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NADPH oxidase is not an essential mediator of oxidative stress or liver injury in murine MCD diet-induced steatohepatitis

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***Background/Aims*: Hepatic oxidative stress is a key feature of metabolic forms of steatohepatitis, but the sources of pro-oxidants are unclear. The NADPH oxidase complex is critical for ROS generation in inflammatory cells; loss of any one component (e.g., gp91^{phox}) renders NADPH oxidase inactive. We tested whether activated inflammatory cells contribute to oxidant stress in steatohepatitis.**

***Methods*: gp91$^{phox-/-}$ and wildtype (*wt*) mice were fed a methionine and choline-deficient (MCD) diet. Serum ALT, hepatic triglycerides, histopathology, lipid peroxidation, activation of NF-κB, expression of NF-κB-regulated genes and macrophage chemokines were measured.**

***Results*: After 10 days of MCD dietary feeding, gp91$^{phox-/-}$ and *wt* mice displayed equivalent hepatocellular injury. After 8 weeks, there were fewer activated macrophages in livers of gp91$^{phox-/-}$ mice than controls, despite similar mRNA levels for MCP and MIP chemokines, but fibrosis was similar. NF-κB activation and increased expression of ICAM-1, TNF-α and COX-2 mRNA were evident in both genotypes, but in gp91$^{phox-/-}$ mice, expression of these genes was confined to hepatocytes.**

***Conclusions*: A functional NADPH oxidase complex does not contribute importantly to oxidative stress in this model and therefore is not obligatory for induction or perpetuation of dietary steatohepatitis.**

***Keywords*: MCD diet; NF-κB; gp91^{phox}; Non-parenchymal cells; Inflammation; Oxidant stress**

1. Introduction

Hepatic oxidative stress is a key feature of non-alcoholic steatohepatitis (NASH) [1–4]. Lipoperoxides are directly pro-inflammatory, and activation of the redox-sensitive pro-inflammatory transcription factor nuclear factor-kappaB (NF-κB) [5,6] occurs in both experimental and clinical forms of steatohepatitis [7,8]. Oxidative stress is therefore a plausible trigger of the transition from simple steatosis to steatohepatitis [9–11], and has been suggested to play a direct role in fibrogenesis [12,13]. Hepatocytes comprise one source of reactive oxygen species (ROS); mitochondria, endoplasmic reticulum (cytochromes P450 [CYP]2E1 and 4A) and peroxisomes are likely subcellular sites [14–16]. Innate or recruited inflammatory cells comprise an alternative source of pro-oxidants. For example, activated Kupffer cells are capable of producing pro-oxidants and other pro-inflammatory mediators [17–19].

The NADPH oxidase complex is the most abundant intracellular source of ROS generated by inflammatory cells [20,21]. It is composed of several subunits; two are membrane-bound (gp91^{phox} and p22^{phox}, together known as cytochrome b_{558}) and three are cytoplasmic (p47^{phox}, p67^{phox} and p40^{phox}). In response to physio-

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logical stimuli, the cytoplasmic complex translocates to cytochrome b_{558}, allowing the complete enzyme complex to catalyse the oxidation of NADPH to $NADP^+$; this results in superoxide production. Through the generation of gene-deleted mice, it has been shown that deficiency of any one of its subunits renders the NADPH oxidase complex inactive [20].

Kupffer cells appear to be key players in the pathogenesis of experimental alcohol-induced liver injury. Thus, their ablation by gadolinium chloride ($GdCl_3$) [22–24], inactivation by dietary glycine [25], administration of NADPH oxidase inhibitors [26], and studies in $p47^{phox-/-}$ gene-deleted mice [27] all attenuate severity of alcohol-induced liver injury. A favoured pathogenic mechanism is that binding of endotoxin to CD14 on Kupffer cells activates NADPH oxidase, thereby increasing production of superoxide. In turn, this activates NF-κB to promote release of TNF-α and other pro-inflammatory and potentially cytotoxic molecules, such as cyclooxygenase-2 (COX-2) [18,28]. It is conceivable that similar mechanisms operate in the pathogenesis of metabolic forms of steatohepatitis. In the present study, we tested the hypothesis that activated inflammatory cells contribute to liver injury in mice fed a lipogenic methionine and choline-deficient (MCD) diet. We administered the MCD diet to $gp91^{phox-/-}$ mice [29], which have a defective NADPH oxidase complex and are therefore unable to activate inflammatory cells, and determined the effects on liver injury, inflammatory recruitment and fibrotic severity.

2. Materials and methods

2.1. Animal procedures and dietary induction of steatohepatitis

Female C57BL/6J (wildtype, *wt*) mice were supplied by the Animal Resource Centre (Canning Vale, WA, Australia). $gp91^{phox}$ gene-deleted mice ($gp91^{phox-/-}$) on a C57BL/6J background (backcrossed 10 times) were obtained from the Centenary Institute of Cancer Medicine and Cell Biology, University of Sydney, Australia. Animal housing conditions, handling procedures and dietary formulation have been described previously [8]. All studies were approved by the Western Sydney Area Health Service Animal Care and Ethics Committee, and conformed to the highest international standards of humane care of animals in biomedical research.

2.2. Preparation of tissue and cellular fractions

At pre-determined times, mice were anaesthetised (ketamine 100 mg/kg and xylazine 20 mg/kg administered i.p.), blood was collected by cardiac puncture, and serum prepared for determination of alanine aminotransferase (ALT). The preparation and storage of liver for histological studies, preparation of nuclear fractions and molecular studies have been reported [8]. In some experiments, hepatic non-parenchymal cells (NPC; Kupffer, endothelial, stellate and recruited inflammatory cells) were separated from hepatocytes by differential centrifugation of a liver cell suspension prepared by *in situ* collagenase perfusion of the liver [8]. Peritoneal cells were obtained by peritoneal lavage [30].

2.3. Morphological studies

Paraffin-embedded tissue was sectioned, stained with haematoxylin & eosin, and graded blindly for hepatic steatosis and necroinflammation (none, mild, moderate, severe, as previously described) [31]. Fibrosis was determined by Sirius Red stain [13]. Polymorphs were stained with nitrosylated pararosanilin (Sigma, St. Louis, MO) containing 0.028% (w/v) naphthol AS-D chloroacetate in 1 M Sorenson's buffer [32]. Activated macrophages were stained by periodic acid-Schiff (PAS) with diastase [33]. Cell counts were expressed as the average number of positive-stained cells per section (three sections counted) at 200× magnification.

2.4. Biochemical assays

Aliquots of peritoneal macrophages were incubated for 15 min at 37 °C in stimulation buffer (123 mM NaCl, 1.23 mM $MgCl_2$, 4.9 mM KCl, 16.7 mM sodium phosphate buffer, pH 7.4, 5 mM glucose, and 0.5 mM $CaCl_2$) containing 2 mM NaN_3, with or without 100 ng/mL phorbol 12-myristate 13-acetate (PMA). After incubation, the cells were pelleted, the supernatant discarded, and the cells resuspended in 100 μL of reaction buffer (50 mM Hepes, pH 7.0, 1 mM DTPA, 2 mM NaN_3, 120 μM cytochrome *c*, 0.05% sodium deoxycholate, and 0.15 mM NADPH), with or without 50 μg/mL superoxide dismutase (SOD). The reaction proceeded for 10 min at room temperature, and was stopped with an equal volume of stop buffer (50 mM Hepes, pH 7.0, 1 mM DTPA, 2 mM NaN_3, 1 mM *p*-chloromercuribenzoate). Absorbance was read at 550 and 468 nm; the difference between A_{550} and A_{468} was taken as indicating cytochrome *c* reduction. Superoxide production was then calculated from the difference in cytochrome *c* reduction in the presence and absence of SOD [34].

Hepatic triglyceride content was determined with the Wako *E*-Test triglyceride kit (Novachem, Cat. No. 432-40201). Total lipoperoxides were measured as thiobarbituric acid-reactive substances (TBARS) in liver homogenate [35].

2.5. Assessment of NF-κB activity

Hepatic nuclear protein was prepared as described [8,36]. 1.75 pmol of the target DNA probe (Promega, Madison, WI) containing the consensus binding sequence for NF-κB was end-labelled with γ-^{32}P ATP by T4 polynucleotide kinase. Following probe purification and β-scintillation counting, 120 fmol of the labelled probe were incubated with 10 μg of nuclear extract in a gel shift binding buffer [36], and DNA–protein complexes were resolved on a 4% native polyacrylamide gel.

2.6. Quantitation of hepatic mRNA levels

RNA was extracted from frozen liver tissue using TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA). Transcript levels of MCP-1, MCP-2, MIP-1α, MIP-1β, MIP-2, ICAM-1, TNF-α, COX-2 and collagen α_1(I) were determined by real-time RT-PCR using primers and probes designed by Primer Express™ design software (Applied Biosystems, Foster City, CA), as listed in Table 1. Amplification was performed in an ABI PRISM™ 7700 Sequence Detector (Applied Biosystems, Foster City, CA), using 5 μL of a 50-μL cDNA reaction incubated with either 1× SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St Louis, MO) or 1× TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The data were analysed with Sequence Detector Systems software (Applied Biosystems, Foster City, CA). Final values were normalised to GAPDH.

2.7. Statistical analyses

All data (means ± standard deviation) were analysed with StatView 4.5 software (Abacus Concepts Inc., Cary, NC). Differences between groups were initially tested using the unpaired Student's *t*-test, and when significant ($P < 0.05$), data were further subjected to ANOVA post hoc analysis.