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The Presence of p47phox in Liver Parenchymal Cells is a Key Mediator in the Pathogenesis of Alcoholic Liver Steatosis

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Abstract

Background—Reactive oxygen species contribute to steatosis and inflammation in alcoholic liver disease (ALD). Here, we evaluated the selective contribution of p47phox, a critical subunit of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase complex, in liver parenchymal cells and in bone marrow (BM)-derived cells.

Methods—Female C57Bl/6 wild type [WT], total body p47phox-deficient knockout [KO] or p47phox chimera mice generated by BM transplantation of p47phox-KO-BM into irradiated WT mice (WT/p47phox-KO-BM mice) received 5% Lieber–DeCarli alcohol or control (pair feeding) diet for 4 weeks.

Results—Alcohol-induced liver steatosis as measured by Oil Red O staining and serum triglyceride up-regulation were prevented in p47phox-KO mice but not in WT/p47phox-KO-BM chimeras compared to WT controls. Serum alanine aminotransferase (ALT) was significantly increased in alcohol-fed WT mice but not in p47phox-KO mice compared to pair-fed controls. There was no protection from alcohol-induced increase in ALT and liver damage in the WT/p47phox-KO-BM mice. Alcohol-induced liver steatosis was accompanied by up-regulation of the lipid droplet-stabilizing protein, adipocyte differentiation-related protein (ADRP), and the fatty acid synthesis-associated genes, fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACACA). Total body deficiency in p47phox but not selective absence of p47phox in BM-derived cells prevented alcohol-induced up-regulation of ADRP, FASN, and ACACA in the liver. Finally, alcohol-induced activation and DNA binding of nuclear factor κ B (NF- κ B), a master regulator of inflammation, were significantly increased after alcohol feeding in WT but not in p47phox-KO mice. Selective deficiency of p47phox in BM-derived cells (WT/p47phox-KO-BM chimera) failed to prevent NF- κ B induction after alcohol feeding.

Conclusions—Total body deficiency in p47phox subunit of NADPH oxidase complex protects mice from alcohol-induced liver steatosis via mechanisms involving ADRP, FASN, and ACACA as well as from alcohol-induced NF- κ B activation. In contrast, selective absence of p47phox in BM-derived cells fails to provide protection via these mechanisms. These results suggest that p47phox in parenchymal cells plays a critical role in the pathogenesis of ALD.

Keywords

p47phox; Nicotinamide Adenine Dinucleotide Phosphate Oxidase; Adipocyte Differentiation–Related Protein; NF-κB; Alcoholic Liver Disease

Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is a membrane-bound enzyme complex that was originally found in phagocytic cells including neutrophils and macrophages where it plays an important role in host defense by generating superoxide with the secondary production of other reactive oxygen species (ROS; Babior, 2004). Normally, the enzyme complex presents in a latent form and, upon stimulation, it is rapidly activated via assembly of its cytosolic and membrane-bound subunits in the active NADPH complex. It is known that the complex consists of the membrane-bound flavocytochrome b558, composed of 1 gp91phox and 1 p22phox subunits, and 4 regulatory cytosolic components, p47phox, p67phox, p40phox, and the small G protein Rac ½ (Bedard and Krause, 2007; Lambeth, 2004).

During the last 2 decades, the use of more sensitive assays revealed the presence of low amounts of ROS in cell types other than phagocytes (Gill and Wilcox, 2006; Hilenski et al., 2004; Infanger et al., 2006; Krause, 2004; Sorce and Krause, 2009). The nonphagocyte NADPH complex is structurally and functionally similar to phagocyte NADPH. However, the nonphagocyte NADPH oxidases are constitutively active, producing relatively low levels of ROS under basal conditions and generating higher levels of oxidants in response to different stimuli (De Minicis et al., 2006).

Despite the constant growth in understanding alcohol metabolism and action, the mechanisms by which alcohol causes cell injury are still not clear. Some of the leading hypotheses of alcohol-induced cell injury include redox state changes (Dey and Cederbaum, 2006; Wu et al., 2006). Several differentially localized and expressed enzymatic systems contribute to ROS formation in the liver, including endothelial NO synthases, cytochrome P450 monooxygenases, and NADPH oxidase (Bailey and Cunningham, 1998; Zima and Kalousova, 2005). In the healthy liver, antioxidant systems such as superoxide dismutase and catalase efficiently remove excess ROS to maintain the normal cell homeostasis. However, in chronic liver diseases there is increased ROS production, resulting in oxidative stress (Arteel, 2003; Hentze et al., 2003; Tirmenstein et al., 2000; Videla, 2009; Zhang et al., 2001).

Hepatotoxic agents (e.g., bile salts) can activate the NADPH oxidase and stimulate death pathways such as CD95-induced signaling to induce apoptosis in hepatocytes (Reinehr et al., 2005). In addition, nonparenchymal cells in the liver such as Kupffer cells and liver macrophages contain the phagocytic form of NADPH oxidase and are believed to mediate hepatocellular damage in alcohol-induced liver injury (Hasegawa et al., 2002; Thakur et al., 2006). Previous studies showed that treatment of mice with gadolinium chloride (GdCl₃) prevents free radical formation and alcohol-induced liver injury in the enteral alcohol feeding model (Adachi et al., 1994). Furthermore, the NADPH oxidase inhibitor, diphenyleneiodonium sulfate, prevented alcohol-induced liver injury almost completely by inhibiting free radical formation in another study (Kono et al., 2001). Finally, hepatic

steatosis observed in wild-type (WT) mice was prevented nearly completely in p47phox knockout [KO] mice (Kono et al., 2000). However, the contribution of NADPH oxidase between liver parenchymal cells and immune cells is yet to be determined in the development of alcoholic liver disease (ALD).

Here, we investigated the possible mechanism of the protection from alcoholic liver steatosis in total body p47phox- KO mice and found that NADPH oxidase in bone marrow (BM)-derived cells did not prevent alcohol-induced steatosis or liver damage. Results from our study suggest that possibly the p47phox/NADPH oxidase complex in parenchymal cells could have the primary significance in alcoholic liver injury.

MATERIALS AND METHODS

Animal Studies

All animals received proper care in agreement with protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. Seven- to 8-week-old C57Bl/6 (WT), total body p47phox-deficient (p47phox-KO) on C57Bl/6 background mice were employed. Animals were fed with the Lieber–DeCarli diet (Dyets, Bethlehem, PA) with 5% (vol/vol) ethanol (EtOH; 36% EtOH-derived calories) for 4 weeks; pair-fed control mice matched the alcohol-derived calories with dextran–maltose. Chimeric p47phox-KO mice were generated by transplanting total body p47phox-deficient (p47phox-KO) BM into irradiated, C57Bl/6 (WT) mice. WT mice with transplanted WT-BM were used as controls. Serum was separated and frozen at –80°C. Livers were snap-frozen in liquid nitrogen, stored in RNA later (Qiagen, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathology.

BM Transplantation

Mice received an intravenous injection of liposomal clodronate (200 μ l intravenously) before irradiation to deplete Kupffer cells (Van Rooijen and Sanders, 1996). BM was collected from the long bones of 14-week-old donor male mice by flushing with a 25G needle and passed through a cell strainer to remove clumps. Female mice were irradiated (9 Gy) from a cesium irradiator (Gammacell 40; Atomic Energy of Canada, Chalk River, Ontario, Canada) and 4 hours later transplanted with 5 million freshly isolated donor BM cells by way of a single tail vein injection. Transplanted mice were housed in microisolator cages and placed on medicated water (Sulfamethoxazole/Trimethoprim; Hi-Tech Pharmacal, Amityville, NY) until engraftment was complete 6 weeks later, as discussed (Hritz et al., 2008b; Li et al., 2007). The engraftment rate for all transplanted mice was >90%, as indicated by polymerase chain reaction (PCR) analysis of peripheral blood 6 weeks posttransplant.

Histopathology

Liver sections were stained with hematoxylin and eosin and analyzed by microscopy as described (Hritz et al., 2008a). The Oil Red O tissue staining of frozen sections embedded in OCT-media was used to quantify the steatosis. The area of positively stained lipid droplets by Oil Red O was calculated using ImageJ software (freely available at www.nih.gov).

Biochemical Assays

Serum alanine aminotransferase (ALT) was determined using a kinetic method (D-Tek, Bensalem, PA). Liver triglyceride (TG) levels were assessed using the L-Type Triglyceride H kit (Wako Chemicals, Richmond, VA).

RNA Analysis

RNA was purified using the RNeasy kit (Qiagen Sciences, Germantown, MD) and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System (Promega, Madison, WI). SybrGreen-based real-time quantitative PCR was performed using the iCycler (Bio-Rad Laboratories, Hercules, CA) as described (Hritz et al., 2008a). mRNA levels were normalized against the housekeeping gene, 18S, in the same sample.

Western Blot and NF- κ B Activity Analysis

Whole-liver lysates or nuclear fractions were extracted from liver, as described (Romics et al., 2004). Samples with equal amounts of protein were separated in a 10% polyacrylamide gel, transferred to nitrocellulose support, probed with specific primary antibodies, and detected using a chemiluminescent/horseradish peroxidase secondary antibody system. Electrophoretic mobility shift assay was performed as we previously described (Mandrekars et al., 1999) using equal amounts (5 μ g) of nuclear protein and a 32 P-labeled nuclear factor κ B (NF- κ B) consensus (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

We implemented a 2-factor design using the 2-way analysis of variance (ANOVA) to determine whether diet (alcohol or control diet) or genotype (p47phox-KO vs. WT, or WT/p47phox-KO-BM vs. WT/WT) affects the studied outcomes. Prior to ANOVA, Levene's test for equal variances was performed to ensure homogeneity of groups. To compare the means between individual groups, we used 1-way ANOVA with post hoc testing using the Tukey-HSD test. Owing to unequal variances, Dunnett-T3 test was used to calculate the intergroup differences in Fig. 5F. These methods were used to analyze data from Figs 1–5. We used the *t*-test to compare the means shown in Fig. 6. SPSS version 15.0 (SPSS Inc., Chicago, IL) was used to perform the statistical analyses. Data are shown as mean $\pm$ standard error of the mean (SEM) and were considered statistically significant at $p < 0.05$.

RESULTS

Total Body p47phox Deficiency but Not Selective Deficiency of p47phox in BM-Derived Cells Protects Mice from Alcohol- Induced Liver Steatosis

The pathomechanism of ALD involves multiple alcohol-induced cellular insults including induction of ROS. The NADPH oxidase system that is expressed both in inflammatory cells and in liver parenchymal cells is one of the central sources of ROS production in ALD (Kono et al., 2000). We found that liver steatosis induced by chronic EtOH feeding was prevented in mice with total body p47phox deficiency, as indicated by both hematoxylin and eosin (Fig. 1A) and Oil Red O staining (Fig. 1B). Analysis of the Oil Red O-positive areas

revealed that both pair-fed and alcohol-fed total body p47phox-KO mice had significantly lower levels of fat staining compared to WT controls (Fig. 1C).

Previous studies suggested that liver macrophages (Kupffer cells) and p47phox expression both contribute to the pathomechanism of ALD (Thakur et al., 2006). Thus, we hypothesized that p47phox in immune cells has a critical role in alcohol-induced liver steatosis. To assess the differential contribution of p47phox expression in liver parenchymal cells and BM-derived cells to ALD, we developed chimeric mice where WT mice received BM transplantation from p47phox-KO mice (WT/p47phox-KO-BM). WT mice transplanted with WT BM served as controls (WT/WT-BM).

In contrast to mice with a total body deficiency of p47phox, we did not observe any protection from liver steatosis in mice deficient in p47phox in BM-derived cells, following alcohol feeding (Fig. 1A,B). Morphometric analysis demonstrated that whereas total body p47phox-KO mice fed with alcohol showed significantly lower lipid accumulation, compared to WT mice (Fig. 1C), the lipid accumulation in livers of alcohol-fed WT/p47phox-KO-BM mice was similar to WT/WT-BM controls (Fig. 1D).

To further evaluate fat accumulation in the liver, we assessed liver TG levels. TG levels mirrored findings from Oil Red O staining. Total body inactivation of p47phox subunit led to significantly lower levels of liver TG compared to WT mice after alcohol feeding (Fig. 2A), whereas there was no significant difference in TG levels between the WT/WT-BM mice and WT/p47phox-KO-BM chimeric mice fed with alcohol (Fig. 2B).

We observed that in the pair-fed groups, both total body and BM-specific p47phox-KO mice showed significantly decreased lipid accumulation than WT mice (Figs 1C,D and 2A,B). This finding would suggest that p47phox deficiency affects baseline lipid accumulation regardless of the cell specificity (total body vs. BM-specific) and that the difference between the total body and BM-specific p47phox-KO groups occurs only when lipid synthesis is induced by alcohol diet.

To confirm this hypothesis, we performed a 2-factor analysis, which showed that diet (alcohol vs. control) was an independent determinant of lipid accumulation in both total body and BM-specific KO models (Table 1). However, genotype (WT vs. p47phox) proved to be an independent factor determining lipid accumulation only in the total body KO model (Table 1). In the BM-transplant model, the effect of genotype on lipid accumulation showed a statistical interaction with the effect of the diet (Table 1), confirming that the effect of the WT/p47phox-KO-BM genotype on lipid accumulation was different for pair- and alcohol-fed mice. Indeed, alcohol-fed WT and WT/WT mice showed approximately 2-fold increase in lipid accumulation, compared to pair-fed controls (Figs 1C,D and 2A,B), and lipid accumulation was also observed in the total body p47phox-deficient mice following alcohol feeding (Figs 1C and 2A). In contrast, the WT/p47phox-KO-BM mice showed a 5- to 20-fold induction in lipid accumulation, compared to pair-fed mice of the same genotype (Figs 1D and 2B). This analysis confirmed that total body and BM-specific deficiency of p47phox result in comparable reduction of baseline lipid accumulation in the liver and that the role for cell specificity of p47phox deletion in lipid accumulation is unraveled only when lipid

synthesis is induced by alcohol diet. Taken together, these findings demonstrated that total body p47phox expression, but not BM-specific p47phox expression, is an independent factor determining alcohol-induced lipid accumulation in the liver. This finding points to a dominant role of parenchymal cell-specific p47phox in lipid accumulation in ALD.

Alcohol-Induced Liver Injury is Prevented by Total Body Deficiency of p47phox, but Not by Selective Lack of p47phox in BM-Derived Cells

In WT and WT/WT mice, alcohol feeding significantly increased serum ALT, a marker of liver injury (Fig. 2C,D). In contrast, alcohol feeding in mice with a total body deficiency of p47phox failed to induce a significant liver injury (Fig. 2C), whereas mice with BM-specific p47phox deficiency showed only a partial protection from alcohol-induced liver injury (Fig. 2D). This finding was confirmed by the 2-factor analysis showing that the effect of alcohol diet on liver injury in the total body p47phox mouse model was dependent on genotype ($p < 0.004$ for interaction in Table 1; Fig. 2C), whereas both diet and genotype were independent factors for liver injury in the BM-specific KO model (Table 1; Fig. 2D). Thus, this analysis showed that although BM-specific deficiency of p47phox ameliorated alcohol-induced liver damage, BM-specific p47phox is not an absolute requirement for alcohol-induced liver damage to occur and suggested that the pathogenic role of p47phox in ALD is specific to liver parenchymal cells.

Chronic EtOH Feeding Stimulates Fatty Acid Synthesis and Stabilization of Lipid Droplets in the Liver in a p47phox- Dependent Manner

Among a variety of different mediators and processes involved in lipid metabolism, there are 2 major metabolic pathways that regulate the fatty acid status in liver: first, free fatty acid synthesis and second, fatty acid β -oxidation (Musso et al., 2009). In our work, we identified several possible genes that could be responsible for the differences in lipid accumulation between the total body and BM-specific p47phox-KO models after chronic EtOH feeding. Adipocyte differentiation-related protein (ADRP), also known as perilipin in adipocytes, is one of the main proteins involved in the stabilization of newly produced lipid droplets within hepatocytes (Magnusson et al., 2006). We observed that alcoholic diet and p47phox-KO genotype were independent factors determining ADRP expression in the total body KO model (Table 1; Fig. 3B). Further analysis showed that following feeding with alcohol, ADRP expression was significantly lower in total body p47phox-KO compared to WT mice (Fig. 3B); there was no significant difference in ADRP expression between pair-fed total body p47phox-KO and WT mice (Fig. 3B). Contrary to the total body KO mice, selective deficiency of p47phox in BM-derived cells did not affect the expression of ADRP ($p = 0.12$ for genotype in Table 1; Fig. 3D).

Investigation of 2 important genes involved at the last stages of fatty acid synthesis machinery, the fatty acid synthase (FASN) and the acetyl-CoA carboxylase (ACACA), also revealed that their expression is determined by the total body, but not the BM-specific p47phox status. FASN catalyzes the synthesis of saturated fatty acids by using the substrates acetyl-CoA and malonyl-CoA. We determined that mRNA levels of FASN were substantially lower in alcohol-fed p47phox-KO mice, compared to WT mice (Fig. 4A). In contrast, WT/p47phox-KO-BM mice demonstrated a significant induction of FASN

following alcohol feeding, and there was no difference between alcohol-fed WT/p47phox-KO-BM and WT/WT mice (Fig. 4B). These results were confirmed by the 2-factor analysis which showed that genotype significantly determined FASN expression only in the total body p47phox model; we did not see any role for genotype in FASN expression in the BM transplant model (Fig. 4A,B; Table 1).

The other important gene, ACACA, which has the main function to provide the malonyl-CoA substrate for the biosynthesis of fatty acids, was significantly increased in alcohol-fed WT mice compared to pair-fed controls (Fig. 4C). We found that total body p47phox-KO mice but not the WT/p47phox-KO-BM mice were protected from EtOH-induced up-regulation of ACACA (Fig. 4D). Similar to FASN, genotype proved to have a significant effect on ACACA expression only in the total body p47phox model, but not in the BM-specific p47phox model (Table 1).

Chronic EtOH Feeding in Mice Leads to the Activation of NADPH Oxidase and Increased NF- κ B Binding in Liver and Occurs Despite Inactivation of p47phox in BM-Derived Cells

It has been shown that alcohol leads to the generation of ROS in immune cells (e.g., Kupffer cells) and that ROS has damaging impact on surrounding tissue (e.g., hepatocytes; Thurman et al., 1998). Thus, we evaluated the expression of different NADPH oxidase subunits that contribute to recruitment and activation of this ROS-generating enzyme in alcohol-fed livers. We observed that alcohol diet was an independent factor that increased the expression of gp91phox and p40phox in WT, WT/WT and p47phox-KO mice, but not in WT/p47phox-BM KO mice (Fig. 5A–F; Table 1). Specifically, total body deficiency of p47phox increased alcohol-induced expression of the NADPH oxidase subunits gp91phox and p40phox, compared to WT mice (Fig. 5A,C). In contrast, BM-specific deficiency of p47phox resulted in a significantly increased baseline expression of gp91phox and p40phox, which did not show further induction by alcohol (Fig. 5B,D).

As we expected, the expression of p47phox was absent in total body p47phox-KO mice, whereas there was a detectable residual expression of p47phox in WT/p47phox-KO-BM mice (Fig. 5E,F). Together with the observations that WT/p47phox-KO-BM were not protected from alcohol-induced liver steatosis and damage, this observation suggests that even low presence of NADPH oxidase in liver parenchymal cells might be sufficient to mediate liver injury in chronic EtOH feeding.

The expression of pro-inflammatory genes is largely regulated by NF- κ B, the master regulator of pro-inflammatory genes (Perkins, 2007). We found up-regulation of NF- κ B DNA binding in the livers of alcohol-fed WT mice but not in total body p47phox-KO mice (Fig. 6). It is important to note that these p47phox total body KO mice were also protected from accumulation of liver fat and from liver injury (Fig. 1). In contrast, chimeras of WT mice with p47phox-KO BM-derived cells showed increased NF- κ B activation in the liver after alcohol feeding and were not protected from the development of steatotic liver and liver injury after alcohol feeding compared to pair-fed mice (Fig. 6B).

DISCUSSION

The current concept on the development of ALD implies involvement of 2 key pathogenetic components: metabolic and inflammatory pathways (Miller et al., 2011; Nath and Szabo, 2009). The first one is represented by a variety of EtOH-induced changes that include redox state changes, production of the reactive product acetaldehyde, damage to mitochondria, direct or membrane effects caused by hydrophobic EtOH, EtOH-induced hypoxia, EtOH induction of CYP2E1, EtOH mobilization of iron, and effects on antioxidant enzymes and chemicals like mitochondrial and cytosolic glutathione (Dey and Cederbaum, 2006). Second, it has been shown that the influence of alcohol on the immune system could play a key role in alcohol-induced liver damage and inflammation (Mandrekar and Szabo, 2009). Among several mediators, ROS has been implicated in the activation of the inflammatory cascade in ALD (Thakur et al., 2006).

In our present work, we showed that NADPH oxidase plays an important role in a mouse model of chronic EtOH feeding because total body inactivation of a critical cytosolic p47phox subunit led to protection from the development of liver steatosis and injury and from NF- κ B activation. Utilizing chimeric mice with p47phox deficiency specific for BM-derived cells, we demonstrated for the first time that protection associated with p47phox deficiency did not depend on the absence of NADPH oxidase in BM-derived immune cells. Rather, our results suggest that the pathogenic role of p47phox is specific for liver parenchymal cells. Our observation complements previous reports where increased activation of the NADPH oxidase system and p47phox was demonstrated in Kupffer cells in the liver after alcohol feeding (Thakur et al., 2006). We found that the possible mechanism explaining the involvement of NADPH oxidase-derived ROS is likely realized through the alcohol-induced stimulation of genes responsible for fatty acid synthesis and stabilization of lipid droplets with ADRP in the liver. Interestingly, despite the fact that NADPH oxidase complex is present in the liver mostly in BM-derived cells (as it could be seen from the analysis of the expression of p47phox mRNA in chimera mice; Fig. 5E,F), selective inactivation of p47phox in immune cells was not sufficient to prevent liver injury and lipid accumulation. These findings allow the speculation that even the relatively small expression of NADPH oxidase complex in parenchymal cells (hepatocytes or liver stellate cells) is important in mediation of the consequences of alcohol action on fat metabolism.

In addition to ROS, gut-derived lipopolysaccharide (LPS) has been proposed to play a central role in ALD (Mandal et al., 2010; Szabo and Bala, 2010). Studies showed that because of inhibition of tight junction protein expression in gut mucosa, alcohol can disrupt gut barrier function, which leads to the increased leakiness of Gram-negative bacterial endotoxin (LPS) from the intestine to systemic circulation (Bode and Bode, 2005; Rao, 2008; Rao et al., 2004). LPS leads to the activation of TLR4 and the stimulation of NF- κ B (Hritz et al., 2008a). In accordance with the results from the liver histology assessment, we found that p47phox total body deficiency was protective from alcohol-induced NF- κ B activation but the NF- κ B increase was not prevented in WT/p47phox-KO-BM chimeras.

Recent studies brought attention to the significance of NADPH oxidase complex in nonphagocytic cells, in which superoxide and its derivatives are playing important roles as

signaling molecules. For example, in a mouse model of myocardial infarction caused by permanent ligation of the left coronary artery, it was shown that cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis were reduced in p47phox-KO mice (Doerries et al., 2007). In another work, cultured vascular smooth muscle cells after isolation from p47phox-KO mice demonstrated decreased superoxide generation compared with WT mice when stimulated with thrombin (Barry-Lane et al., 2001). Furthermore, a cell signaling role for endogenous ROS produced by NADPH oxidase was confirmed by the study of airway structure/mechanical properties and airway smooth muscle (ASM) function when an impaired ASM relaxation was found in p47phox-KO mice (Chitano et al., 2008).

Recently, it was shown that specific inactivation of the p47phox subunit in liver parenchymal cells through the generation of chimera mice led to much more significant reduction in liver fibrosis model compared to chimera mice with p47phox-KO-BM-derived cells (Kupffer cells) and WT endogenous liver cells (60% vs. 25%, correspondingly; De Minicis et al., 2010). Our present results corroborate the importance of p47phox in liver parenchymal cells. Our finding of protection from ALD-related steatosis and NF- κ B activation in the total body p47phox-KO but not in the chimeras with p47phox deficiency only in BM-derived immune cells suggests that p47phox expression in liver parenchymal cells plays the critical role in the induction of steatosis in this model.

The effects of selective inhibition of p47phox in liver parenchymal cells may shed lights on the cell-specific effects of p47phox in the future.

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Fig. 1.

Total body p47phox knockout (p47phox-KO) but not cell-specific deficiency of p47phox protects mice from alcohol-induced liver steatosis. C57Bl/6 (wild type [WT]) female mice received Lieber–DeCarli alcohol or pair-fed diet for 4 weeks. **(A)** Photomicrographs prepared from H&E-stained liver sections from ethanol (EtOH)-fed or pair-fed mice. **(B)** Photomicrographs prepared from Oil Red O-stained frozen liver sections from EtOH-fed or pair-fed mice. **(C, D)** Oil Red O-positive area was quantified for the comparison of steatotic changes between mouse groups. There were 6 to 8 mice/group. PF, pair-fed; BM, bone marrow.

Fig. 2.

Total body p47phox deficiency more efficiently reduced alcohol-induced liver injury than selective lack of p47phox expression in bone marrow (BM)-derived cells. Mice received alcohol or pair-fed diet as described in Fig. 1. (A, B) Liver triglycerides were measured using the L-Type Triglyceride H kit. (C, D) Serum alanine aminotransferase (ALT) was quantified as an index of liver injury in sera from ethanol (EtOH)-fed or pair-fed mice. There were 6 to 8 mice/group. KO, knockout; PF, pair-fed; WT, wild type.

Fig. 3.

Chronic ethanol (EtOH) feeding stimulates stabilization of lipid droplets in the liver in a p47phox-dependent manner. Whole-liver lysates were prepared and used for the measurement of adipocyte differentiation-related protein (ADRP) in (A) WT and p47phox-KO groups or (C) in WT/WT-BM and WT/p47phox-KO-BM groups. Western blot (WB) ADRP band densitometry is shown in the corresponding graphs (B, D). BM, bone marrow; KO, knockout; PF, pair-fed; WT, wild type.

Fig. 4.

Chronic ethanol (EtOH) feeding stimulates fatty acid synthesis in the liver in a p47phox-dependent manner. **(A, B)** Fatty acid synthase (FASN) and **(C, D)** acetyl-CoA carboxylase (ACACA) mRNA expression in livers of mice after 4 weeks of 5% (vol/vol) EtOH or pair feeding. There were 6 to 8 mice/group. BM, bonemarrow; KO, knockout; PF, pair-fed; WT, wild type.

Fig. 5.

Chronic ethanol (EtOH) feeding in mice leads to the activation of NADPH oxidase. (**A, B**) gp91phox or (**C, D**) p40phox subunit mRNA in EtOH-fed or pair-fed mouse livers was quantified by real-time polymerase chain reaction. (**E, F**) p47phox mRNA expression in livers of mice after 4 weeks of 5% (vol/vol) EtOH or pair feeding. There were 6 to 8 mice/group. BM, bone marrow; KO, knockout; PF, pair-fed; WT, wild type.

Fig. 6.

Chronic ethanol (EtOH) feeding in mice leads to the increased nuclear factor κ B (NF- κ B) binding in liver and occurs despite inactivation of p47phox in bone marrow (BM)-derived cells. (A) NF- κ B DNA-binding activity was measured after equal loading of liver nuclear extracts using the electrophoretic mobility shift assay (EMSA) and a NF- κ B-specific probe; 10-fold excess of unlabeled NF- κ B-oligo was used for competition (cold comp); densitometry results of EMSA are shown as mean $\pm$ SE in the graph below (B). BM, bone marrow; KO, knockout; PF, pair-fed; WT, wild type.

Two-Way ANOVA of the Effect of Diet and Genotype on the Outcomes Investigated in the Study

Table 1

Outcome	WT vs. p47phox-KO mice (global deficiency model)				WT/WT vs. WT/p47phox-KO-BM (BM-specific model)			
	Figure	Diet	Genotype	Interaction	Figure	Diet	Genotype	Interaction
Liver Oil Red O-positive area	1C	<0.001	<0.001	0.266	1D	<0.001	<0.001	<0.001
Liver triglycerides	2A	<0.001	<0.001	0.502	2B	<0.001	0.039	0.088
Serum ALT	2C	<0.001	<0.001	0.004	2D	<0.001	0.002	0.094
Liver ADRP	3B	<0.001	0.002	0.906	3D	<0.001	0.12	0.981
Liver FASN	4A	0.078	0.005	0.121	4B	<0.001	0.243	0.123
Liver ACACA	4C	0.005	<0.001	0.191	4D	<0.001	0.861	0.743
Liver gp91phox	5A	<0.001	<0.001	0.329	5B	<0.001	<0.001	0.033
Liver p40phox	5C	<0.001	<0.001	0.247	5D	<0.001	<0.001	0.044
Liver p47phox	5E	—	—	—	5F	—	—	—

Numbers in the table represent *p*-values. Values < 0.05 for diet and genotype indicate their independent effect on the outcomes. *p*-Values < 0.05 for interaction indicate interdependency of diet and genotype. “Diet” refers to the comparison of pair-fed and alcohol feeding. “Genotype” refers to the comparison of WT vs. p47phox-KO mice in the total body deficiency model and to the comparison of WT/WT vs. WT/p47phox-KO-BM mice in the BM-specific model. No 2-way statistics was calculated for Fig. 5E because there was zero expression of liver p47phox in the p47phox-KO mice, and for data in Fig. 5F that showed significant differences in variance between groups on Levene’s test for homogeneity of variance. Outcomes are sorted in the order they appear in the text.

ACACA, acetyl-CoA carboxylase; ADRP, adipocyte differentiation-related protein; ALT, alanine aminotransferase; BM, bone marrow; FASN, fatty acid synthase; KO, knockout; WT, wild type.