BBA - Molecular Basis of Disease 1863 (2017) 3170–3182

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

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

Growth arrest and DNA damage-inducible 45α protects against nonalcoholic steatohepatitis induced by methionine- and choline-deficient diet☆,☆☆

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ARTICLE INFO

Keywords:
Oxidative stress
ER stress
Lipidomics
Glycerophospholipid metabolism
Burned-out NASH

ABSTRACT

Growth arrest and DNA damage-inducible 45 α (Gadd45α) is a stress-inducible protein that plays an important role in cell survival/death and DNA repair, but its contribution to the development of nonalcoholic steatohepatitis (NASH) has not been investigated. C57BL/6 *Gadd45a*-null and wild-type (WT) mice were treated with a methionine and choline-deficient diet (MCD) for eight weeks and phenotypic changes examined. *Gadd45a*-null mice had more severe hepatic inflammation and fibrosis, higher levels of mRNAs encoding pro-inflammatory, pro-fibrotic, and pro-apoptotic proteins, and greater oxidative and endoplasmic reticulum (ER) stress compared with WT mice. Indeed, *Gadd45a* mRNA was induced in response to ER stress in primary hepatocytes. Lipidomic analysis of NASH livers demonstrated decreased triacylglycerol (TG) in MCD-treated *Gadd45a*-null mice, which was associated with increased mRNAs encoding phospholipase D (*Pld1/2*), phosphatidic acid phosphatase type 2A, and choline/ethanolamine phosphotransferase 1 (*Cept1*), involved in the phosphatidylcholine-phosphatidic acid-diacylglycerol cycle, and decreased mRNAs encoding fatty acid (FA)-binding protein 1 (*Fabp1*) and FA transport protein 5. Treatment of cultured primary hepatocytes with tumor necrosis factor α, transforming growth factor β, and hydrogen peroxide led to the corresponding induction of *Fabp1*, *Pld1/2*, and *Cept1* mRNAs. Collectively, Gadd45α plays protective roles against MCD-induced NASH likely due to attenuating cellular stress and ensuing inflammatory signaling. These results also suggest an interconnection between hepatocyte injury, inflammation and disrupted glycerophospholipid/FA metabolism that yields a possible mechanism for decreased TG accumulation with NASH progression (i.e., "burned-out" NASH).

1. Introduction

Nonalcoholic steatohepatitis (NASH) is a major chronic liver disease defined by the presence of macrovesicular steatosis, hepatocyte ballooning, and hepatic inflammation regardless of no ethanol consumption. Similar to alcoholic steatohepatitis, NASH can progress to liver cirrhosis, hepatocellular carcinoma, and hepatic failure. It was documented that the accumulation of toxic lipids, such as saturated fatty acid (FA), free cholesterol, and diacylglycerol (DG) in hepatocytes augmented oxidative stress and endoplasmic reticulum (ER) stress, leading to enhanced inflammatory signaling, hepatocyte apoptosis, and Kupffer cell/stellate cell activation [1,2]. Therefore, oxidative and ER stress are thought to be key contributors to the progression from steatosis to steatohepatitis.

Abbreviations: NASH, nonalcoholic steatohepatitis; FA, fatty acid; DG, diacylglycerol; ER, endoplasmic reticulum; Gadd45, growth arrest and DNA damage-inducible 45; DMBA, dimethylbenzanthracene; WT, wild-type; ATF, activating transcription factor; MCD, methionine- and choline-deficient diet; UPLC-ESI-QTOFMS, ultraperformance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry; PCA, principal component analysis; OPLS, supervised orthogonal projection to latent structure; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; qPCR, quantitative polymerase chain reaction; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TG, triglyceride; NEFA, non-esterified fatty acid; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; GSH, glutathione; TNFα, tumor necrosis factor α; TGFβ, transforming growth factor β; ROS, reactive oxygen species; Dr5, death receptor 5; Bim, BCL2-like 11; PLD, phospholipase D; PA, phosphatidic acid; PPAP, PA phosphatase; PE, phosphatidylethanolamine; CEPT, choline/ethanolamine-phosphotransferase 1; FABP, FA-binding protein; SPT, serine palmitoyltransferase; PKC, protein kinase C

☆ Potential conflict of interest: The authors have declared that no conflict of interest exists.
☆☆ Financial support: Supported by the National Cancer Institute Intramural Research Program and U54 ES16015.

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http://dx.doi.org/10.1016/j.bbadis.2017.08.017
Received 10 May 2017; Received in revised form 12 August 2017; Accepted 15 August 2017
Available online 24 August 2017
0925-4439/ Published by Elsevier B.V.

Growth arrest and DNA damage-inducible 45 α (Gadd45α) is a 17–18 kDa protein that is linked to several cellular events, such as cell survival, DNA repair, chromatin assembly, and genome stability [3,4]. For example, Gadd45α is markedly induced after the administration of dimethylbenzanthracene (DMBA), a genotoxic carcinogen in mice, and decreased DNA repair, increased mutation frequency, and enhanced tumorigenesis were observed in DMBA-treated *Gadd45a*-null mice compared with wild-type (WT) mice [5]. Additionally, Gadd45α is regulated by p53 and stress-responsive transcription factors, such as activating transcription factor (ATF) 2 [4,6]. These findings indicate a possible link between Gadd45α and chronic liver injury, but its contribution to the pathogenesis of NASH remains unclear.

To address this issue, *Gadd45a*-null and WT mice were treated with methionine- and choline-deficient diet (MCD), a conventional NASH-inducible diet in rodents, for eight weeks.

2. Materials and methods

2.1. Mice and treatment

All studies were conducted according to Institute of Laboratory Animal Resource guidelines and approved by the National Cancer Institute Animal Care and Use Committee. The mice were housed in a specific pathogen-free environment controlled for temperature and light (25 °C, 12-h light/dark cycle) and maintained with NIH31 regular chow and tap water *ad libitum*. The MCD and control MCS diets were purchased from Dyets Inc. (Bethlehem, PA; #518810 and #518754, respectively). The compositions of these diets were described previously [7,8]. Before starting the experiments, the NIH31 chow was replaced with control MCS for acclimatization. After five-day acclimatization, male C57BL/6NCr WT and *Gadd45a*-null mice at 8–12 weeks of age (n = 6–8/group) [5] were moved to new cages and the respective diet was given for eight weeks. To examine time course of *Gadd45a* mRNA expression in MCD-induced NASH, liver tissues obtained from male C57BL/6NCr WT mice fed a MCS or MCD for three days and one, two, and four weeks reported previously [7–9] were used (n = 5–7/group). Throughout all experiments, mice were weighed and killed after a four- to six-hour fasting. Blood was collected using Serum Separator Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and centrifuged for 10 min at 8000*g* at 4 °C. Liver was isolated, weighed, and divided into the two parts. One part of the liver tissue (a neighboring lobe to gallbladder) was immediately soaked in 10% neutral formalin for histological examination. Sera and the remaining liver were immediately frozen in liquid nitrogen and kept at −80 °C until use.

2.2. Serum metabolomic analysis

Samples were prepared and serum metabolomics was performed using ultraperformance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) as described previously [7,8,10]. All samples were analyzed in a randomized fashion and MassLynx software (Waters Corp., Milford, MA) was used to acquire the chromatogram and mass spectrometric data. Centroided and integrated chromatographic mass data were processed by MarkerLynx (Waters) to generate a multivariate data matrix. Pareto-scaled MarkerLynx matrices including information on sample identity were analyzed by principal component analysis (PCA) and supervised orthogonal projection to latent structure (OPLS) analysis using SIMCA-P + 12 (Umetrics, Kinnelon, NJ). The ions were searched using METLIN metabolite database. The identity of ions was confirmed by tandem mass spectrometry MS/MS fragmentation patterns. The abundance of metabolites was determined by calculating peak area, normalized to that of chlorpropamide, and expressed as fold changes relative to that of MCS-treated WT mice.

2.3. Liver lipidomic analysis

Approximately 25 mg of liver tissue was homogenized in H_2O/methanol (300 μL/400 μL) containing chlorpropamide and aminopimelic acid (final concentrations of 5 μM and 10 μM, respectively). The homogenates were added to 800 μL of chloroform containing heptadecanoyl-lysophosphatidylcholine (17:0-LPC, 2 μM), heptadecanoyl-phosphatidylcholine (17:0-PC, 1 μM), heptadecanoyl-sphingomyelin (2 μM), and heptadecanoyl-ceramide (2 μM), incubated at 37 °C while shaking for 20 min, and then centrifuged at 10,000*g* for 20 min. Organic phases were carefully collected, evaporated under nitrogen flow around 1 h, and dissolved with 100 μL of methanol/chloroform (1:1). After 50-fold dilution with injection buffer (isopropanol:acetonitrile:H_2O = 2:1:1), samples were subjected to using UPLC-ESI-QTOFMS. The samples were separated and analyzed using a Waters Acquity UPLC system coupled to a Waters Synapt HDMS Quadrupole-Time of Flight (Q-TOF) mass spectrometer operating under the following conditions: capillary volts 2.8 kV, sample cone 30 V, source temperature 150 °C, desolvation temperature 400 °C, cone and desolvation gas flow 50 and 850 L/h, respectively. Data was acquired in centroid mode in both positive and negative electrospray ionization modes, using sulfadimethoxine (m/z 311.0814 +, 309.0658 −) as the lock mass. Mass range acquired was 100–1200 m/z at 0.3 s scans. Chromatography was carried out using a Waters Acquity CSH C18 column (2.1 × 100 mm) under acidic conditions buffered with 10 mM ammonium formate using the following compositions: (A) 60% acetonitrile in water; (B) 10% acetonitrile in isopropanol. The following gradient (6 = linear, 1 = ballistic) was used: initial conditions 60% (A) to 80% (A) at 6.5 min (6), to 50% (A) at 2.1 min (1), to 46% (A) at 12.0 min (6), to 30% (A) at 12.1 min (1), to 1% (A) at 18.0 min (6), to 60% (A) at 18.1 min (6), held for 5 min for column equilibration before the next injection. Total run time was 21 min. Flow rate was maintained at 0.4 mL/min throughout the run and column temperature was maintained at 55 °C. All samples were injected at 5 μL, using partial loop with needle overfill. Centroided and integrated chromatographic mass data were processed by MarkerLynx (Waters) to generate a multivariate data matrix. Pareto-scaled MarkerLynx matrices including information on sample identity were analyzed by PCA and supervised OPLS analysis using SIMCA-P + 12 (Umetrics, Kinnelon, NJ). The OPLS loading scatter S-plot was used to determine the lipids that contributed significantly to the separation between MCD-treated WT and *Gadd45a*-null mice. The lipid metabolite structures were determined using the METLIN metabolite database. The identity of LPC in the liver was confirmed by tandem mass spectrometry MS/MS fragmentation patterns (Supplementary Fig. 1). The abundance of lipid metabolites was measured by calculating peak area, normalized to that of chlorpropamide, and expressed as fold changes relative to that of MCS-treated WT mice.

2.4. Quantitative polymerase chain reaction (qPCR) analysis

After extraction of total RNA from liver tissue using a TRIzol Reagent (Invitrogen, Carlsbad, CA), cDNA was generated from 1 μg RNA with a SuperScript II™ Reverse Transcriptase kit and random oligonucleotides (Invitrogen) and qPCR was performed using SYBR green PCR master mix and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) [7–9]. The primer pairs were designed sing qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/) and listed in Supplementary Table 1. The mRNA levels were normalized to those of 18S ribosomal RNA and expressed as fold changes relative to those of MCS-treated WT mice.

2.5. Histological analysis

Small pieces of liver tissue were fixed in 10% neutral formalin, dehydrated by serial ethanol/xylene, and embedded in paraffin. The