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Proinflammatory Adipokine Leptin Mediates Disinfection Byproduct Bromodichloromethane-induced Early Steatohepatic Injury in Obesity

Suvarthi Das^{1,#}, Ashutosh Kumar^{2,#}, Ratanesh Kumar Seth¹, Erik J Tokar³, Maria B. Kadiiska², Michael P Waalkes³, Ronald P Mason², and Saurabh Chatterjee^{1,*}

¹Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia SC 29208

²Free Radical Metabolism Group, Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

³Inorganic Toxicology Group, National Toxicology Program Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Abstract

Today's developed world faces a major public health challenge in a rise in the obese population and an increased incidence in fatty liver disease. There is a strong association among diet induced obesity, fatty liver disease and development of nonalcoholic steatohepatitis but the environmental link to disease progression remains unclear. Here we demonstrate that in obesity, early steatohepatic lesions induced by the water disinfection byproduct bromodichloromethane are mediated by increased oxidative stress and leptin which act in synchrony to potentiate disease progression. Low acute exposure to bromodichloromethane (BDCM), in diet-induced obesity produced oxidative stress as shown by increased lipid peroxidation, protein free radical and nitrotyrosine formation and elevated leptin levels. Exposed obese mice showed histopathological signs of early steatohepatic injury and necrosis. Spontaneous knockout mice for leptin or systemic leptin receptor knockout mice had significantly decreased oxidative stress and TNF- α levels. Co-incubation of leptin and BDCM caused Kupffer cell activation as shown by increased MCP-1 release and NADPH oxidase membrane assembly, a phenomenon that was decreased in Kupffer cells isolated from leptin receptor knockout mice. In obese mice that were BDCM-exposed, livers showed a significant increase in Kupffer cell activation marker CD68 and, increased necrosis as assessed by levels of isocitrate dehydrogenase, events that were decreased in the absence of leptin or its receptor. In conclusion, our results show that exposure to the disinfection byproduct BDCM in diet-induced obesity augments steatohepatic injury by

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*Author for correspondence: Dr. Saurabh Chatterjee, Ph.D. ¹Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, University of South Carolina, Columbia 29208 USA. schatt@mailbox.sc.edu; Tel: 803-777-8120; Fax: 803-777-3391.

#Suvarthi Das and Ashutosh Kumar contributed equally to the work.

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potentiating the effects of leptin on oxidative stress, Kupffer cell activation and cell death in the liver.

Keywords

Adipocytokines; Kupffer cell; oxidative stress; lipid peroxidation; tyrosine nitration; necrosis; tumor necrosis factor; OB/OB mice

Introduction

Childhood and adolescent rates of obesity and overweight have assumed pandemic proportions in the last decade. Risk factors such as diet composition, excess caloric intake, decreased exercise, genetics, and the built environment are perceived as causes of obesity and associated co-morbidities (Farooqi, 2011) (Rius *et al.*, 2012). Obesity is considered to be a low inflammatory condition, following a number of studies in the last decade (Johnson *et al.*, 2012). The increased blood levels of proinflammatory cytokines and adipokines such as leptin influence the inflammatory state in obesity (Ikejima *et al.*, 2001). Obesity-associated co-morbidities include lung, liver and cardiovascular complications (Rius *et al.*, 2012). In parallel, it is perceived that with increased obesity there is also an increase in liver disorders such as non-alcoholic steatohepatitis, cirrhosis and in extreme cases hepatocarcinoma (Shen *et al.*, 2012). Several hypotheses have been put forward for the progression of fatty liver to inflammatory liver disease in obesity. The two-hit hypothesis and the multi-factorial hypothesis point to a secondary assault apart from obesity to be a relevant cause for liver diseases (Day and James, 1998; Tilg and Moschen, 2010). In recent literature, we and others have shown that CCl₄ exposure, albeit in lower doses, can sensitize the obese liver to hepatotoxicity and development of early steatohepatitic injury (Ikejima *et al.*, 2001; Chatterjee *et al.*, 2012b). A recent study from Cave M *et al.* shows the involvement of the built environment in the development of liver diseases in humans (Cave *et al.*, 2010). The study explicitly shows the association of polychlorinated biphenyls and heavy metals to non-alcoholic steatohepatitis in American adults. Lately the involvement of cytochrome p450s in the metabolism of these environmental toxicants has been an active area of research (Abdelmegeed *et al.*, 2012; Leung and Nieto, 2012). The role of CYP2E1 has been extensively studied and it has been found to have a close association with reactive oxygen species generation and development of steatohepatitic injury (Parola and Marra, 2011). Our research has shown previously that free radical metabolism of the model toxin CCl₄ by CYP2E1 causes the release of damage-associated molecular patterns (DAMPS) and increased release of leptin from both hepatic and adipose tissues (Chatterjee *et al.*, 2012a; Chatterjee *et al.*, 2012b). These studies assume significance because both reactive oxygen species (ROS) and leptin have been found to exacerbate the progression of steatosis to steatohepatitis in obesity (Parola and Marra, 2011), (Chatterjee *et al.*, 2012a). Similar to CCl₄, which produces trihalomethyl radicals upon metabolism by CYP2E1, several trihalomethanes have been found to be metabolized by CYP2E1 to produce noxious free radicals (Tomasi *et al.*, 1985; Lilly *et al.*, 1997; Mezyk *et al.*, 2006). The disinfection byproducts of drinking water, which are formed from reaction with the bromine in water following chlorine disinfection, produce ROS (Lilly *et al.*, 1997). Although direct exposure to high doses of environmental toxins is rare, low exposures from the environment are far more common. These doses may be well-tolerated by normal healthy individuals but can be potential risk factors for inflammatory liver injuries such as steatohepatitis in obese persons. This risk assumes significance in terms of public health because there is presently an alarming rise in obesity in the United States alone. The National Toxicological program identifies several disinfection byproducts for drinking water that are hepatotoxic (Lilly *et al.*, 1997; Keegan *et al.*, 1998; 2011) and are bioactivated by liver cytochrome p450 enzymes

leading to generation of free radicals (Tomasi *et al.*, 1985; Lilly *et al.*, 1997). The resulting oxidative stress from the reductive metabolism of bromodichloromethane (BDCM) can lead to amplification of hepatotoxicity by triggering host innate immune responses. Innate immune events such as activation of macrophages through secretion of an array of cytokines can amplify the risk of developing full-blown inflammatory diseases like early steatohepatic injury leading to fibrosis and cirrhosis if exposed on a chronic basis.

Leptin is a 167-amino acid protein discovered in 1994 by positional cloning of the ob gene (Kamada *et al.*, 2008; Rius *et al.*, 2012). Though it is considered to be an anorexigenic hormone, its levels are elevated in obesity as a result of resistance to its actions in the hypothalamus, a condition called central resistance (Konner and Bruning, 2012). Leptin is thought to contribute, in part, to NASH development in obesity through its proinflammatory actions on sinusoidal epithelial cells and Kupffer cells (Ikejima *et al.*, 2001; Li *et al.*, 2002; Krawczyk *et al.*, 2009; Wang *et al.*, 2009). Recent lines of evidence support the role of elevated levels of leptin found in obesity in generating reactive oxygen and reactive nitrogen species and subsequent free radical formation (De Minicis *et al.*, 2008). Free radical production appears to contribute to the progression of steatohepatitis and its resultant hepatotoxicity (Handy *et al.*, 2011). The presence of high levels of leptin in obesity certainly makes it a prime candidate for amplifying the risk of NASH progression as both a first and second hit, which not only satisfies the two-hit hypothesis, but also is in line with the multi-hit paradigm (Tilg and Moschen, 2010). Our own studies have demonstrated that leptin mediates the effect on NASH progression through peroxynitrite formation and Kupffer cell activation in a toxin model of NASH.

Our present study tests the hypothesis that low levels of BDCM exposure, which have been studied for the formation of dihalomethyl radicals in ESR studies, produces ROS in the obese liver and the resultant oxidative stress links early steatohepatic injury. We also examine the role of leptin in mediating the progression of simple steatosis to early steatohepatic injury following acute BDCM exposure. We show here, for the first time the direct role of BDCM in inducing steatohepatic injury in the obese mouse liver through the involvement of increased leptin, resulting in Kupffer cell activation, TNF- α -release, and cell death.

Materials and Methods

Materials

The spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Dojindo Molecular Technologies, Kumamoto, Japan. Bromodichloromethane, Collagenase Type IV, apocynin and gadolinium chloride (GdCl₃) were purchased from Sigma Chemical Company, St Louis, MO. Tempol was purchased from Sigma. Mouse recombinant leptin and neutralizing antibody to mouse leptin were purchased from R&D Systems (R&D Systems Inc, Minneapolis, MN). All other chemicals were of analytical grade and were purchased from Sigma Chemical Company or Roche Molecular Biochemicals (Mannheim, Germany). All aqueous solutions were prepared using water passed through a Picopure 2UV Plus system (Hydro Services and Supplies, Inc., RTP, NC) equipped with a 0.2 μ m pore size filter.

Obese mice

Custom DIO adult male, pathogen-free, 10-week-old mice with a C57BL/6J background (Jackson Laboratories, Bar Harbor, Maine) were used as models of diet-induced obesity. The mice were fed with 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 to a cage before any experimental use. Mice that contained the disrupted OB gene (leptin) (B6.V-Lep^{ob}/J) (Jackson Laboratories) and disrupted DB gene (leptin receptor) (B6.BKS(D)-Lepr^{db}/J) 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 both at NIEHS and the University of South Carolina at Columbia.

Induction of liver injury in obese mice

DIO mice or high fat-fed gene-specific knockout mice at 16 weeks were administered BDCM (2.0 mmoles/kg, diluted in olive oil) through the intraperitoneal route. BDCM was administered once to the mice for inducing acute liver injury, at a dose which was lower than the dose which caused hepatocellular necrosis in normal mice or rats (1987). Tissues and blood were collected at 6 h, 24 h and 48 h following administration of BDCM. The endpoint was selected to assess early steatohepatic injury.

Isolation of Kupffer cells

Kupffer cells were isolated as per the protocol by Froh, et al. (Current Protocols in Toxicology, 14.4.1–14.4.12, 2002) with some modifications. Briefly, mice were anesthetized and their livers were perfused initially by sterile HBSS and later by 0.03% collagenase at a flow rate of 5 ml/minute. Following perfusion, the liver was dissected and incubated with 0.03% collagenase for another 30 minutes at 37°C. The tissue was minced and cells were extracted with a syringe piston. The cell suspension was filtered through a 75 µm cell strainer (BD Falcon). The resultant single cell suspension was centrifuged at 50g for 3 minutes to obtain parenchymal cells (mostly hepatocytes). The supernatant was further centrifuged at 650xg and the pellet was resuspended in 10 ml of HBSS. The suspension was loaded on a Percoll gradient and centrifuged at 1500 g for 15 minutes to obtain the Kupffer cells. The isolated cells were plated onto 35mm plastic, glass bottomed dishes for adherence. Qualitative screening for Kupffer cells was carried out with immunoreactivity against the CD68 antibody. Cultures with >80% CD68-positive cells were used for experiments.

Kupffer cell culture

10⁵ isolated Kupffer cells were cultured in 24 well plates with 5 mM BDCM and 100 µg/ml recombinant leptin. Following 24 h incubation, the supernatant was collected for analysis of cytokines, and cell lysates were used for DMPO nitron adduct and nitrotyrosine immunoreactivity.

Enzyme-linked immunosorbent assay

Immunoreactivity for DMPO nitron adducts and nitrotyrosine was detected in liver homogenates and Kupffer cell lysates using standard ELISA (Chatterjee *et al.*, 2009). TNF-α and MCP-1 release was estimated in serum using ELISA kits from BD Pharmingen and Thermo Scientific, respectively, following the manufacturer's protocols.

Histopathology

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

TUNEL Assay

Liver tissue from mice were fixed overnight in 10% neutral-buffered formalin (Sigma Aldrich, Missouri, USA), and paraffin-embedded sections (5 μ m thickness, courtesy Instrumentation Resource Facility, School of Medicine, University of South Carolina, USA) prepared from them were used for TUNEL staining. TACS 2TdT DAB in situ Apoptosis Detection Kits were supplied by Trevigen Inc. Maryland, USA. Stained sections were imaged under high power (40X, 25.2X magnification) using BX43 microscope and DP73 camera from Olympus America Inc., Pennsylvania, USA.

Western Blotting

100 mg of tissue from each liver sample was homogenized in 1ml of RIPA buffer (Sigma Aldrich) using a Dounce homogenizer. Homogenate was centrifuged, the supernatant was diluted 1:10 and used for Western blotting. We used Novex (Invitrogen, California, USA) 4 – 12% bis-tris gradient gel. Rabbit anti-mouse primary antibodies against isocitrate dehydrogenase (IDH) and β -actin (positive control); and goat anti-rabbit HRP-conjugated secondary antibody were obtained from AbCam plc (Massachusetts, USA). The dilutions used were: anti-IDH – 1:1000, anti- β -actin – 1:500, secondary antibody – 1:10000. A Trans – Blot Turbo transfer system (Bio-Rad Laboratories Inc., California, USA) and pre-cut nitrocellulose blotting membrane/filter paper sandwiches (Bio-Rad) were used to perform a 7-minute transfer. Pierce ECL Western Blotting substrate (Thermo Fisher Scientific Inc., Illinois, USA) was used. BioMax MS Films and cassettes (with intensifying screen) were from Kodak.

Quantitative Real-Time Polymerase Chain Reaction Analysis (qRT-PCR)

Gene expression levels in tissue samples were measured by quantitative real-time polymerase chain reaction analysis. Tissue samples (85–100 mg) were taken from each liver and individually homogenized using a Tissue Tearor variable speed homogenizer. Total RNA was isolated from homogenates with the use of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and purified with the use of RNeasy mini-kit columns (Qiagen, Valencia, CA). Purified RNA was reverse transcribed to complementary DNA with the use of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primers. Gene-specific primers were designed with the use of Primer Express 3.0 software (Applied Biosystems, Foster City, CA).

The ABsolute SYBR Green ROX Mix (ABgene, Rockford, IL) was used for amplification. Amplification conditions were as follows: 15 minutes at 95°C, followed by 40 cycles of 95°C for 1 minute and 60°C for 1 minute. Cycle threshold (Ct) values for the selected genes were normalized to values for 18S in the same sample. For each liver, tissue samples were collected and tested separately with triplicate Ct values collected each time. DIO mouse liver was used as the control for comparison with all other livers. Ct values for all controls were set at 100%. The sequences for the primers used for RT-PCR are provided in 5' to 3' orientation.

TNF: Forward – AGCCACGTCGTAGCAAACCACCAA

Reverse – AACACCCATTCCCTTCACAGAGCAAT

Mcp-1: Forward - CTTCTGGGCCTGCTGTTCA

Reverse – CCAGCCTACTCATTGGGATCA

CD68: Forward- GCTACATGGCGGTGGAGTACAA

Reverse – ATGATGAGAGGCAGCAAGATGG

Immuno-spin trapping of DMPO-protein radicals from cell lysates using ELISA

Kupffer cell lysates for use in ELISA for the detection of DMPO-protein nitron adducts were prepared using a cell sonicator. Since buffers contain traces of metals and other contaminants that could form DMPO-protein radicals, we compared chelexed phosphate buffer (pH 7.4) containing 100 μ M DTPA and RIPA buffer. Results from the pilot experiments found no significant difference in the chemiluminescence signal from these experiments, and all subsequent lysates were prepared in RIPA buffer (0.05 g sodium deoxycholate, 100 μ L Triton X-100 and 10 μ L of 10% SDS in 10 mL of 0.1 M PBS) containing protease inhibitors (Complete, Mini Protease Inhibitor Cocktail Tablets, Roche Applied Science, Indianapolis, IN). Following a 30 min incubation of the samples in ice, they were centrifuged at 20 000 $\times$ g for 20 min. The soluble material (supernatant) was stored at 4° C until use.

Confocal laser scanning microscopy (Zeiss LSM 510 UV Meta)

Livers were fixed in 10% neutral buffered formalin and soaked in 30% sucrose for 24 h. The frozen sections (10 μ m) were cryo-cut using a frozen tissue processor (Leica Instruments, Bannockburn, IL) at the immunohistochemistry core facility at NIEHS. Tissue slices were then treated with 0.5% SDS (5 minutes) for antigen retrieval, permeabilized, and blocked (2% nonfat dry milk, Pierce Biomedical, Rockford, IL). An antibody specific to DMPO nitron adducts and Alexafluor 568 goat anti-rabbit secondary antibody (Molecular Probes, now Invitrogen, Carlsbad, CA) were used as primary and secondary antibodies, respectively. Anti-nitrotyrosine antibody was from Abcam. Confocal images were taken on a Zeiss LSM510-UV meta (Carl Zeiss, Inc., Oberkochen, Germany) using a Plan-NeoFluor 40X/1.3/40xOil DIC objective with different zoom levels.

Macrophage depletion by GdCl₃

Mice were injected with gadolinium chloride (10 mg/kg) through the i.v. route, 24 h prior to BDCM treatment, (Farooqi, 2011).

Statistical analyses

All in vivo experiments were repeated three times with 3 mice per group (N=3; data from each group of three mice were pooled). All in vitro experiments were repeated three times, and the statistical analysis was carried out by analysis of variance (ANOVA) followed by the Bonferroni posthoc correction for intergroup comparisons. 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

Bromodichloromethane acute exposure sensitizes obese liver to increased free radical stress

We have shown previously that free radical metabolism of a "second hit" like CCl₄ produces lipid peroxidation and protein radical formation in obesity (Chatterjee *et al.*, 2012b). BDCM is known to be metabolized by CYP2E1 and produces dihalomethyl radicals (Lilly *et al.*, 1997). To investigate the role of BDCM exposure in causing increased free radical stress in obesity, lipid peroxidation was analyzed in the obese liver. First, 4-hydroxynonenal, (4-HNE) a peroxidation product of lipids was measured in whole liver homogenates. Results showed that there was a significant increase in 4-HNE adducts in the DIO+BDCM group as compared to the lean control, lean control + BDCM and DIO groups at 6 h post-exposure (P<0.05) (Fig. 1A). Next, we analyzed the effect of lipid peroxidation on protein radical formation and tyrosine nitration in the obese liver. Results showed that there was a

significant increase in the protein radical-adduct formation in the DIO+BDCM group as compared to the lean control, lean control+BDCM and DIO groups ($P<0.05$) (Fig. 1B). The protein radical adducts were localized in the centrilobular region of the liver (Zone 3), a typical hallmark of radical-based liver injury (Lilly *et al.*, 1997) (Fig. 1C). To find whether the protein radicals were primarily due to tyrosine radicals, we analyzed immunoreactivity against nitrotyrosine with confocal laser scanning microscopy. The DIO+BDCM group had greater immunoreactivity to 3-nitrotyrosine in the centrilobular region than lean control mice treated with BDCM (Fig. 1D). The CYP2E1 protein levels also increased significantly in the DIO+BDCM group compared to the lean control, and DIO only groups (Supplementary Fig. 2). The HNE adducts and protein radical formation was abrogated following use of the CYP2E1 inhibitor diallylsulfide (data not shown). Since higher CYP2E1 levels and resultant metabolic oxidative stress by CYP2E1 yielded noxious free radicals following BDCM exposure as shown here, the results indicate potentiation of free radical formation in obesity.

BDCM acute exposure causes increased leptin levels and contributes to early steatohepatic lesions in obesity

We have shown previously that CYP2E1 metabolism and increased free radical formation lead to heightened leptin levels in liver (Chatterjee *et al.*, 2012a). To determine whether BDCM exposure led to increased leptin mRNA in the liver and adipose tissues, qRT-PCR analysis was performed with both liver and adipose tissues in mice exposed to BDCM. Results showed that BDCM treatment significantly increased leptin mRNA levels in both liver and adipose tissues of DIO mice as compared to lean control, lean control+BDCM and DIO only mice ($P<0.05$) (Fig. 2A). BDCM exposure also significantly elevated plasma AST, ALT and LDH levels in DIO+BDCM mice as compared to lean control, lean control+BDCM and DIO only mice ($P<0.05$) (Fig. 2B). This result also emphasizes liver injury with significant necrotic cell death as indicated by elevated LDH levels (Fig. 2B). To characterize the early steatohepatic lesions in obesity following BDCM exposure, liver histopathology was scored as per the histology activity index (HAI) index. Results showed that there was significant elevation of HAI scores in DIO+BDCM mice compared to lean controls (Table 1). Examination of liver tissue revealed significant leukocyte infiltration and ballooning degeneration at 48 h post-BDCM exposure (Fig. 2C). Though hepatocyte degeneration and binucleated hepatocytes were fewer than in full blown steatohepatitis, it is likely that BDCM exposure caused an early shift towards steatohepatitis of obesity.

Increased leptin in obesity mediates protein radical and nitrotyrosine formation in vivo and in isolated Kupffer cells

Leptin, a proinflammatory adipokine, has been shown to participate in the inflammatory process associated with steatohepatic lesions in obesity (Ikejima *et al.*, 2001; Munzberg, 2010). Leptin resistance is key to the progression of disease in obesity (Munzberg, 2010). We have shown that CYP2E1 metabolism of CCl_4 elevates serum leptin levels (Chatterjee *et al.*, 2012a). To investigate whether increased leptin in obesity and following BDCM exposure contributes to protein radical formation and free radical stress, we used two mouse models: 1) mice with a spontaneous knockout of the leptin gene and 2) leptin receptor knockout models ($\text{lepr}^{-/-}$). DMPO-nitron adduct formation, which exemplifies protein radical adducts, was significantly decreased in OB/OB mice and leptin receptor knockout mice compared to DIO mice treated with BDCM (Fig. 3A), as was 3-nitrotyrosine immunoreactivity (Fig. 3B). To show the involvement of Kupffer cells in leptin-mediated protein radical formation and the mechanisms that underlie redox stress, we incubated isolated Kupffer cells from DIO mice with recombinant leptin and BDCM. We found that Kupffer cells from DIO mice that were incubated with leptin and exposed to BDCM had significantly elevated protein radical formation compared to Kupffer cells from mice

deficient in the leptin receptor or to Kupffer cells that were treated with excess DMPO (spin trap for free radicals), apocynin (an NADPH oxidase inhibitor), or Tempol (a Superoxide Dismutase-mimetic compound) (Fig. 3C). DMPO when used in excess of 20–40 micromolar, has been found to act as a scavenger of free radicals (Chatterjee *et al.*, 2011). Excess DMPO (100 mM) was thus used to scavenge free radicals in this experiment. Confocal laser scanning images also showed decreased protein radical formation in isolated Kupffer cells from leptin-receptor-deficient mice compared to the DIO and DIO+BDCM-treated groups (Fig. 3D). The results clearly showed that leptin was fundamental in the potentiation of the free radical stress mediated by lipid peroxidation and superoxide release from either CYP2E1 or NADPH oxidase (Kupffer cells) in obese mice.

Increased leptin and BDCM metabolism in obesity activate Kupffer cells and cause p67 translocation, a measure of NADPH oxidase membrane assembly and activation

It has been shown by us and others that adipocytokine leptin activates Kupffer cells in acute and chronic liver injury (Wang *et al.*, 2009; Tosello-Trampont *et al.*, 2012), (Chatterjee *et al.*, 2012a). Kupffer cell activation is marked by an increased release of TNF- α and monocyte chemoattractant protein-1 (MCP-1) (Lilly *et al.*, 1997; Miura *et al.*, 2012; Tosello-Trampont *et al.*, 2012). Kupffer cells have functional NADPH oxidase and release a flux of superoxide upon activation (Chatterjee *et al.*, 2012a). We found that isolated Kupffer cells, incubated with BDCM and leptin, exhibited a significant elevation of TNF- α levels in the supernatant at 24 h compared to cells isolated from DIO-only mice ($P < 0.05$), while apocynin, Tempol and excess DMPO significantly decreased the release of TNF- α compared to BDCM and leptin incubated cells ($P < 0.05$) (Fig. 4A). Furthermore, cells isolated from leptin receptor knockout mice had a significant decrease in TNF- α levels compared to the DIO+BDCM +Leptin group (Fig. 4A). qRT-PCR analysis showed a significant increase in CD68, the activation marker for Kupffer cells, in the DIO+BDCM group compared to lean controls, lean+BDCM group and the DIO group. The expression of CD68 was significantly lower in leptin receptor knockout and leptin knockout mouse livers than in the DIO+BDCM group ($P < 0.05$) (Fig. 4B). GdCl₃-treated livers of the DIO+BDCM group also showed a significant decrease in CD68 mRNA expression as compared to the DIO+BDCM group, showing that the CD68 expression originated strictly in the macrophages ($P < 0.05$) (Fig. 4B). TNF- α and MCP-1 protein levels in the livers of DIO+BDCM mice increased significantly compared to lean controls and DIO groups, as measured by Western blot analysis (Fig. 4C) and band quantification (Supplementary Fig. 1). Protein levels of these proteins decreased significantly in leptin receptor knockout and leptin knockout mice livers compared to the DIO+BDCM group ($P < 0.05$). For its activation and production of superoxide, the P67 phox subunit of NADPH oxidase needs to translocate to the membrane to align with the GP91 phox subunit. Western blot analysis of membrane fractions of liver homogenates of DIO mice that were treated with BDCM and fed with a high fat diet had a significant increase in p67 translocation as compared to lean controls, DIO, leptin receptor knockout and leptin knockout mice ($P < 0.05$) (Fig. 4D). These results clearly show the synergistic effect of leptin and BDCM exposure in promoting Kupffer cell activation and NADPH oxidase membrane assembly, two crucial events in the progression of steatosis to early steatohepatic lesions in obesity.

BDCM exposure synergizes with leptin in promoting non-programmed cell death and progression of steatosis to early steatohepatic lesions

One of the key events in early steatohepatic injury is necrosis and degeneration of hepatocytes (Handy *et al.*, 2011). To investigate the role of BDCM exposure to high fat-fed mice in cell death, we measured levels of a hepatocyte necrotic marker, isocitrate dehydrogenase. Western blot analysis indicated that isocitrate dehydrogenase increased significantly in the DIO+BDCM group as compared to the lean control, DIO only and lean

control+BDCM groups ($P<0.05$) (Fig. 5A and 5B). Leptin receptor knockout or leptin knockout liver homogenates had significant decrease in the isocitrate dehydrogenase levels compared to the DIO+BDCM group ($P<0.05$) (Fig. 5A and 5B). To evaluate whether late stage apoptosis followed BDCM treatment and was influenced by increased leptin levels, an in situ TUNEL assay was performed. Immunohistochemistry for TUNEL labeling showed more apoptosis in DIO mice than in DIO+BDCM, leptin receptor knockout or leptin knockout mice (Fig. 5C). The number of TUNEL-positive nuclei was significantly higher in DIO mice than in DIO+BDCM, leptin receptor knockout or leptin knockout mice ($P<0.05$) (Fig. 5D). These results assume significance because programmed cell death induction as compared to necrosis might be viewed as a survival mechanism of hepatocytes in toxin-mediated injury (Tang *et al.*, 2012). Furthermore, the finding of significant increases in isocitrate dehydrogenase in the DIO+BDCM group (Fig. 5A) confirmed that BDCM and leptin acted synergistically to cause necrotic injury and non-programmed cell death.

Discussion

Acute BDCM exposure in mice that were fed a high fat diet led to increased oxidative stress, increased expression of leptin, liver inflammation through Kupffer cell activation, NADPH oxidase membrane assembly, cytokine release and non-programmed cell death following the reductive metabolism of this water disinfection byproduct. These events following BDCM exposure are hallmarks of early steatohepatic injury in the liver and are consistent with the two-hit hypothesis and the multi-hit paradigm (Day and James, 1998; Tilg and Moschen, 2010). The observations showed for the first time that an underlying condition of obesity can potentiate the risk of developing steatohepatitis following exposure to disinfection byproducts of drinking water. Since more than 30% of the US population is obese, the results underline a serious public health problem not only in this country but in developed and developing nations where use of chlorine as a disinfectant is largely used.

In spite of stricter regulation of BDCM levels in drinking water, chlorine is a widely used disinfection agent across the civilized world. The general population is exposed to trihalomethanes through consumption of treated drinking water, beverage, and food products, inhalation of contaminated air, and dermal contact with treated water (Keegan *et al.*, 1998; Torti *et al.*, 2001). BDCM reductive metabolism by CYP2E1 causes free radical production and has been shown to generate PBN-specific free radicals by ESR (Tomasi *et al.*, 1985). CYP2E1 has been the primary cyp450 isoform to metabolize BDCM (Lilly *et al.*, 1997). We hypothesized that BDCM exposure and the resultant free radical generation starts an inflammatory cascade linking oxidative stress and the innate immune response of the liver. We found that BDCM formed 4-HNE adducts, a lipid peroxidation product at 6 h followed by protein free radical formation and stable nitrotyrosine adducts in hepatocytes and Kupffer cells (Fig. 1A). Interestingly, following free radical generation, BDCM exposure also significantly increased leptin mRNA expression in both adipose tissue and livers of mice fed a high fat diet (DIO) (Fig. 2A). This data was important to finding a strong correlation between obesity, BDCM-induced oxidative stress and altered histopathology in the liver. Increased leptin in DIO mice coupled with higher release of this adipokine following CCl₄-induced NASH was found to cause immune cell activation and necrosis in the liver (Chatterjee *et al.*, 2012a). Similarly our finding that BDCM caused an increase in leptin mRNA in liver of obese mice, over and above the high leptin levels found in obesity was crucial to finding a causal effect in BDCM-induced early steatohepatic lesions.

To prove that the BDCM-leptin interplay caused early NASH-like symptoms, we used both OB/OB mice, which have a spontaneous knockout for leptin, and DB/DB mice that have a systemic deletion of the leptin receptor isoform. The cellular effects of leptin are mediated

by the leptin receptor isoforms Ob (long) and Ob (short), which belong to the IL-6 family of receptors and activate the JAK-STAT pathway for their downstream effects (Gorska *et al.*, 2010). Leptin is known to mediate oxidative stress by activating NADPH oxidase and inducing formation of peroxynitrite (De Minicis *et al.*, 2008); (Chatterjee *et al.*, 2012a). Leptin also mediates Kupffer cell activation and thus triggers the release of innate immune mediators and T cell proliferation, through its actions in the liver and other peripheral tissues (Shen *et al.*, 2005; Kamada *et al.*, 2008; Lafrance *et al.*, 2010). Our observations of increased release of leptin following BDCM exposure allowed us to probe the significance of leptin in early steatohepatic lesions in BDCM-exposed mice. Both whole liver homogenates and isolated Kupffer cells produced protein free radicals as measured by the immuno-spin trapping technique (Fig. 3). These protein free radicals further stabilized to form nitrotyrosine adducts, which are formed either as a product of peroxynitrite metabolism or in a direct reaction of peroxidases in the presence of nitric oxide (Souza *et al.*, 2008). Nitrotyrosine formation is correlated with the immune pathogenesis of many diseases. The significant formation of 3-nitrotyrosine indicated a prominent role for the interplay of BDCM and leptin in steatohepatic lesions in DIO mice. Isolated Kupffer cells that were incubated with leptin and BDCM showed increased oxidative stress, but oxidative stress was significantly decreased in the presence of apocynin, an NADPH oxidase inhibitor, or Tempol, an SOD mimetic compound, or excess DMPO (Fig. 3C). Kupffer cells that lacked the leptin receptor isoforms had a significant decrease in oxidative stress, suggesting that the presence of leptin and leptin receptor signaling coupled with BDCM were strongly associated with reactive oxygen species generation in Kupffer cells (Fig. 3C and D). Published literature indicates that oxidative stress in Kupffer cells leads to the release of proinflammatory cytokines and chemokines that are central to inflammatory disease pathogenesis in fatty liver disease (De Minicis *et al.*, 2008; Farrell *et al.*, 2012). We and others have shown that Kupffer cell activation by leptin results in a poor outcome in fatty liver disease, leading to NASH-like symptoms (Wang *et al.*, 2009; Tosello-Tramont *et al.*, 2012) (Chatterjee *et al.*, 2012a). The release of proinflammatory cytokines and chemotactic proteins such as TNF- α and MCP-1 from Kupffer cells leads to activation of inflammatory pathways that mediate disease progression in the liver (Farrell *et al.*, 2012). Our results showed that BDCM-leptin synchrony *in vivo* and *in vitro* led to the release of TNF- α and MCP-1 (Fig. 4). Furthermore, there was a significant increase in CD68 protein in liver homogenates following BDCM exposure, suggesting the activation of Kupffer cells (Fig. 4). Kupffer cell activation was also confirmed by the use of GdCl₃-induced depletion of resident macrophages. The GdCl₃-treated group showed a significant decrease in CD68 expression compared to the DIO+BDCM group (Fig. 4B). Kupffer cell activation in liver disease due to either alcohol treatment or NASH pathophysiology is crucial for disease progression. It has been presumed that inflammatory events in the Kupffer cells primarily trigger increased antigen presentation, T-cell proliferation and amplification of the inflammatory cascade (Wang *et al.*, 2009; Chatterjee *et al.*, 2012b). Our results of increased oxidative stress, TNF- α and MCP-1 release, and increased CD68 in the liver confirm the Kupffer cell activation in BDCM exposure and show that it is leptin dependent.

Kupffer cell activation and the resultant inflammatory events that follow in liver injury lead to cell death. There is evidence that damage-associated molecular patterns have resulted in hepatocyte necrosis in CCl₄-mediated early steatohepatitis (Chatterjee *et al.*, 2012b). Leptin, which is found in higher concentrations in obesity, due to central leptin resistance, may contribute to the cell death patterns in the liver. NASH histopathology shows that hepatocyte necrosis is a key event in the pathogenesis of disease progression (Kim and Younossi, 2008; Chatterjee *et al.*, 2012b; Farrell *et al.*, 2012). We argued that BDCM-induced early steatohepatic injury will cause hepatic cell death and is regulated by leptin and involves its receptor. We also hypothesized that the absence of leptin would prevent non-programmed cell death in the hepatic microenvironment. Our results showed a significant increase in

apoptotic nuclei in the DIO group, whereas the DIO+BDCM group and the groups lacking leptin or its receptor showed decreased apoptosis in the liver (Fig. 5C). Interestingly, the levels of Isocitrate Dehydrogenase, a hepatocyte marker of necrosis, increased significantly in the DIO+BDCM group compared to DIO alone or the groups that were devoid of leptin or the leptin receptor (Fig. 5A and 5B). These results assumed significance because apoptosis and other programmed cell death patterns like autophagy are increasingly being recognized as cell survival mechanisms (Tang *et al.*, 2012). Necrosis, as seen in the DIO+BDCM group, is a significant event in the steatohepatitis of obesity and can also be perceived as leptin dependent in BDCM-exposed early steatohepatic lesions.

Taken together, our results show that BDCM exposure causes early steatohepatic lesions in high fat diet-induced obesity and is dependent on higher leptin levels in obesity but is exacerbated by the direct effects of BDCM on both adipose tissue and liver in producing higher expression of this adipokine. Though leptin is perceived as an anorexigenic hormone, a profound rise is seen in its levels in obesity due to central leptin resistance. The levels of leptin might rise further if environmental contaminants like BDCM directly cause more hepatic and adipose tissue expressions of this molecule, and thus sensitize the obese liver to heightened tissue injury. These events of hepatic tissue damage can lead to early steatohepatic lesions and can predispose the obese liver to more severe liver disease forms such as NASH and cirrhosis. Future studies of chronic exposure to low doses of BDCM might shed light on the disease progression of NASH and other inflammatory complications of the liver such as autoimmune hepatitis and hepatocellular carcinoma. Thus, this first ever report on BDCM-exposed early steatohepatic lesions in obesity might lead to answers to more complex public health questions on the environmental link to disease progression in obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- BDCM acute exposure sensitizes liver to increased free radical stress in obesity
- BDCM-induced higher Leptin contributes to early steatohepatic lesions
- Increased Leptin mediates protein radical and 3-Nitrotyrosine formation
- BDCM exposure in obesity activates Kupffer cells and NADPH Oxidase
- BDCM/Leptin synergy promotes necrotic cell-death and augments steatohepatitis

Fig. 1. Diet-induced obesity potentiates oxidative stress following BDCM exposure in mice. A. Immunoreactivity of 4-hydroxynonenal (4-HNE) adducts in mice liver homogenates as detected by ELISA. B. Immuno-spin trapping of DMPO nitrene adducts, a measure of protein radical formation in mice liver homogenates following BDCM exposure, as detected by ELISA. C. Immunolocalization of DMPO nitrene adducts in the centolobular region (Zone III) following BDCM exposure. Anti-DMPO immunoreactivity is shown by red staining, as detected by confocal laser scanning microscopy. D. Immunolocalization of 3-nitrotyrosine adducts, detected by confocal laser scanning microscopy in mice exposed to BDCM as shown by green staining. The nuclear stain (DAPI) is shown in blue. Results are expressed as mean $\pm$ SE. Statistical significance was tested using ANOVA, with Bonferroni posthoc correction. *($P < 0.05$)

Fig. 2.

Diet-induced obesity and BDCM co-exposure increases leptin expression and contributes to early steatohepatic lesions. A. Leptin mRNA expression in adipose and liver tissues in mice exposed to BDCM. Results are shown as an index of fold increase over the lean control group. B. Serum levels of liver enzymes, markers of tissue injury and necrosis. Serum levels of AST, ALT and LDH, a marker of tissue necrosis, are shown in mice that were exposed to BDCM. C. HE-stained paraffin-embedded liver tissue section of mice exposed to BDCM. Arrowheads indicate increased leukocyte infiltration, a marker of an inflammatory microenvironment. Results are expressed as mean $\pm$ SE. Statistical significance was tested using ANOVA, with Bonferroni posthoc correction. *($P < 0.05$)

Fig. 3. BDCM exposure-induced oxidative stress is mediated by leptin in diet-induced obese mice and in isolated Kupffer cells. **A.** Immunoreactivity of protein radical adducts as assessed by ELISA in liver homogenates from BDCM-exposed diet-induced obese mice, leptin receptor knockout and leptin knockout mice. **B.** Immunoreactivity of protein nitrotyrosine adducts as assessed by ELISA in liver homogenates from BDCM-exposed diet-induced obese mice, leptin receptor knockout and leptin knockout mice. **C.** Kupffer cell protein radical adducts following co-incubation of BDCM and leptin, in the presence or absence of the NADPH oxidase inhibitor apocynin or the superoxide dismutase mimetic Tempol or in the presence of an excess of the spin trap DMPO or in cells isolated from leptin receptor deficient mice. **D.** Immunolocalization of DMPO-nitrosonitrite adducts in isolated Kupffer cells from diet-induced obese mice and leptin receptor knockout mice, as assessed by confocal laser

scanning microscope. Anti-DMPO staining is marked by red and nuclear stain in blue. Results are expressed as mean $\pm$ SE. Statistical significance was tested using ANOVA, with Bonferroni posthoc correction. *(P<0.05)

Fig. 4.

BDCM co-exposure in diet-induced obese mice causes Kupffer cell activation and is mediated by leptin. A. TNF- α levels in supernatants of isolated Kupffer cells following co-incubation with BDCM and leptin in the presence or absence of the NADPH oxidase inhibitor apocynin or the superoxide dismutase mimetic Tempol or in the presence of an excess of the spin trap DMPO or in cells isolated from leptin receptor deficient mice. B. CD68 mRNA expression as assessed by quantitative real-time PCR. C. Western blot analysis of TNF- α and MCP-1 in liver homogenates of BDCM-exposed diet-induced obese mice or mice that lacked the leptin receptor or leptin. D. Membrane translocation of NADPH oxidase subunit P67 following BDCM exposure in diet-induced obese mice and in mice that lack either the leptin receptor or leptin. Results are expressed as mean $\pm$ SE. Statistical significance was tested using ANOVA, with Bonferroni posthoc correction. *($P < 0.05$)

Fig. 5. BDCM and leptin mediated cell death mechanisms in the diet-induced obese liver. A. Western blot analysis of isocitrate dehydrogenase, a hepatocyte necrosis marker in liver homogenates. Lanes 1–8 represent lean, lean+BDCM, DIO, DIO+BDCM, DIO internal control, Leptin receptor knockout, OB/OB and DIO+BDCM (reference control) respectively. B. Band analysis of Western blots shown in A. C. Histological examination of paraffin-embedded liver tissue sections following TUNEL staining of BDCM-exposed diet-induced obese mice in the presence or absence of either leptin or the leptin receptor. D. Numerical estimate of TUNEL positive nuclei in paraffin-embedded liver tissue sections when assessed in 10x magnification from BDCM-exposed DIO mice and in the absence of leptin or leptin receptor.

Table 1

Assessment of inflammation scores using the Histology Activity Index. H&E slides were analyzed for each group, namely, Lean, Lean+BDCM, DIO and DIO+BDCM and ranked blindfoldedly. Inflammation was ranked based on the leukocyte infiltration and presence of necrosis with a progressive rank from 0 to 10, with 0 being minimal on the scale. Results are expressed as mean $\pm$ SE.

Histological Activity Index Scorep				
Groups	Lean Control	Lean Control+BDCM	DIO	DIO+BDCM
Confluent Necrosis:	0	1	1	3 $\pm$ 1
Parenchymal Injury	0	1	1	3 $\pm$ 1
Portal Inflammation:	0	1	1	4 $\pm$ 2
Grade (Inflammation):	4 $\pm$ 1	7 $\pm$ 3	8 $\pm$ 2	15 $\pm$ 4
Stage (Fibrosis):	0	0	1	1