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Leptin is key to peroxynitrite-mediated oxidative stress and Kupffer cell activation in experimental non-alcoholic steatohepatitis

Saurabh Chatterjee[1,2,*], Douglas Ganini[2], Erik J. Tokar[3], Ashutosh Kumar[2], Suvarthi Das[1], Jean Corbett[2], Maria B. Kadiiska[2], Michael P. Waalkes[3], Anna Mae Diehl[4], Ronald P. Mason[2]

[1]*Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, University of South Carolina, Columbia, SC 29208, USA;* [2]*Free Radical Metabolism Group, Laboratories of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA;* [3]*Inorganic Toxicology Group, Division of National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA;* [4]*Division of Gastroenterology, Duke University, Durham, NC 27707, USA*

Background & Aims: Progression from steatosis to steatohepatitic lesions is hypothesized to require a second hit. These lesions have been associated with increased oxidative stress, often ascribed to high levels of leptin and other proinflammatory mediators. Here we have examined the role of leptin in inducing oxidative stress and Kupffer cell activation in CCl_4-mediated steatohepatitic lesions of obese mice.

Methods: Male C57BL/6 mice fed with a high-fat diet (60% kcal) at 16 weeks were administered CCl_4 to induce steatohepatitic lesions. Approaches included use of immuno-spin trapping for measuring free radical stress, gene-deficient mice for leptin, p47 phox, iNOS and adoptive transfer of leptin primed macrophages *in vivo*.

Results: Diet-induced obese (DIO) mice, treated with CCl_4 increased serum leptin levels. Oxidative stress was significantly elevated in the DIO mouse liver, but not in *ob*/*ob* mice, or in DIO mice treated with leptin antibody. In *ob*/*ob* mice, leptin supplementation restored markers of free radical generation. Markers of free radical formation were significantly decreased by the peroxynitrite decomposition catalyst FeTPPS, the iNOS inhibitor 1400W, the NADPH oxidase inhibitor apocynin, or in iNOS or p47 phox-deficient mice. These results correlated with the decreased expression of TNF-alpha and MCP-1. Kupffer cell depletion eliminated oxidative stress and inflammation, whereas in macrophage-depleted mice, the adoptive transfer of leptin-primed macrophages significantly restored inflammation.

Conclusions: These results, for the first time, suggest that leptin action in macrophages of the steatotic liver, through induction of iNOS and NADPH oxidase, causes peroxynitrite-mediated oxidative stress thus activating Kupffer cells.

Keywords: Adipocytokines, Kupffer cell; Oxidative stress; Tyrosine nitration; NADPH oxidase; *ob*/*ob* mice.

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* Corresponding author. Address: Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, University of South Carolina, Columbia, SC 29208, USA. Tel.: +1 803 777 8120; fax: +1 803 777 3199.

E-mail address: schatt@mailbox.sc.edu (S. Chatterjee).

Introduction

Leptin's role as a proinflammatory adipocytokine has gained attention in non-alcoholic steatohepatitis. Circulating leptin levels are elevated in obesity and NASH. Leptin-induced cytokine release, especially of IL-1 and TNF-α, has been shown in microglia and monocytes [1,2]. Leptin acts on Kupffer cells, the resident macrophages, by binding to its functional receptor in the liver and inducing the release of TNF-α, TGF-beta and IL-15 [3–5].

Despite wide-ranging reports of leptin's role in inflammation and release of inflammatory mediators, its role in inducing oxidative stress in the liver remains unclear. There are reports regarding leptin-induced reactive oxygen species formation by different cell types, including endothelial cells, cardiomyocytes and hepatic stellate cells [6–8]. These studies focused on reactive oxygen species formation but the mechanisms of free radical species generation and their link to exacerbated inflammation through Kupffer cell activation are not completely understood. Since leptin is known to induce both NADPH oxidase and iNOS, the resultant superoxide and nitric oxide can react at a diffusion-controlled rate to produce peroxynitrite, a strong physiological oxidant. Peroxynitrite can form several free radical species including $^{\cdot}OH$, $^{\cdot}CO_3$ and $^{\cdot}NO_2$ radicals, depending on the pathophysiological microenvironment [9–11].

Based on the available studies on the role of leptin in oxidative stress induction and inflammation in steatohepatitis, we hypothesized that leptin-induced peroxynitrite and its ensuing free radical formation play a major role in early liver injury in obesity. Here we show that CCl_4 administration in diet-induced obese mice increases circulating levels of leptin; we also demonstrate that heightened levels of leptin contribute significantly to the pathogenesis of the resultant liver damage by activating NADPH oxidase, inducing iNOS, and activating release of TNF-α and MCP-1 from Kupffer cells by peroxynitrite-dependent mechanisms. Furthermore, we prove that leptin exerts its free radical formation and proinflammatory effects mainly by acting on macrophages and Kupffer cells of obese mice.

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Materials and methods

Obese mice

Custom DIO adult male, pathogen-free, with a C57BL6/J background (Jackson Laboratories, Bar Harbor, Maine) were used as models of diet-induced obesity. The mice were fed a high-fat diet (60% kcal) from 6 weeks until 16 weeks. All experiments were conducted in the 16-week age group. Age-matched lean controls were fed with a diet having 10% kcal fat. The animals were housed, one animal per cage before any experimental use. Mice bearing the disrupted *OB* gene (leptin) (B6.V-Lep<ob>/J) (Jackson Laboratories), disrupted *p47* phox (B6.129S2-Ncf1^{tm1shl} N14) (Taconic, Cranbury, NJ) genes, or disrupted *NOS2* (B6.129P2-*Nos2*^{tm1Lau}/J, Jackson Laboratories; C57BL6 background) were fed with a high-fat diet and treated identically to DIO obese mice. Mice had *ad libitum* access to food and water and were housed in a temperature-controlled room at 23–24 °C with a 12-h light/dark cycle. All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals, and the experiments were approved by the institutional review board.

Induction of liver injury in obese mice

DIO mice or high-fat-fed gene-specific knockout mice at 16 weeks were administered carbon tetrachloride (0.8 mmoles/kg, diluted in olive oil) through the intraperitoneal route. This model is a free radical-based mechanistic model for non-alcoholic steatohepatitis [12].

Administration of allopurinol, FeTPPS and 1400W

Allopurinol, a specific inhibitor of xanthine oxidase, was administered in a single bolus dose of 35 mg/kg through the i.p. route, 30 min prior to carbon tetrachloride treatment [13]. In other studies, the iNOS inhibitor 1400W was administered through the intraperitoneal route at a dose of 10 mg/kg, 1 h before carbon tetrachloride treatment, using an intraperitoneal route [14]. FeTPPS was administered at 30 mg/kg, 1 h prior to CCl_4 treatment [10,15].

Administration of mouse recombinant leptin and leptin neutralization

ob/*ob* mice received recombinant leptin (100 μg/mice) twice daily for 5 days prior to CCl_4 administration through the intraperitoneal route. Leptin antibody was used to neutralize the circulating leptin in DIO mice. DIO mice were treated for 2 days prior to CCl_4 administration either with 100 μg of control mouse IgM or with mouse leptin Abs intraperitoneally in a total volume of 100 μl of PBS [16].

Isolation of Kupffer cells

Kupffer cells were isolated as per the protocol by Froh *et al.* [17]. Qualitative screening for Kupffer cells was carried out with immunoreactivity against a CD68 antibody. Cultures with >80% CD68-positive cells were used for the experiments.

Enzyme-linked immunosorbent assay

Immuno-spin trapping, a method for detection of free radical formation, was used, and immunoreactivity for DMPO nitrone adducts and nitrotyrosine was detected in liver homogenates and Kupffer cell lysates using standard ELISA [10].

Western blot analysis

Liver homogenates were resolved in 4–10% Bis-Tris gels using SDS–PAGE, and subjected to Western blot analysis.

Histopathology

For each animal, sections of the liver were collected and fixed in 10% neutral buffered formalin. For histological examinations, formalin-fixed liver sections were stained with hematoxylin/eosin (H&E) and observed under a light microscope.

Real-time reverse transcription–polymerase chain reaction analysis

Gene expression levels in tissue samples were measured by real-time reverse transcription–polymerase chain reaction analysis as described in Supplementary material.

Confocal laser scanning microscopy (Zeiss LSM 510 UV meta)

Frozen tissue sections after formalin fixation were analyzed by confocal microscopy, Zeiss LSM710-UV meta (Carl Zeiss, Inc., Oberkochen, Germany), using a Plan-NeoFluor 40×/1.3/40× Oil DIC objective with different zoom levels.

Macrophage depletion by $GdCl_3$ and liposomal clodronate

Mice were injected with gadolinium chloride (10 mg/kg) through the i.v. route 24 h prior to CCl_4 treatment, as described by Rai *et al.* [18]. Liposomal clodronate was injected through intravenous injections at a dose of 4 μl/g of mice (Clophosome™; Formumax, Pao Alto, CA), 24 h prior to CCl_4 treatment.

Adoptive transfer of leptin primed cells

Mouse non-parenchymal cells (mostly Kupffer cells) were isolated as per Froh *et al.* [17]. Cells were washed and plated in 35 mm^2 dishes using 10% FBS containing DMEM with mouse recombinant leptin (500 ng/ml). The dose was selected on the basis of the concentration used by Wang *et al.* (10–100 nmoles/L) [4]. The cells were harvested at 18 h and 1×10^6 cells/mouse were injected through the tail vein into macrophage depleted mice. The recipient mice were macrophage-depleted by the administration of the macrophage toxin gadolinium chloride.

Statistical analyses

All *in vivo* experiments were repeated three times with 3 mice per group (N = 3; data from each group of three mice was pooled). The statistical analysis was carried out by analysis of variance (ANOVA) followed by a *post hoc* test. Quantitative data from Western blots, as depicted by the relative intensity of the bands, were analyzed by performing a Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Increased leptin levels cause oxidative and nitrosative stress in DIO-steatohepatitic mice

Our results indicated that diet-induced obese mice had significantly higher leptin levels as compared to lean control mice (Fig. 1A), which is in line with human studies [19]. Our previous study established a free radical-based mechanistic model of non-alcoholic steatohepatitis where low-dose CCl_4 administration induced non-alcoholic steatoheaptitis in obese mice [12]. Furthermore, DIO mice treated with CCl_4 had significantly higher serum leptin levels when compared to both untreated DIO and CCl_4-treated lean control mice (Fig. 1A). The study showed that leptin deficiency significantly decreased protein radical formation. We found that when CCl_4-treated leptin deficient mice and CCl_4-treated DIO mice were administered a neutralizing antibody against leptin, they had significantly decreased protein DMPO nitrone adduct formation, a measurement of free radical formation on proteins (Fig. 1B), compared to DIO mice treated with CCl_4 only [20]. Treating leptin deficient mice with a seven-day course of recombinant leptin increased their protein radical formation in response to CCl_4 administration to levels that were comparable to those in wild type DIO mice (Fig. 1B).

Since protein 3-nitrotyrosine formation originates from tyrosyl radicals reacting with reactive nitrogen species [21], we

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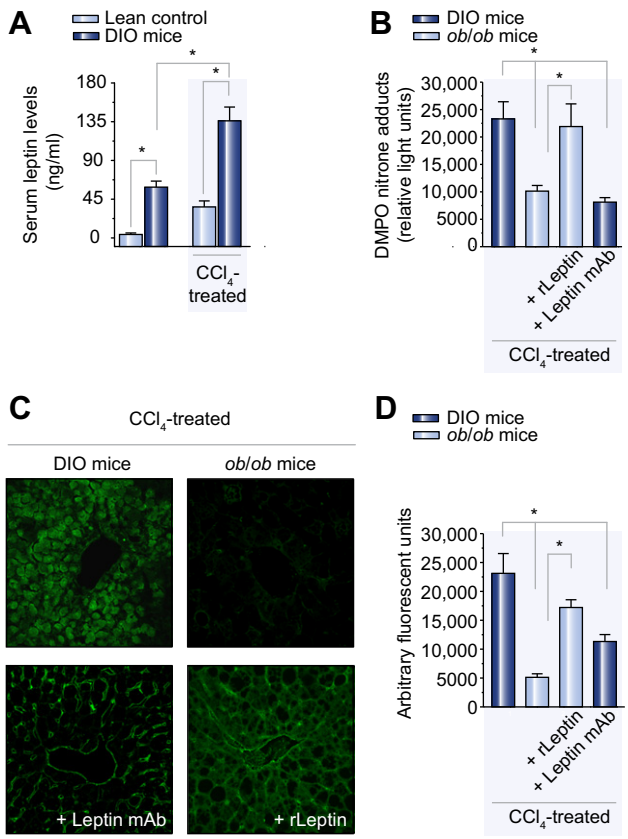

Fig. 1. Increased leptin levels cause free radical and nitrosative stress in DIO-steatohepatitic mice. Lean control or high-fat fed diet induced obese (DIO) mice were treated with either olive oil (vehicle) or CCl_4. (A) Serum levels at 24 h post CCl_4. (B) Liver homogenates from DIO, *ob*/*ob*, leptin supplemented and DIO mice injected with leptin antibody were subjected to immuno-spin trapping and anti-DMPO immunoreactivity was measured using ELISA. (C) Frozen liver slices were analyzed for 3-nitrotyrosine immunoreactivity. (D) Relative fluorescence intensities of 3-nitrotyrosine immunoreactivity from mouse livers (n = 4). $^{*}p$ <0.05.

probed nitrotyrosine formation as a stable post-translational oxidative modification. Results from laser scanning confocal microscopy showed both sinusoidal and centrolobular 3-nitrotyrosine immunoreactivity in DIO mouse livers treated with CCl_4 and in leptin deficient mice treated with CCl_4 and recombinant leptin (Fig. 1C). However, leptin deficient mice and DIO mice administered leptin monoclonal antibody, similarly treated with CCl_4, had 3-nitrotyrosine immunoreactivity primarily in the sinusoids.

Finally, quantification of fluorescent intensities of 3-nitrotyrosine immunoreactivity from these groups showed that when CCl_4-treated leptin deficient mice and CCl_4-treated DIO mice were administered a neutralizing antibody against leptin, they had significantly decreased 3-nitrotyrosine formation (Fig. 1D) compared to DIO mice treated with CCl_4. However, nitrotyrosine reactivity increased significantly following a seven-day course of recombinant leptin treatment.

Serum levels of leptin might have originated from both the adipose tissue and liver since Western blot analysis of leptin showed significant increases in both liver and adipose tissue following CCl_4 treatment (Supplementary Fig. 2A).

Leptin augments proinflammatory cytokines that are markers of Kupffer cell activation

Oxidative stress can play a significant role in Kupffer cell activation [12,22,23]. Kupffer cell activation can be marked by increased release of TNF-α and monocyte chemoattractant protein-1 (MCP-1). Thus we analyzed leptin-induced effects on TNF-α and MCP-1 in DIO mice treated with CCl_4. We found that CCl_4-treated leptin deficient mice and CCl_4-treated DIO mice, administered neutralizing antibody against leptin, had significantly decreased TNF-α and MCP-1 levels (Supplementary Fig. 1A) compared to DIO mice treated with CCl_4 alone. However, pre-treating leptin deficient mice with a seven-day course of recombinant leptin restored their post-CCl_4 TNF-α and MCP-1 levels to those of wild type DIO mice that were treated with CCl_4 (Supplementary Fig. 1B). Liver activation of NFκB was also increased in DIO mice treated with CCl_4 as seen from increased translocation of p65 unit to the nucleus (Supplementary Fig. 2C).

Peroxynitrite from NADPH oxidase and iNOS activity is a key regulator of Kupffer cell activation in steatohepatitic injury

When leptin deficient mice and DIO mice, administered a monoclonal antibody against leptin, were treated with CCl_4, their levels of *p47 phox* and *iNOS* mRNA expression were significantly less (Fig. 2A and B) compared to DIO mice treated with CCl_4. When leptin deficient mice were pretreated with recombinant leptin before CCl_4 exposure, expression of *p47 phox* mRNA increased significantly from the leptin deficient level to a point near to, but less than that of (hyperleptinemic) DIO mice treated with CCl_4 (Fig. 2A), while *iNOS* mRNA expression also increased significantly when compared to leptin deficient mice identically treated, and was comparable to that in DIO mice treated with CCl_4 (Fig. 2B). The significant increase in mRNA expression of both *p47 phox* and *iNOS* in DIO mice was observed 24 h following treatment with CCl_4, thus coinciding with the increase in protein radical formation and nitrotyrosine formation. Since we observed a significant leptin-dependent increase in both *p47 phox* and *iNOS* mRNA expression, we explored the role of peroxynitrite in the formation of protein radicals and tyrosine nitration. Peroxynitrite is a key oxidant species, and nitric oxide formed from iNOS reacts with superoxide, in a diffusion controlled rate, to form peroxynitrite. We used the peroxynitrite decomposition catalyst FeTPPS administered *in vivo* to assess the role of peroxynitrite as reported by Chatterjee *et al.* [15] and others [24,25]. In DIO mice treated with CCl_4 and administered FeTPPS, protein radical formation and tyrosine nitration were significantly decreased (Fig. 2C and D). They also decreased significantly when mice were administered the NADPH oxidase inhibitor apocynin or the iNOS inhibitor 1400W. When fed with a high-fat diet and treated with CCl_4, mice that lacked the NADPH oxidase subunit p47 phox or iNOS had significantly decreased protein radical formation and tyrosine nitration compared to DIO mice treated with CCl_4 (Fig. 2C and D).

Confocal laser scanning microscopy showed decreased protein radical adducts and nitrotyrosine immunoreactivity in the centrolobular regions of CCl_4-treated mouse livers, after administration of FeTPPS, compared to CCl_4-treated DIO mice after vehicle treatment only (without FeTPPS) (Fig. 2E). FeTPPS also had a significant effect in reducing both mRNA and protein levels of proinflammatory cytokines that exacerbate sterile inflammation in

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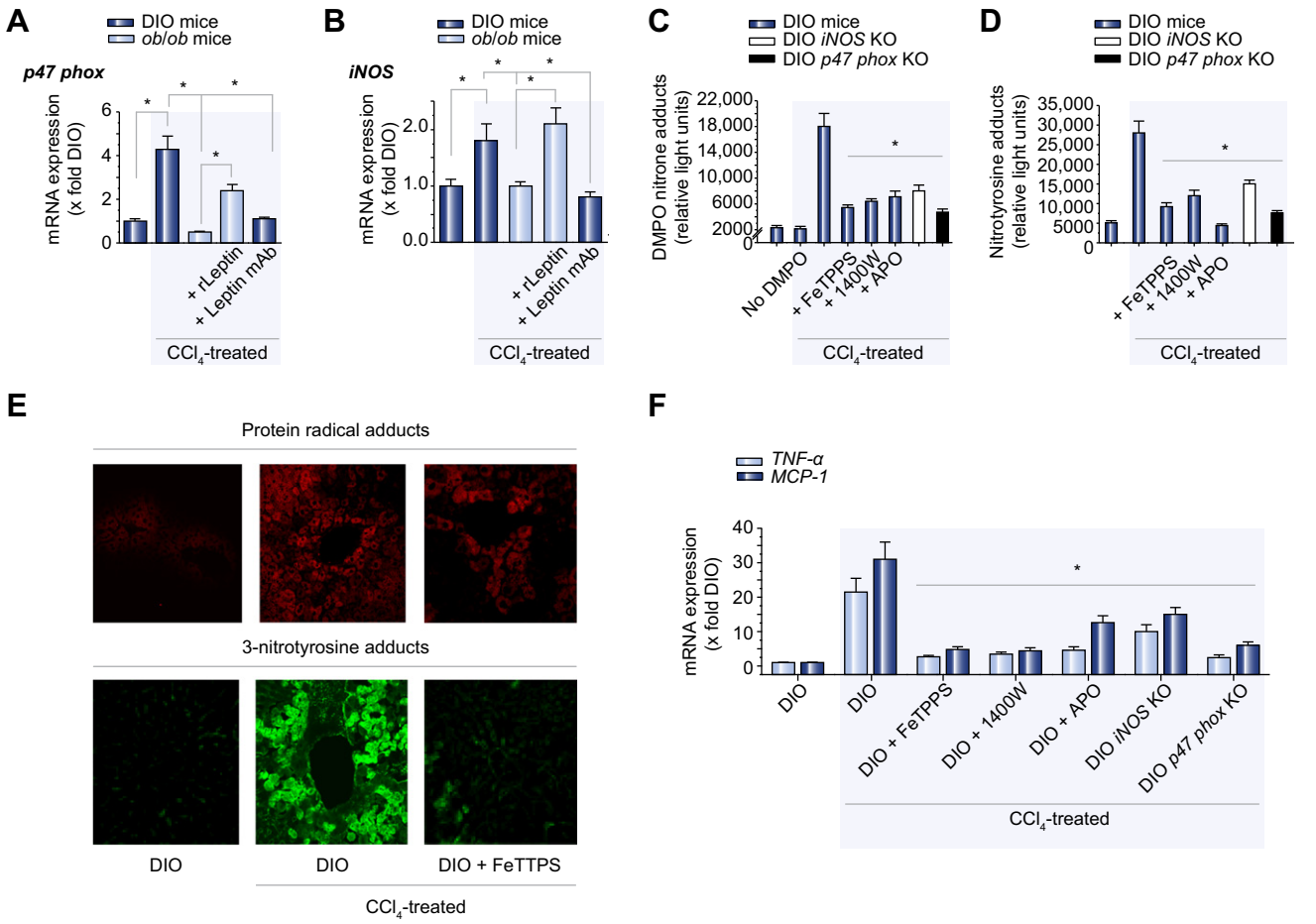

Fig. 2. Peroxynitrite from NADPH oxidase and iNOS activity is a key regulator in Kupffer cell activation in steatohepatitic injury. (A) Quantitative real-time PCR analysis of *p47 phox* and (B) *iNOS* mRNA expression in DIO, DIO + CCl4, *ob*/*ob* mice, with or without leptin supplementation, and DIO + CCl4 mice treated with leptin monoclonal antibody. (C) Liver homogenates from CCl_4-treated DIO mice, either challenged with the peroxynitrite decomposition catalyst FeTPPS or iNOS inhibitor 1400W or NADPH oxidase inhibitor apocynin; or using *iNOS* and *p47 phox* knockout mice, were assayed for anti-DMPO immunoreactivity, and (D) for 3-nitrotyrosine immunoreactivity using ELISA. (E) Localization of DMPO nitrone adducts (red, upper panel) or nitrotyrosine adducts (green, lower panel) was analyzed by confocal laser scanning microscopy of liver slices from CCl_4-treated mice, with or without the peroxynitrite decomposition catalyst FeTPPS, and is a representative of liver slices collected from 4 mice (n = 4). (F) Inhibitors of peroxynitrite decrease macrophage activation. Liver homogenates were assayed for *TNF-α* and *MCP-1* mRNA expression. The figure represents data from 3 mice/group and $^*p < 0.05$.

steatohepatitis. In CCl_4-treated DIO mice administered FeTPPS, both *TNF-α* and *MCP-1* mRNA expression and protein levels were significantly decreased, compared to DIO mice treated with CCl_4 alone (Figs. 2F and 3A–D). Similarly, pharmacologically inhibiting either iNOS or NADPH oxidase or using *p47 phox* and *iNOS* gene-deleted mice, resulted in significantly decreased *TNF-α* and *MCP-1* mRNA and protein levels post-CCl_4 treatment, compared to DIO mice treated with CCl_4 (2F and 3A–D).

Leptin-mediated protein radical and 3-nitrotyrosine formation and release of the proinflammatory cytokines TNF-α and MCP-1 are Kupffer cell and macrophage dependent.

Resident liver macrophages (primarily Kupffer cells) and infiltrating neutrophils and other immune cells that exhibit macrophage-like function are found to express both the vascular form of NADPH oxidase and iNOS. Consistent with that concept, we found that protein radical formation and tyrosine nitration were significantly reduced upon administration of the macrophage toxin $GdCl_3$ or liposomal clodronate to DIO mice before treatment with CCl_4 (Fig. 4A and B; Supplementary Fig. 6), and they were not restored upon further supplementation of recombinant leptin. However, they were significantly increased after adoptive transfer of leptin-primed Kupffer cells into DIO mice treated with CCl_4 or *ob*/*ob* mice (Fig. 4A and B; Supplementary Fig. 6). Since TNF-α and MCP-1 release from macrophages contribute to steatohepatitic lesions in obesity following treatment with CCl_4, it was important to explore whether the leptin effect on release of the cytokines was also macrophage dependent. Results indicated that administration of macrophage toxin $GdCl_3$ to DIO mice before treatment with CCl_4, significantly decreased TNF-α and MCP-1 release (Fig. 4C and D; Supplementary Fig. 6). These results thus clearly suggested that leptin was acting through macrophages in the CCl_4-treated DIO mouse liver in exacerbating the steatohepatitic lesions in these mice.

Leptin contributes to steatohepatitic lesions in CCl_4-treated DIO mice

To examine the effect of leptin in inducing steatohepatitic lesions following CCl_4 administration in DIO mice, we compared the histopathology of steatohepatitis in leptin deficient mice and mice fed with a high-fat diet. DIO mice with higher leptin levels,

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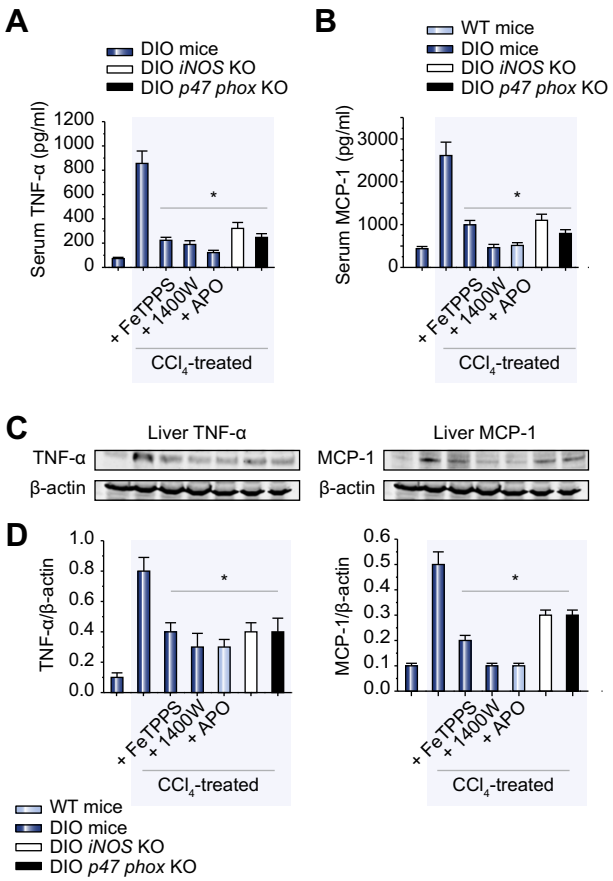

Fig. 3. Leptin-induced oxidative stress and peroxynitrite formation activate Kupffer cells. Serum and liver tissue (A and C) TNF-α and (B and D) MCP-1 analyses following blockade of peroxynitrite formation. The figure represents data from 3 mice/group and $^{*}p$ <0.05.

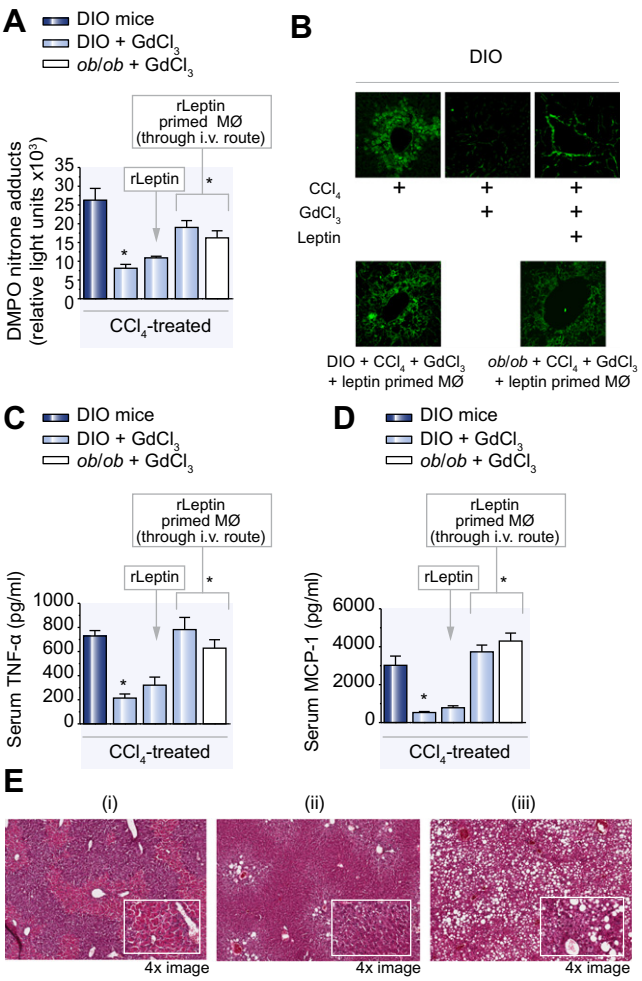

Fig. 4. Leptin action through liver macrophages and Kupffer cells is responsible for peroxynitrite chemistry and subsequent free radical formation in steatohepatitc injury. Macrophage toxin gadolinium chloride was used to deplete liver macrophages and was followed by CCl_4 treatment in DIO mice. In certain experimental groups, which had their macrophages depleted, either only leptin or leptin primed macrophages were infused through tail vein. (A) Whole liver homogenates from these mice were then analyzed for anti-DMPO immunoreactivity by ELISA. (B) Liver slices of mice from the above experimental set-up and adoptive transfer of leptin or leptin primed macrophages were subjected to confocal laser scanning microscopy to analyze 3-nitrotyrosine immunoreactivity and localization. Analysis of serum TNF-α (C), and MCP-1 (D) was assayed by ELISA. The figure represents data from 3 mice/group and $^{*}p$ <0.05 was considered statistically different. (E) H&E of the liver of obese, CCl_4-treated mice administered either saline (i) or FeTPPS (ii) or CCl_4-treated *ob*/*ob* mice (iii). The images represent both a 4× magnification and a 20× magnification (inset). MØ, macrophage.

treated with CCl_4 had centrolobular necrosis and showed markers of early steatohepatitic injury (Fig. 4Ei). When DIO mice that received FeTPPS (Fig. 4Eii), or leptin deficient mice (*ob*/*ob*) (Fig. 4Eiii) were administered CCl_4, they showed steatosis, periportal necrosis and occasional necrotic areas around the central vein (Zone III), with little or no leukocyte accumulation compared to wild type DIO mice treated with CCl_4 (Fig. 4Ei), which had centrilobular necrosis, infiltration of leukocytes, and ballooned hepatocytes. This result suggests that leptin contributes significantly in inducing early inflammatory lesions in DIO mice treated with CCl_4. Consistent with this result, serum ALT levels in DIO mice significantly increased compared to leptin deficient mice administered CCl_4 (Supplementary Fig. 2B). Kupffer cell aggregation near the perivenular regions, a hallmark of early steatohepatitic lesions, was also evident by confocal microscopy of CD68 positive cells (Supplementary Fig. 7). Histological Activity Index score was found to be significantly higher for DIO mice treated with CCl_4 when compared to *ob*/*ob* mice (Supplementary Table 1).

Discussion

This is the first report that shows that leptin-mediated protein radical formation, tyrosine nitration and activation of Kupffer cells are caused by peroxynitrite formation, and demonstrates that this process exacerbates CCl_4-induced steatohepatitic lesions in diet-induced obesity. We found that exposing mice with DIO to a potential "2nd hit" from CCl_4 caused a twofold increase in circulating leptin levels (Fig. 1). This result was central to our subsequent investigations, which established that leptin-induced formation of peroxynitrite was key to drive the ensuing inflammatory processes. Higher leptin levels, as a result of CCl_4 administration in DIO mice, might be due to the release of IL-beta, as shown previously by others [26]. Since diet-induced obese mice that were treated with CCl_4 had higher leptin levels (2-fold higher than vehicle-treated obese mice), and leptin is known to cause

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oxidative stress [27], we investigated the contribution of leptin to the protein radical formation and tyrosine nitration in early steatohepatitis. In *ob*/*ob* mice and DIO mice, treatment with a neutralizing antibody against leptin significantly reduced protein radical formation, whereas leptin supplementation for 7 days restored higher protein radical formation and tyrosine nitration, suggesting that leptin is responsible for the redox imbalance in the liver in early steatohepatitic injury (Fig. 1B–D). This result assumes significance since leptin is known to cause oxidative stress in different tissues and cells in response to inflammatory stress [6–8]. Furthermore, leptin-mediated reactive oxygen species generation was found to activate stellate cell proliferation, a known phenomenon in steatohepatitic injury [8]. However, little is known with regard to the redox mechanisms by which Kupffer cells promote liver inflammation and fibrosis in obesity. Our results indicated that in addition to leptin's involvement in protein radical formation and tyrosine nitration, it is also involved in activating Kupffer cell release of TNF-α and MCP-1 (Supplementary Fig. 1), but how the cytokine release and Kupffer cell activation are related to the redox processes initiated by leptin remained unknown at this point.

Therefore, to establish the mechanism of free radical formation by leptin action, we analyzed the mRNA expression of enzymes that are significant contributors to production of free radical species in inflammatory microenvironments. We found that levels of expression of inducible *NOS* and *p47 phox* mRNA were significantly elevated in early steatohepatitic injury and leptin-supplemented *ob*/*ob* mice (Fig. 2A–B). In addition, *ob*/*ob* mice had less induction of mRNA for these enzymes. This data suggested that nitric oxide and superoxide radicals play a significant role in the protein radical formation process by leptin. Furthermore, since protein radical formation and tyrosine nitration were localized in Kupffer cells [12], there was a possibility that Kupffer cells, which are known to express both NADPH oxidase and inducible NOS, might form peroxynitrite from superoxide and nitric oxide, respectively, at a diffusion-controlled rate. Such a reaction is feasible since both species are found in the same cell at the same time. Our results showed that the peroxynitrite decomposition catalyst FeTPPs, the NADPH oxidase inhibitor apocynin and the iNOS inhibitor 1400W, all significantly inhibited protein radical formation and tyrosine nitration (Fig. 2C and D). High-fat fed mice, depleted of iNOS and the NADPH oxidase subunit p47 phox, and treated with CCl_4, showed significant decreases in protein radical formation and tyrosine nitration, thus confirming the role of these enzymes in the formation of peroxynitrite.

The role of peroxynitrite formation in the activation of Kupffer cells was established by examining subsequent *TNF-α* and *MCP-1* mRNA expression in the presence of FeTPPS and inhibitors of iNOS and NADPH oxidase, as well as the use of gene-deficient mice for both *iNOS* and *p47 phox* (Fig. 2F). The radicals that are generated from peroxynitrite form stable post-translational modifications of proteins that can affect their function and contribute to the activation of inflammatory pathways [15]. In the present model of early steatohepatitic injury, Kupffer cell activation (there was an increase in both CD68 and F4/80 immunoreactivity in liver tissues of CCl_4-administered DIO mice as compared to DIO and *ob*/*ob* mice; Supplementary Fig. 3.) and production of the inflammatory cytokines TNF-α and MCP-1 might have resulted from loss or gain of function of the proteins that modulated the induction response of these genes; however, this point remains speculative at present. Though there may be multiple sources of these cytokines, including stellate cells, Kupffer cells were found to be a principal source of MCP-1 as shown in Supplementary Fig. 4B.

Although much of our attention in this study was focused on the ability of either resident macrophages or infiltrating leukocytes to promote leptin-induced formation of peroxynitrite, the data so far is incomplete without further confirmatory evidence of their involvement in leptin-induced redox changes, inflammation, and progression of steatohepatitic injury. To establish the site of leptin action, we first depleted DIO mice of macrophages by pre-treating them with $GdCl_3$ and then adoptively transferred leptin-primed Kupffer cells into both DIO mice and leptin deficient *ob*/*ob* mice. Results showed that protein radical formation, tyrosine nitration, and release of proinflammatory cytokines were restored in macrophage-depleted mice only when leptin primed Kupffer cells were adoptively transferred (Fig. 4). Furthermore, supplementation with leptin only (without macrophages) in these mice had no effect. Similarly, macrophage-depleted mice that were leptin deficient exhibited increased protein radical formation, tyrosine nitration and release of proinflammatory cytokines, following adoptive transfer of leptin-primed macrophages, suggesting the essential roles of both leptin and macrophages in potentiating redox-mediated steatohepatitic injury (Fig. 4). Our results also establish that leptin and macrophages are symbiotic in their actions in promoting inflammation in the fatty liver, and it might be speculated that the process involves multiple signaling mechanisms and intermediary molecules [28].

Finally, we report a leptin-mediated oxidative stress mechanism for inflammatory events promoted by the formation of peroxynitrite in macrophages of the steatohepatitic liver.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.11.035.

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