAT3 (Figure 4) and increased expression of IL-10 dependent genes, including HO-1 and SOCS-3 mRNA (Figure 5).

Very little is known about the impact of acute or chronic ethanol on IL-10 expression and signaling. After chronic ethanol exposure, plasma IL-10 concentrations are reduced in mice and IL-10 deficient mice exhibit an even greater sensitivity to LPS after ethanol feeding compared to wild type mice (4). Short term/acute ethanol exposure increases IL-10 expression by monocytes in human subjects, as well as in mice in response to LPS. When human subjects consume a single dose of alcohol, the production of IL-10 by isolated monocytes in response to LPS is increased compared to controls (25). This increase can be prevented by inhibiting HO-1 by pre-treatment with zinc protoporphyrin (26). Taken together with the current data, it appears that while chronic ethanol exposure decreases circulating concentrations of IL-10 (4), both short term/acute and chronic ethanol exposure contribute to an enhanced IL-10 expression in monocytes/macrophages in response to immunoregulatory signals, such as LPS or gAcrp.

IL-10 binds to a heterodimeric IL-10R, which undergoes transphosphorylation and then activates the Jak1/STAT3 pathway (27). Activation of STAT3 is essential for IL-10-dependent signaling (2). Chronic ethanol feeding increased IL-10 stimulated phosphorylation of JAK1 and STAT3 in Kupffer cells. Furthermore, inhibition of STAT3 signaling via chemical inhibitors or via siRNA knock-down ameliorated IL-10-dependent expression of HO-1 (Figure 6). Reports in the literature suggest that the impact of chronic ethanol on the regulation of STAT3 is complex, and is likely to have ligand- and cell-type specific effects. Exposure of primary cultures of hepatocytes to ethanol suppresses IL-6-stimulated STAT3 activation (28). Gao and colleagues have identified cell specific roles for STAT3 in hepatocytes compared to monocytes/macrophages in the liver (29). Expression of STAT3 in hepatocytes had a negative impact on liver injury and promoted inflammation, while expression of STAT3 in monocytes/macrophages suppressed inflammation during

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ethanol exposure (29). The anti-inflammatory role of STAT3 in monocytes/macrophages during chronic ethanol exposure is consistent with our identification of a critical contribution of STAT3 in Kupffer cells in mediating the anti-inflammatory effects of gAcrp.

Accumulating evidence suggests that HO-1 plays an important anti-inflammatory role in chronic inflammatory diseases and protects cells from oxidative insult (15). Heme oxygenase catalyzes the initial and rate limiting step in oxidative degradation of heme, yielding equimolar amounts of biliverdin IXα, carbon monoxide, and free iron (30). There are three isoforms of HO: HO-2 and HO-3 are constitutive forms, while HO-1 (also known as heat shock protein 32) is an inducible isozyme, with high expression levels in spleen and Kupffer cells (31). HO-1 is a stress-responsive protein whose expression is upregulated by a broad spectrum of inducers, including heme, heavy metals, nephrotoxins, cytokines, endotoxins and oxidative stress. Interestingly, HO-1 expression was not increased by chronic ethanol exposure alone in either isolated Kupffer cells (Figure 5) or mouse liver (Figure 8). However, literature suggests that HO-1 expression in response to ethanol may be dependent on the age of the animals studied (32,33). In Kupffer cells, pharmacological inhibition of HO-1 or siRNA knock-down of HO-1 expression completely ameliorated the ability of gAcrp to inhibit LPS-stimulated TNF-α expression. Pharmacological induction of HO-1 in mice reduced LPS-stimulated TNF-α expression in the livers of ethanol-fed mice to that of pair-fed controls. Taken together, these data demonstrated a critical role for HO-1 in dampening the pro-inflammatory response to LPS both in Kupffer cells and in vivo.

In summary, these data provide strong evidence for an essential role of IL-10/STAT3/HO-1 in mediating the anti-inflammatory function of gAcrp, demonstrating that gAcrp-dependent responses utilize two critical anti-inflammatory pathways. Importantly, after chronic ethanol exposure, Kupffer cells exhibit an increased sensitivity to the anti-inflammatory effects of both gAcrp and IL-10 and induction of HO-1 in vivo protects mice from the sensitizing effects of ethanol on LPS-stimulated TNF-α expression. The identification of HO-1 as a downstream effector of gAcrp provides an exciting path for the design and development of novel therapeutic approaches for the resolution of chronic inflammation associated with alcoholic liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ALD</td>
<td>Alcoholic liver disease</td>
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<tr>
<td>AdipoR</td>
<td>adiponectin receptor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BV</td>
<td>biliverdin IX</td>
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<td>CO</td>
<td>carbon monoxide</td>
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<td>CoPP</td>
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</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>gAcrp</td>
<td>globular adiponectin</td>
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EtOH  ethanol  
Fcy  Fc-gamma receptor  
fl-Acrp  full-length adiponectin  
HO-1  heme oxygenase-1  
IgG  immunoglobulin G  
IL-1β  interleukin 1β  
IL-6  interleukin 6  
IL-10  interleukin 10  
IL-10R  interleukin 10 receptor  
JAK1  janus kinase 1  
LPS  lipopolysaccharide  
MAPK  mitogen activated protein kinase  
PBS  phosphate buffered saline  
PMSF  phenylmethylsulphonyl fluoride  
ROS  reactive oxygen species  
Scrb siRNA  scrambled small interfering RNA  
SDS  sodium dodecyl sulfate  
SEM  standard error of the mean  
SOCS-3  suppressor of cytokine signaling-3  
STAT3  signal transducers and activators of transcription protein 3  
TNF-α  tumor necrosis factor α  
TNFR-1  tumor necrosis factor receptor superfamily, member 1A  
ZnPP  zinc protoporphyrin  

References


Figure 1. IL-10 siRNA prevents globular adiponectin (gAcrp)-induced suppression of TNF-α mRNA expression in LPS-stimulated Kupffer cells

Kupffer cells isolated from pair- and ethanol-fed rats were transfected or not with 2.0 μg of IL-10 siRNA or scrambled siRNA and then cultured with or without 1 μg/ml gAcrp for 18 h. Kupffer cells were then stimulated with 100 ng/ml of LPS for 60 min and TNF-α and 18S mRNA measured by qRT-PCR. In control experiments, siRNA knock-down of IL-10 decreased gAcrp-induced IL-10 mRNA expression after treatment with gAcrp for 5 h (see Supplementary Figure 1A). Knock-down efficiency did not differ between Kupffer cells from pair- and ethanol-fed rats. Values represent means ± SEM, n=4, *p<0.05 ethanol-fed compared to pair-fed, +p<0.05 compared to cells not treated with gAcrp.
Figure 2. Chronic ethanol feeding increases the sensitivity of Kupffer cells to adiponectin-stimulated IL-10 protein and mRNA expression
A) Kupffer cells isolated from pair- and ethanol-fed rats were cultured with 0–1000 ng/ml gAcrp for 18 h and IL-10 peptide secreted into the media measured by ELISA. Values represent means ± SEM, n=7, *p<0.05 ethanol-fed compared to pair-fed, +p<0.05 compared to cells not treated with gAcrp. B/C) Kupffer cells isolated from pair- and ethanol-fed rats were cultured overnight and then treated with 1 μg/ml gAcrp (B) or 1 μg/ml full-length adiponectin (C) for 0–5h and the quantity of IL-10, β-actin and 18S mRNA measured by qRT-PCR. Expression of IL-10 mRNA was normalized to β-actin or 18S and then expressed relative to expression in Kupffer cells from pair-fed rats not treated with gAcrp. Values represent means ± SEM, n=6–8 in B and 4 in C, *p<0.05 ethanol-fed compared to pair-fed, +p<0.05 compared to cells not treated with adiponectin. D) Kupffer cells were transfected or not with 2.0 μg of adiponectin R1 (AdipoR1), adiponectin R2 (AdipoR2) siRNA or scrambled siRNA and then cultured for 18 h. Kupffer cells were then stimulated or not with 1 μg/ml gAcrp for 5 h and IL-10 and 18S mRNA measured by qRT-PCR. siRNA knock-down decreased expression of AdipoR1 and AdipoR2 mRNA equally in Kupffer cells from pair- and ethanol-fed rats (see Supplementary Figure 1D/E). Values represent means ± SEM, n=4, *p<0.05 ethanol-fed compared to pair-fed, +p<0.05 compared to cells not treated with gAcrp.
Figure 3. IL-10 receptor A (IL-10RA) expression in Kupffer cells after chronic ethanol feeding
Kupffer cells isolated from pair- and ethanol-fed rats were treated with or without 1 μg/ml gAcrp for 18 h. and cell surface expression of IL-10RA (solid line), relative to isotype controls (dotted line), was measured by flow cytometry. Kupffer cells were harvested by gentle scraping and the cells collected by centrifugation at 300 × g for 10 minutes. The pellet was washed with PBS and resuspended in 100 μL PBS+ 0.1% sodium azide and then blocked with 1.0 μg anti-mouse CD32/CD16 Fcγ Block antibodies for 15 min at 4°C. Then the cells were stained with ~0.5 μg fluorochrome conjugated IL-10 receptor A (PE-conjugated IL-10 RA) or isotype control (PE-conjugated IgG1) diluted in PBS containing 0.1% sodium azide for 30 min. Cells were then washed twice with PBS and resuspended in 0.5 ml wash buffer (final concentration ~10^6 cells in 0.5 ml) and held on ice until flow cytometric measurements were performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry systems, Mountain View, CA). The percentage of IL-10RA positive cells (50 ± 3 for pair-fed and 53 ± 3 for ethanol-fed) and the median fluorescence intensity (20 ± 8 for pair-fed and 26 ± 13) did not differ between Kupffer cells from pair- and ethanol-fed rats. Values represent means ± SEM, n=4. Traces shown are representative of four experiments.
Figure 4. Chronic ethanol feeding increases IL-10-stimulated phosphorylation of JAK1 and STAT3 in rat Kupffer cells
Kupffer cells isolated from pair-and ethanol-fed rats were cultured overnight and then stimulated with 10 ng/ml IL-10 for 0–30 min. Kupffer cells were then washed twice in ice-cold PBS and lysed at 4°C in 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS containing 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM PMSF, 10 μg/ml aprotinin and protease inhibitor (Complete-EDTA free™). After 30 min, lysates were centrifuged at 16,000 × g for 15 min at 4°C. 20 μg of cellular extract was separated on 10% SDS-PAGE gels and then transferred to PVDF membranes for detection. Membranes were blocked in BSA and probed with antibodies against phospho-JAK1 and phospho-STAT-3. Membranes were then probed with antibodies to total JAK1 (data not shown), STAT3 and heat shock cognate protein 70 (hsc70), as a loading control. A) Representative images. B–C) Values represent means ± SEM, n=8–9, *p<0.05 ethanol-fed compared to pair-fed, +p<0.05 compared to cells (time 0) not treated with IL-10.
Figure 5. Chronic ethanol feeding increases gAcrp-mediated induction of HO-1 and SOCS-3 mRNA and HO-1 protein expression in rat Kupffer cells

Kupffer cells isolated from pair- and ethanol-fed rats were cultured with 1μg/ml gAcrp for 18h and quantity of A) heme-oxygenase-1 (HO-1) or B) suppressor of cytokine signaling-3 (SOCS-3) and 18S mRNA measured by qRT-PCR. HO-1 and SOCS-3 mRNA were normalized to 18S mRNA and values are expressed relative to Kupffer cells from pair-fed rats not treated with gAcrp. Values represent means ± SEM, n=4, values with different superscripts are significantly different from each other, p<0.05. C) Kupffer cells isolated from pair-and ethanol-fed rats were cultured with 1 μg/ml gAcrp for 18h and HO-1 and SOCS-3 protein measured by Western blot. Images are representative of three independent experiments. gAcrp increased HO-1 protein by 1.66 ± 0.13 in pair-fed and 5.28 ± 1.73 (p<0.05 for ethanol-fed only, n=4).
Figure 6. Adiponectin induces HO-1 mRNA expression through an IL-10- and STAT3-dependent pathway

A) Kupffer cells isolated from pair- and ethanol-fed rats were transfected or not with 2.0 μg of IL-10 siRNA or scrambled siRNA and then cultured with or without 1 μg/ml gAcrp for 18 h. HO-1 and 18S mRNA were measured by qRT-PCR. HO-1 mRNA was normalized to 18S mRNA and values are expressed relative to Kupffer cells from pair-fed rats not treated with gAcrp. Values represent means ± SEM, n=3, +p<0.05 compared to gAcrp-treated cells not transfected with siRNA or transfected with scrambled siRNA.

B) Kupffer cells isolated from pair- and ethanol-fed rats were pretreated with 10 μM JSI-124, an inhibitor of STAT3 signaling, or vehicle (DMSO) for 30 min and then cultured with or without 10 ng/ml IL-10 for 18 h. HO-1 and 18S mRNA were measured by qRT-PCR. HO-1 mRNA was normalized to 18S mRNA and values are expressed relative to Kupffer cells from pair-fed rats not treated with IL-10. Values represent means ± SEM, n=3, +p<0.05 compared to IL-10-treated cells not treated with inhibitor.

C) Kupffer cells isolated from pair- and ethanol-fed rats were transfected or not with 2.0 μg of STAT3 siRNA or scrambled siRNA and then cultured with or without 10 ng/ml IL-10 for 18 h. HO-1 and 18S mRNA were measured by qRT-PCR. HO-1 mRNA was normalized to 18S mRNA and values are expressed relative to Kupffer cells from pair-fed rats not treated with gAcrp. Values represent means ± SEM, n=3, +p<0.05 compared to gAcrp-treated cells not transfected with siRNA or transfected with scrambled siRNA.
Figure 7. HO-1 mediates the inhibitory effects of gAcrp on LPS-stimulated TNF-α expression

A) Kupffer cells isolated from pair- and ethanol-fed rats were cultured with or without 0.5 μM zinc protoporphyrin (ZnPP) in the presence or absence of 1μg/ml gAcrp for 18h. Kupffer cells were then stimulated with 100 ng/ml LPS for 1h and TNF-α and 18S mRNA measured by qRT-PCR. Values represent means ± SEM, n=4, +p<0.05 compared to control cells not treated with gAcrp.

B) Kupffer cells isolated from pair- and ethanol-fed rats were transfected or not with 2.0 μg of HO-1 siRNA or scrambled siRNA and then cultured with or without 1 μg/ml gAcrp for 18 h. Kupffer cells were then stimulated with 100 ng/ml of LPS. TNF-α and 18S mRNA were measured by qRT-PCR. Values represent means ± SEM, n=5, +p<0.05 compared to cells within a treatment group not cultured with gAcrp.
Figure 8. Induction of HO-1 decreases LPS-stimulated TNF-α expression in vivo after chronic ethanol feeding to mice

A/B Mice were allowed free access to increasing concentrations of ethanol as part of a complete liquid diet to a maximum concentration of 32% of kcal over 25 days or pair-fed control diets (see Supplemental materials for detailed ethanol exposure protocol). Mice were then injected or not with 5 mg/kg cobalt protoporphyrin (CoPP) or vehicle (saline). After 24 h, mice were injected with 0.7 μg/kg LPS or saline. Expression of HO-1 (A) and TNF-α mRNA (B) was measured by qRT-PCR after 60 min and normalized to 18S mRNA. HO-1 protein was measured by Western blot (representative images are shown in the inset to panel A). Values represent means ± SEM, n=4–7 (A) values with difference superscripts are
significantly different from each other (B) *p<0.05 compared to pair-fed, +p<0.05 compared to LPS treated without treatment.