Research Article

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Activation of p53 enhances apoptosis and insulin resistance in a rat model of alcoholic liver disease

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Background & Aims: Chronic ethanol consumption in the Long–Evans (LE) rat has been associated with hepatic p53 activation, and inhibition of the insulin/PI3K/AKT signal transduction cascade due to increased expression of PTEN. We hypothesize that p53 activation and altered insulin signaling may influence the susceptibility of rats to ethanol-induced liver damage. Furthermore, p53 not only activates programmed cell death pathways and suppresses hepatocellular survival signals, but also promotes gluconeogenesis to increase systemic insulin resistance due to a novel metabolic function.

Methods: Fischer (F), Sprague–Dawley (SD) and LE rats were fed ethanol-containing or control liquid diet for 8 weeks. Histopathological and biochemical changes were assessed.

Results: Here, we demonstrate that chronic ethanol feeding in rats promotes p53 activation, hepatic steatosis, oxidative stress, PUMA, and PTEN expression, which contribute to hepatocellular death and diminished insulin signaling in the liver. Such changes are pronounced in the LE, less prominent in SD, and virtually absent in the F rat strain. More importantly, there is activation of Tp53-induced glycolysis and apoptosis regulator (TIGAR) in the ethanol-fed LE rat. This event generates low hepatic fructose-2,6-bisphosphate (Fru-2,6-P_2) levels, reduced lactate/pyruvate ratio and may contribute to increased basal glucose turnover and high residual hepatic glucose production during euglycemic hyperinsulinemic clamp.

Conclusions: p53 activation correlates with the susceptibility to ethanol-induced liver damage in different rat strains. p53 not only orchestrates apoptosis and suppresses cell survival, but by activating TIGAR and decreasing hepatic Fru-2,6-P_2 levels it promotes insulin resistance and therefore, contributes to the metabolic abnormalities associated with hepatic steatosis.

Introduction

Alcoholic liver disease (ALD) is a disorder governed by gene–environment interactions [1]. A myriad of genetic factors have been linked to increased susceptibility towards ethanol-induced liver damage, many of which are involved in the regulation of insulin signaling, oxidative stress, and apoptosis [2]. Hepatic insulin resistance, micro- and macrovesicular steatosis, increased oxidative stress, hepatocellular death, and suppressed cell survival signaling are the cardinal features of ALD produced by 8 weeks of ethanol feeding in the LE rat [3,4]. Chronic ethanol exposure impaired survival mechanisms in the liver by constitutive inhibition of insulin/PI3K signaling and downstream AKT activity, an effect that was mediated by increased PTEN expression and function [4]. Robust nuclear accumulation of p53 during chronic ethanol feeding was also observed [4]. It is established that PTEN is a downstream target of p53 [5] promoting hepatic insulin resistance [3]. However, the role of p53 in ALD has not been fully elucidated. Activation of p53 can be critical in ethanol-induced hepatocyte apoptosis, since genetic ablation of p53 abrogates ethanol-induced liver damage [6]. Furthermore, 4 weeks of ethanol feeding in rats was shown to increase mRNA abundance and acetylation of p53 [7]. It was recently demonstrated that the lifespan regulator $p66^{shc}$ that is indispensable for p53-dependent apoptosis [8] may have a pivotal role in ethanol-induced liver damage [9].

We compared rat strain susceptibility to ALD in the context of p53 activation [4]; this approach allowed us to correlate p53 alterations with liver damage. We demonstrated that p53 may

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Abbreviations: LE, Long–Evans; PTEN, phosphatase and tensin homologue deleted on chromosome 10; F, Fischer; SD, Sprague–Dawley; PUMA, p53 upregulated modulator of apoptosis; TIGAR, Tp53-induced glycolysis and apoptosis regulator; Fru-2,6-P_2, fructose-2,6-bisphosphate; ROS, reactive oxygen species; ALD, alcoholic liver disease; ALT, alanine aminotransferase; ADH, ethanol dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DCF, 2′,7′-dichlorodihydrofluorescin diacetate; LX-PCR, long-extension polymerase chain reaction; mtDNA, mitochondrial DNA; EMSA, electrophoretic mobility shift assay; SREBP1c, sterol regulatory element-binding protein 1c; CYP2E1, cytochrome P450 2E1; IRS-2, insulin receptor substrate 2; PARP, poly (ADP-ribose) polymerase; ISOL, in situ oligo ligation; SCO2, synthesis of cytochrome c oxidase 2; HGP, hepatic glucose production; Ra, rate of glucose appearance; Rd, rate of glucose disappearance.

have a broader role in the pathogenesis of ALD than previously determined; p53 not only orchestrated hepatocellular death and suppressed cell survival, but also by upregulating TIGAR, caused perturbations in hepatic glucose metabolism that contributed to insulin resistance. Thus, p53 activation may be important in oxidative stress, apoptosis, impaired survival signaling, liver regeneration [10], and sustaining systemic and hepatic insulin resistance. Investigation of such pathways, in different rat strains, led to the hypothesis that ethanol-induced p53 expression may be crucial for the development of ALD.

Materials and methods

Animals and treatment

The 150–200 gram, male Fischer, Sprague–Dawley and Long–Evans rats (Harlan Laboratories, Indianapolis, IN) were fed *ad libitum* with ethanol-containing (ethanol-derived calories were increased from 13% to 36% in the first two weeks) or isocaloric control liquid diet (Bioserv, Frenchtown, NJ) for eight weeks (following initial adaptation). Body weights and food intake were recorded weekly. After 8 weeks, the animals were fasted for 4 h and sacrificed. Blood and liver samples were collected. Five animals from each group were used for hepatocyte isolation. The Lifespan Animal Welfare Committee of Rhode Island Hospital, Providence, RI, approved all animal experiments.

Histological studies and image analysis

Histological changes in the liver were assessed by routine hematoxylin–eosin, Oil Red O, Masson's trichrome and Sirius red collagen staining. Slides were scanned with Aperio ScanScope CS (Aperio Technologies Inc., Vista, CA). Area measurements were performed with iVision software (BioVision Technologies, Exton, PA). Positive Oil Red O staining was defined through intensity thresholding. Apoptosis was assessed by Apoptag® ISOL Dual Fluroescence Apoptosis Detection Kit (Millipore, Billerica, MA). Cells stained for apoptosis were visually counted and recorded. Results were expressed as number of apoptotic cells per square millimeter.

Chemicals

All chemicals used in these studies were purchased from Sigma (Sigma–Aldrich, St. Louis, MO), unless otherwise specified.

Biochemical assays

Serum alanine aminotransferase (ALT) levels were measured by using a commercially available kit (Thermo Fisher Scientific Inc., Waltham, MA). Liver lysates were used to measure triglyceride, lactate, and pyruvate with corresponding kits (BioVision Inc., Mountain View, CA). Serum alcohol was measured by Analox GM7 analyzer (Analox Instruments, Lunenburg, MA). Alcohol dehydrogenase (ADH) activity in liver lysates was measured with an assay kit (Biomedical Research Center, SUNY Buffalo, Buffalo, NY). Fru-2,6-P_2 content was measured by the previously described endpoint enzymatic assay [11]. ADH, triglyceride, lactate, pyruvate, and hepatic Fru-2,6-P_2 assays were normalized to the protein content of the liver lysates. Protein concentration was determined by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc./Pierce, Waltham, MA).

Assessment of basal glucose kinetics and euglycemic hyperinsulinemic clamp in rats

The euglycemic hyperinsulinemic clamp in rats has been carried out as previously described [12]. The detailed description of these methods can be found in Supplementary material.

Caspase-3 assay

Caspase-3 activity was measured by the CPP32/Caspase-3 Fluorometric Assay Kit (BioVision Inc., Mountain View, CA) following the manufacturer's instructions. The optical density values were normalized to the protein content of the lysates.

Hepatocyte isolation and cell fractionation

Hepatocyte isolation was carried out as previously described [13]. Nuclear and cytosolic proteins were separated by using NE-PER Kit (Thermo Fisher Scientific, Inc./Pierce, Waltham, MA) from fresh liver tissue. The purity of these fractions was assessed by performing Western-blots with nuclear lamin A (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and cytosolic β-actin (Sigma–Aldrich, St. Louis, MO) or GAPDH (Cell Signaling Technology Inc., Danvers, MA) antibodies.

Measurement of intracellular ROS

Intracellular ROS formation was assessed by using 2′,7′-dichlorodihydrofluorescin (DCF) diacetate (Invitrogen Corporation, Carlsbad, CA), as previously described [14].

Long-extension polymerase chain reaction (LX-PCR) to detect mitochondrial DNA (mtDNA) damage

DNA was extracted from flash-frozen liver tissue samples by using the EZ1® DNA Tissue Kit with BioRobot EZ1 (Qiagen Inc., Valencia, CA). The LX-PCR and rat mtDNA specific primer sequences have been previously described [15]. PCR products less than 16-kb were considered damaged/rearranged mtDNA species.

p53 and Sterol Regulatory Element-Binding Protein 1c electrophoretic mobility shift assays

The LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc./Pierce, Waltham, MA) was used to assess the *in vitro* binding activity of p53 and Sterol Regulatory Element-Binding Protein 1c (SREBP1c). 5′-end biotin labeled oligonucleotides with the corresponding unlabeled controls were obtained from Sigma (Sigma–Aldrich, St. Louis, MO). The sequences of the p53 and SREBP1c consensus oligonucleotides are available upon request. The binding reaction and subsequent steps were carried out following the manufacturer's instructions.

Western-blot analysis

Whole tissue lysate was prepared from livers, as previously described [14]. Protein lysates were separated on 6–15% SDS–PAGE and transferred onto PVDF membrane (Thermo Fisher Scientific Inc./Pierce, Waltham, MA). The following primary antibodies were used: CYP2E1 (Abcam Inc., Cambridge, MA), p53, PUMA-α, PTEN, p110 subunit of PI3K, AKT1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), IRS-2, p85 subunit of PI3K (Millipore, Billerica, MA), p-AKT^{S473} (R&D Systems, Inc., Minneapolis, MN), p-AKT^{T308}, cleaved-PARP (Cell Signaling Technology Inc., Danvers, MA), TIGAR (Lifespan Biosciences Inc., Seattle, WA).

Statistical analysis

Data are presented as means ± SEM and analyzed by using GraphPad Instat® software (GraphPad Software, Inc., La Jolla, CA); unpaired Student's *t* or Tukey–Kramer tests were executed unless otherwise stated. Data pertaining to basal glucose metabolism and euglycemic hyperinsulinemic clamp were analyzed by ANOVA followed *post hoc* by Student–Neuman–Keuls test for multiple group comparisons. Statistical consideration related to weight gain in the various experimental groups can be found in Supplementary Material. Differences among the groups were considered significant when $p < 0.05$.

Results

Hepatic steatosis and ALT elevation

The level of steatosis and hepatocellular damage, as measured by ALT levels, induced by chronic ethanol feeding was compared in three rat strains. We compared the ethanol-sensitive LE rat [4] to two other frequently used laboratory rat strains (F and SD) to assess the differences in ethanol consumption, blood ethanol levels, ethanol metabolism, and weight gain during feeding. Experiments were repeated three times, using 5–10 animals per group