Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

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

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

Received 7 March 2012; received in revised form 16 November 2012; accepted 20 November 2012; available online 1 December 2012

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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.

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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