Free Radical Biology and Medicine 65 (2013) 680–692

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Increased carbonylation of the lipid phosphatase PTEN contributes to Akt2 activation in a murine model of early alcohol-induced steatosis

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ARTICLE INFO

Article history:
Received 1 November 2012
Received in revised form
25 June 2013
Accepted 4 July 2013
Available online 17 July 2013

Keywords:
Alcoholic liver disease
PTEN
Steatosis
Lipid peroxidation
Free radicals

ABSTRACT

The production of reactive aldehydes such as 4-hydroxynonenal (4-HNE) is a key event in the pathogenesis of alcoholic liver disease (ALD), which ranges from simple steatosis to fibrosis. The lipid phosphatase PTEN plays a central role in the regulation of lipid metabolism in the liver. In this study, the effects of chronic ethanol feeding and carbonylation on the PTEN signaling pathway were examined in a 9-week mouse feeding model for ALD. Chronic ethanol consumption resulted in altered redox homeostasis as evidenced by decreased GSH, decreased Trx1, and increased GST activity. Both PTEN expression and PTEN phosphorylation were significantly increased in the livers of ethanol-fed mice. Carbonylation of PTEN increased significantly in the ethanol-fed mice compared to pair-fed control animals, corresponding to decreased PTEN 3-phosphatase activity. Concomitantly, increased expression of Akt2 along with increased Akt phosphorylation at residues Thr^{308}, Thr^{450}, and Ser^{473} was observed resulting in increased Akt2 activity in the ethanol-fed animals. Akt2 activation corresponded to a decrease in cytosolic SREBP and ChREBP. Subsequent LC/MS/MS analysis of 4-HNE-modified recombinant human PTEN identified Michael addition adducts of 4-HNE on Cys^{71}, Cys^{136}, Lys^{147}, Lys^{223}, Cys^{250}, Lys^{254}, Lys^{313}, Lys^{327}, and Lys^{344}. Computational-based molecular modeling analysis of 4-HNE adducted to Cys^{71} near the active site and Lys^{327} in the C2 domain of PTEN suggested inhibition of enzyme catalysis via either stearic hindrance of the active-site pocket or prevention of C2 domain-dependent PTEN function. We hypothesize that 4-HNE-mediated PTEN inhibition contributes to the observed activation of Akt2, suggesting a possible novel mechanism of lipid accumulation in response to increased reactive aldehyde production during chronic ethanol administration in mice.

Introduction

Steatosis is an early pathologic consequence of both nonalcoholic steatohepatitis (NASH) and chronic alcoholic liver disease (ALD). In general, steatosis is considered relatively benign. However, continued hepatic insults from toxins such as ethanol lead to a transition from mild steatosis to more dynamic and advanced hepatic phenotypes including steatohepatitis, fibrosis, and ultimately cirrhosis. The production of reactive aldehydes has been implicated in both NASH and ALD [1–5]. A well-documented marker for increased oxidative stress in cells is the presence of elevated levels of reactive aldehydes such as 4-hydroxynonenal (4-HNE) that originate from peroxidation of lipids including free or membrane-associated polyunsaturated fatty acids [6]. 4-HNE is a potent electrophile that will react with nucleophilic functional groups in DNA as well as Cys, Lys, and His residues within proteins. Many proteins have been documented to be targets for modification by 4-HNE, including protein disulfide isomerase, heat shock protein 90, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and Akt2 [7–10]. Consistent with the potent electrophilic properties of 4-HNE, proteins modified by this biogenic aldehyde exhibit compromised function.

PTEN is a dual-specificity phosphatase possessing both lipid and protein phosphatase activity and is a member of the protein tyrosine phosphatase (PTP) family of phosphatases [11,12]. PTEN is a tumor suppressor via its ability to regulate Akt, and loss-of-function mutations in PTEN lead to abnormal growth and proliferation as seen in patients with Cowden syndrome [13]. PTEN negatively regulates Akt activation through its ability to dephosphorylate the 3-position phosphate from phosphatidylinositol

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; ChREBP, carbohydrate response element-binding protein; 4-HNE, 4-hydroxy-2-nonenal; NASH, nonalcoholic steatohepatitis; PI3K, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTP, protein tyrosine phosphatase; SREBP, sterol-response element-binding protein; Trx, thioredoxin; TrxR, thioredoxin reductase

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.07.011

3,4,5-trisphosphate (PtdIns(3,4,5)P_3) to produce phosphatidylinositol 4,5-bisphosphate (PIP_2). Production of PIP_2 prevents the membrane lipid binding of the PH domain of Akt thereby preventing subsequent phosphorylation and kinase activation [14].

PTPs contain a signature HCX_5R motif within their active site [15]. The presence of a nucleophilic cysteine residue within the active site allows for regulation of PTEN by reactive oxidative species and oxidative stress. In addition to the aforementioned 4-HNE, both hydrogen peroxide and reactive nitrogen species have been shown to modify and inactivate PTEN [16,17]. In addition, PTEN has also been demonstrated to be a target of glutathionylation leading to a decrease in activity [18]. Inactivation of PTEN leads to sustained Akt activation in both cellular and animal models. Hepatocyte-specific deletion of PTEN leads to insulin hypersensitivity, steatohepatitis, and increased occurrence of hepatocellular carcinoma in mice [19]. Initiation of steatosis and hepatocyte proliferation was linked with increased Akt1/2 activation and Akt-dependent downstream activation of sterol-response element-binding protein 1c (SREBP1c) and PPARγ [20]. In demonstrating the direct link between PTEN and Akt2, concurrent hepatospecific deletion of Akt2 with PTEN deletion led to a decrease in steatosis [21,22].

In this report, we detail the effects of chronic ethanol on carbonylation of PTEN. Our results demonstrate that chronic ethanol administration leads to an increase in carbonylation of PTEN with an accompanying decrease in PTEN activity and subsequent increase in Akt2 activity. Thus, a correlation between inhibition of PTEN, increase in Akt2 activity, and increased steatosis is observed in ethanol-fed mice. Furthermore, using LC/MS/MS and in silico-based computational modeling, we identify a potential mechanism for 4-HNE-mediated inhibition of PTEN activity. Consequently, this report provides new insight into the mechanisms by which lipid peroxidation products such as 4-HNE affect intracellular signaling during chronic ethanol administration.

Materials and methods

Animal model and dietary information

All animal care and use procedures were in accordance with the University of Colorado Institutional Animal Care and Use Committee. Male C57BL/6 J mice (The Jackson Laboratory, Bar Harbor, ME, USA), ages 6–8 weeks, were divided into groups of 12 and pair-fed a modified Leiber–DeCarli diet (Bio-Serv, Frenchtown, NJ, USA) for a total of 9 weeks [23]. The diet consisted of 45% fat-derived calories and 16% protein-derived calories and the remaining balance comprised either ethanol- or maltose dextrin-derived calories. Ethanol content was increased on a weekly basis from week 1 to 7 starting with 2% v/v at week 1 with ethanol concentrations peaking at 6.5% by week 7 (35.0% ethanol-derived calories) and remaining constant at 6.5% until sacrifice at week 9. The pair-fed control animals were isocalorically matched with carbohydrate calories. Food consumption was monitored daily and body weights were measured once per week. Upon completion of the study, animals were anesthetized via intraperitoneal injection with sodium pentobarbital and euthanized by exsanguination. Blood was collected from the inferior vena cava and plasma was separated via centrifugation at 4 °C and assayed for alanine aminotransferase (ALT) activity (Sekisui Diagnostics, Lexington, MA, USA). Excised livers were weighed and subcellular fractions obtained via differential centrifugation as previously described [6]. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus and were performed in accordance with published National Institutes of Health guidelines.

Western blotting

Proteins from either whole liver extracts or subcellular fractions were subjected to standard SDS–PAGE and transferred to polyvinylidene difluoride (GE Healthcare, Piscataway, NJ, USA) as previously described [7]. Membranes were blocked for 60 min with a Tris-buffered saline solution containing 1% Tween 20 (TBST) and 5% nonfat dry milk and probed overnight with primary antibodies directed against $pSer^{380}$ PTEN, $pSer^{473}$ Akt, $pThr^{308}$ Akt, $pThr^{450}$ Akt, total Akt1, total Akt2, total PTEN (Cell Signaling, Danvers, MA, USA); SREBP, carbohydrate-response element-binding protein (ChREBP), Trx2, TrxR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma–Aldrich, St. Louis, MO, USA); and voltage-dependent anion-selective channel (VDAC), Trx1, and TrxR2 (Abcam, Cambridge, MA, USA). A horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was then applied and membranes were developed using ECL-Plus Reagent (GE Healthcare). Chemiluminescence was visualized using film (Thermo Fisher, San Jose, CA, USA).

Histological analysis

Sections of freshly excised liver tissue were placed in 10% neutral-buffered formalin (Sigma–Aldrich) for 16 h and washed in 70% ethanol for 1 h followed by incubation in 70% ethanol overnight. Samples were then processed, embedded in paraffin, and mounted to slides by the University of Colorado at Denver Histology Core. Sections were stained with hematoxylin and eosin (H&E) or immunohistochemical characterization was performed using a rabbit polyclonal antibody directed against $pSer^{473}$Akt (Cell Signaling), ChREBP (Santa Cruz), 4-HNE, or malondialdehyde (MDA) as previously described (7). Images of H&E-stained and immunohistochemically stained liver sections were captured on an Olympus BX51 microscope equipped with a 4 megapixel Macrofire digital camera (Optronics, Goleta, CA, USA) using PictureFrame Application 2.3 (Optronics).

Triglycerides

Liver triglycerides were determined using a 2:1 chloroform: methanol extract of whole liver sections. Triglyceride content was then quantified using a commercially available kit from Genzyme Diagnostics, (Prince Edward Island, Canada). Protein concentrations were determined using a Lowry protein assay from Bio-Rad (Hercules, CA, USA).

Glutathione S-transferase, glutathione peroxidase, and glutathione reductase activity

Glutathione *S*-transferase (GST), glutathione peroxidase (GPX), and glutathione reductase (GSR) activities were performed as previously described [24].

PTEN and Akt activity assays

PTEN, total Akt, Akt1, and Akt2 were immunoprecipitated from 150 μg of whole liver extracts and activity assays were performed as previously described [7,8]. For in vivo identification of PTEN studies, total PTEN was immunoprecipitated from 4.0 mg of whole cell hepatic extracts from control and ethanol-fed animals. Immunoprecipitation was performed using 100 mg of rabbit polyclonal anti-PTEN antibodies. Antibody was bound to Aminolink Plus immunoprecipitation columns (Pierce, Rockford, IL, USA) and immunoprecipitation performed according to the manufacturer's instructions.