Nuclear Factor-Eythroid 2–Related Factor 2 Prevents Alcohol-Induced Fulminant Liver Injury

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Background & Aims: **The transcription factor nuclear factor-eythroid 2–related factor 2 (*Nrf2*$^{-/-}$) is essential for protecting cells against xenobiotic and oxidative stress. Increased oxidative stress has been implicated in the pathophysiology of many diseases including ethanol-induced liver disease. Therefore, the role of *Nrf2*$^{-/-}$ in ethanol-induced liver injury was investigated.** **Methods:** **Wild-type and *Nrf2*$^{-/-}$ mice were fed with the ethanol diet, followed by examination of liver pathology, mortality, and ethanol metabolism.** **Results:** ***Nrf2*$^{-/-}$ mice displayed a dramatically increased mortality associated with liver failure when fed doses of ethanol that were tolerated by WT mice. *Nrf2*$^{-/-}$ mice showed a significantly reduced ability to detoxify acetaldehyde, leading to an accumulation of the toxic metabolite. Loss of *Nrf2*$^{-/-}$ caused a marked steatosis in livers of ethanol-fed mice, and Srebp1 was identified as a candidate transcription factor responsible for lipogenic enzyme induction. Furthermore, ethanol consumption led to a progressive depletion of total and mitochondrial reduced glutathione, which was associated with more pronounced structural and functional changes to mitochondria of *Nrf2*$^{-/-}$ mice. In addition, ethanol feeding elicited an aggravated inflammatory response mediated by Kupffer cells in *Nrf2*$^{-/-}$ mice as shown by an increased tumor necrosis factor-α secretion and activation of the interleukin-6/Stat-3 pathway. Together these changes lead to a vicious cycle of accumulating hepatocellular damage, ultimately leading to liver failure and death of *Nrf2*$^{-/-}$ mice.** **Conclusions:** **Our data establish a central role for *Nrf2*$^{-/-}$ in the protection against ethanol-induced liver injury.**

Alcohol-induced liver disease (ALD) progresses from early steatosis, inflammation, and necrosis (together described as steatohepatitis), to fibrosis and cirrhosis in some people. The progression of liver injury after chronic alcohol abuse is a multifactorial process involving a number of genetic and environmental factors. Factors that link ethanol intake to the onset and progression of liver injury still are poorly understood. Several pathways have been shown to play a role in ALD, including production of acetaldehyde, induction of cytochrome P450 2E1, and direct damage to mitochondria.[1] Acetaldehyde is a highly reactive intermediate that accounts for most of the toxic effects of ethanol. A correlation between the accumulation of acetaldehyde within the liver and cell injury has been observed.[2,3] Furthermore, it has been shown that acetaldehyde promotes hepatic lipid accumulation, which is considered the first step in the development of ALD. Progression beyond steatosis, however, requires a second hit that promotes inflammation and cell death. In this regard, the endotoxin-derived activation of Kupffer cells has been implicated in the pathogenesis of ALD. On priming, these cells make several pro-oxidants that increase the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α. The pivotal role of TNF-α in ALD is shown by experiments in *TNF-R1*$^{-/-}$ mice that are resistant to the toxic effects of ethanol feeding.[4]

It has been reported previously that nuclear factor-eythroid 2–related factor 2 (*Nrf2*$^{-/-}$) is induced in livers of mice treated with ethanol.[5] *Nrf2*$^{-/-}$ is a transcriptions factor belonging to the cap-n-collar family of activators that share a highly conserved basic leucine zipper structure[6] and protect cells against oxidative stress. *Nrf2*$^{-/-}$ is expressed constitutively and ubiquitously in several tissues and is responsible for the low expression level of its target genes. In cells exposed to oxidative stress, *Nrf2*$^{-/-}$ is activated and further drives the transcription of its target genes that are essential for a coordinated antioxidative stress response. The importance of *Nrf2*$^{-/-}$ in the liver is shown by the marked increase in sensitivity of *Nrf2*$^{-/-}$ mice to acetaminophen-induced liver toxicity.[7] Interestingly, regulation of *Nrf2*$^{-/-}$ cellular glutathione

Abbreviations used in this paper: ALD, alcohol-induced liver disease; EtOH, ethanol; GSH, reduced glutathione; IL, interleukin; LPS, lipopolysaccharide; *Nrf2*$^{-/-}$, nuclear factor-eythroid 2–related factor 2; PCR, polymerase chain reaction; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

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and other antioxidants also is important for optimal activation of nuclear factor $\kappa\beta$ and TNF-α in response to lipopolysaccharide (LPS). Disruption of *Nrf2*$^{-/-}$ leads to a dramatically increased mortality during experimental sepsis owing to an inappropriate innate immune response.[8] In this study, we investigated the potential role of *Nrf2*$^{-/-}$ in alcoholic liver injury. We report that *Nrf2*$^{-/-}$ is a critical regulator of ALD that dramatically improves survival after alcohol intake.

Materials and Methods

Mice

Male *Nrf2*$^{-/-}$ mice on the C57BL/6 background were provided by M. Yamamoto. As controls, age-matched male C57BL/6 mice were obtained from Charles River Laboratories (Sulzfield, Germany). Animal experiments were approved by the district government of Lower Saxony, Germany. All mice (6–8 weeks of age) were fed a control Lieber–deCarli liquid diet containing maltose-dextrin for an initial 3-day period (Bioserv, Frenchtown, NJ). The ethanol (EtOH)-fed group was introduced to the diet gradually by increasing the EtOH content of the diet by 2.1% (vol/vol) every third day until the mice were consuming diets containing 6.3% (vol/vol) EtOH. The diet was not changed thereafter. Control mice were pair-fed an isocalorically identical volume of EtOH-free diet daily for 12 days. Most *Nrf2*$^{-/-}$ mice became moribund after 4–7 days on the 6.3% EtOH diet. These mice were combined in one group (for each ethanol-fed *Nrf2*$^{-/-}$ mouse in this group [n = 10], an EtOH-fed wild-type [WT] mouse was euthanized and analyzed). In a second set of experiments, mice received one dose of EtOH (5 g/kg body weight, diluted 25:75; vol/vol in water) by gavage (n = 4). Ethanol was administered once daily by gastric intubation for 4 consecutive days, during which time mice were permitted ad libitum consumption of water and standard pellet show.

Mice that appeared healthy on the EtOH diet (4 days on 6.3% EtOH diet) and controls received a single intraperitoneal injection of 100 μg of LPS (Sigma) and were euthanized at 1.5 and 6 hours post-LPS injection. To investigate the effect of antioxidants, mice were treated with NAC (Sigma, Taufkirchen, Germany) (500 mg/kg body weight intraperitoneally) twice daily during ethanol feeding.

Aminotransferase and TNF-α/Interleukin-6 Determinations

For aminotransferase analysis, animal blood was drawn and used for determination of aminotransferase activities (AU 400; Olympus, Hamburg, Germany). TNF-α and interleukin (IL)-6 were determined with the respective mouse enzyme-linked immunosorbent assay set (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Carbonylation Assay

The levels of modified proteins were quantified by measurement of protein carbonyl content using the Protein Oxidation Detection Kit (Calbiochem, Temecula, CA).

8-Oxo-Deoxyguanosine Analysis

Genomic DNA samples were treated with Micrococcus nuclease and phosphodiesterase, and analyzed for the presence of 8-hydroxydeoxyguanosine by high-performance liquid chromatography–electrochemical detection. 8-hydroxydeoxyguanosine and deoxyguanosine were separated on a LiChrocart 250-4 (Merck, Darmstadt, Germany) high-performance liquid chromatography and quantified with an Agilent Technologies electrochemical detector (Santa Clara, CA).

Antibodies

Antibodies against inducible nitric oxide synthase were purchased from BD Biosciences, against CD68 from AbD Serotec (Düsseldorf, Germany), against Ho-1 from Stressgen (Victoria, Canada), against MnSod and p47 from Millipore (Bedford, MA), against Nqo-1 from Abcam (Cambridge, MA), and against c-Jun and p-Jnk from Cell Signaling (Danvers, MA). Antibodies against glutamate-cysteine ligase catalytic subunit and glutamate-cysteine ligase modifier subunit kindly were provided by T. J. Kavanagh (University of Washington, Seattle, WA), and the antibody against cytochrome P450 2E1 was provided by D. R. Koop (Oregon Health and Science University, Portland, OR).

Western Blot Analysis

Liver tissue was homogenized in cell lysis buffer containing complete protease inhibitor (Roche, Mannheim, Germany), centrifuged for 10 minutes at 16,000×*g*, and resuspended in Laemmli sample buffer. Protein extracts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). Western blotting was performed as previously described.[9] Ponceau red staining was performed with each blot to show equal protein loading of the samples using routine protocols.

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed using the in situ cell death detection kit (Roche) according to the manufacturer's instructions. H&E staining was performed using a standard protocol for formalin-fixed sections. Immunohistochemistry was performed on frozen sections or on formalin-fixed sections.