Toxicology and Applied Pharmacology 269 (2013) 297–306

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Proinflammatory adipokine leptin mediates disinfection byproduct bromodichloromethane-induced early steatohepatitic injury in obesity ☆

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ARTICLE INFO

*Article history:*
Received 11 December 2012
Revised 5 February 2013
Accepted 6 February 2013
Available online 22 February 2013

*Keywords:*
Adipocytokines
Kupffer cell
Oxidative stress
Lipid peroxidation
Tyrosine nitration
Necrosis
Tumor necrosis factor
OB/OB mice

ABSTRACT

Today's developed world faces a major public health challenge in the rise in the obese population and the 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 steatohepatitic 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 steatohepatitic 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 steatohepatitic injury by potentiating the effects of leptin on oxidative stress, Kupffer cell activation and cell death in the liver.



## 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 $CCl_4$ exposure, albeit in lower doses, can sensitize the obese liver to hepatotoxicity and development of early steatohepatitic injury (Chatterjee et al., 2012b; Ikejima et al., 2001). A recent study from M. Cave 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-alchoholic steatohepatitis in American adults. Lately the involvement of cytochrome p450s in the metabolism

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http://dx.doi.org/10.1016/j.taap.2013.02.003

of these environmental toxicants has been an active area of research (Abdelmegeed et al., 2012; Leung and Nieto, 2013). 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 $CCl_4$ 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,b). 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 (Chatterjee et al., 2012a; Parola and Marra, 2011). Similar to $CCl_4$, which produces trihalomethyl radicals upon metabolism by CYP2E1, several trihalomethanes have been found to be metabolized by CYP2E1 to produce noxious free radicals (Lilly et al., 1997; Mezyk et al., 2006; Tomasi et al., 1985). 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 (Anon., 2011; Keegan et al., 1998; Lilly et al., 1997) and are bioactivated by liver cytochrome p450 enzymes leading to generation of free radicals (Lilly et al., 1997; Tomasi et al., 1985). 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 steatohepatitic 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; Krawczyk et al., 2009; Li et al., 2002; 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 steatohepatitic injury. We also examine the role of leptin in mediating the progression of simple steatosis to early steatohepatitic injury following acute BDCM exposure. We show here, for the first time the direct role of BDCM in inducing steatohepatitic 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 ($GdCl_3$) 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 μm 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 mmol/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 (Anon., 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 steatohepatitic injury.

*Isolation of Kupffer cells.* Kupffer cells were isolated as per the protocol of 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/min. Following perfusion, the liver was dissected and incubated with 0.03% collagenase for another 30 min 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 50 *g* for 3 min to obtain parenchymal cells (mostly hepatocytes). The supernatant was further centrifuged at 650 ×*g* 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 min to obtain the Kupffer cells. The isolated cells were plated onto 35 mm 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.* $10^5$ 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-nitrone adducts and nitrotyrosine immunoreactivity.