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Increased Carbonylation of the Lipid Phosphatase PTEN contributes to Akt2 Activation in a Murine Model of Early Alcohol-induced Steatosis

C.T. Shearn¹, R.L. Smathers¹, D.S. Backos¹, P. Reigan¹, D.J. Orlicky², and Dennis R. Petersen^{1,*}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Denver Anschutz Medical Campus, Aurora, CO 80045

²Department of Pathology, School of Medicine, University of Colorado Denver Anschutz Medical Campus, Aurora, CO 80045

Abstract

The production of reactive aldehydes such as 4-hydroxynonenal (4-HNE) is a key event in the pathogenesis of alcoholic liver disease 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 the present 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 phosphorylation was 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 Thr³⁰⁸, Thr⁴⁵⁰ and Ser⁴⁷³ 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 Cys⁷¹, Cys¹³⁶, Lys¹⁴⁷, Lys²²³, Cys²⁵⁰, Lys²⁵⁴, Lys³¹³, Lys³²⁷ and Lys³⁴⁴. Computational based molecular modeling analysis of 4-HNE adducted to Cys⁷¹ near the active site and Lys³²⁷ in the C2 domain of PTEN suggest inhibition of enzyme catalysis via either steric hindrance of the active site pocket or by 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.

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To whom correspondence should be addressed:; Dennis R. Petersen, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Denver Anschutz Medical Campus, 12850 East Montview Blvd Box C238, Building V20 Room 2131, Ph. 303-724-3397, Fax 303-724-7266, Dennis.Petersen@ucdenver.edu.

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Introduction

Steatosis is an early pathologic consequence of both non-alcoholic steatohepatitis (NASH) and chronic alcoholic liver disease (ALD). In general, steatosis is considered relatively benign. However, continued hepatic insults from toxins such as ethanol leads 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 the 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, HSP90, 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's syndrome [13]. PTEN negatively regulates Akt activation through its ability to dephosphorylate the 3-position phosphate from phosphatidylinositol (3,4,5) trisphosphate (PIP₃) to produce phosphatidylinositol (4,5) bisphosphate (PIP₂). Production of PIP₂ prevents the membrane lipid binding of the PH domain of Akt thereby preventing subsequent phosphorylation and kinase activation [14].

PTP phosphatases contain a signature HCX₅R 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. Besides 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 SREBP1c and PPAR γ [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/6J mice (The Jackson Laboratory, Bar Harbor, ME), aged 6–8 weeks, were divided into groups of 12 and pair-fed a modified Leiber-DeCarli diet (Bio-Serv, Frenchtown, NJ) for a total of 9 weeks [23]. The diet consisted of with 45% fat-derived calories, 16% protein-derived calories and the remaining balance comprised of either ethanol or maltose dextrin-derived calories. Ethanol content was increased on a weekly basis from weeks 1–7 starting with 2% v/v at week1 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, P.E.I., Canada). 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 PVDF (GE Healthcare, Picataway, NJ) as previously described [7]. Membranes were blocked for 60 minutes with a tris-buffered saline solution containing 1% Tween–20 (TBST) and 5% non-fat dry milk and probed overnight with primary antibodies directed against pSer³⁸⁰ PTEN, pSer⁴⁷³ Akt, pThr³⁰⁸ Akt, pThr⁴⁵⁰ Akt, total Akt1, total AKT2, total PTEN (Cell Signaling, Danvers, MA), SREBP, ChREBP, Trx2, TrxR1 (Santa Cruz Biotechnology, Santa Cruz, CA), β -actin (Sigma Aldrich, St. Louis, MO), VDAC, Trx1, TrxR2 (ABCam, Cambridge, MA). A horseradish peroxidase conjugated secondary (Jackson ImmunoResearch Inc. West Grove, PA) was then applied and membranes developed using ECL-Plus Reagent (GE Healthcare). Chemiluminescence was visualized using film (Thermofisher).

Histological analysis

Sections of freshly excised liver tissue were placed in 10% neutral buffered formalin (Sigma Aldrich) for 16 hours, washed in 70% ethanol for 1 hr followed by incubation in 70% ethanol overnight. Samples were then processed, embedded in paraffin and mounted to slides by the UC Denver Histology Core. Sections were either stained with hematoxylin and eosin (H&E) or Immunohistochemical characterization was performed using a rabbit polyclonal antibody directed against pSer473Akt (Cell Signaling), ChREBP (Santa Cruz), 4-HNE or 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 four megapixel Macrofire© digital camera (Optronics, Goleta, CA) using the PictureFrame© Application 2.3 (Optronics, Goleta, CA).

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

Diagnostic Research Inc. Protein concentrations were determined using a Lowry Protein Assay from Bio-Rad (Hercules, CA).

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 4.0mg of whole cell hepatic extracts from control and ethanol fed animals. Immunoprecipitation was performed using 100mg of rabbit polyclonal anti-PTEN antibodies. Antibody was bound to Aminolink plus immunoprecipitation columns (Pierce) and immunoprecipitation performed according to the manufacturer's instructions.

LC/MS/MS analysis of 4-HNE modified PTEN

Adduction and trypsin digest of recombinant human PTEN by 4-HNE—Ten µg of recombinant human PTEN (Cayman Chemical, Ann Arbor MI) was treated with 4-HNE at molar ratios of 1:1, 5:1 and 10:1 for 30 min at 37°C. 0.5 micrograms were removed and loaded onto an SDS-PAGE gel and Western blotted using anti-4-HNE polyclonal antibodies. To verify PTEN adduction, membranes were then stripped for 15 min using Restore™ stripping buffer (Pierce, Rockford, IL), washed twice in TBST and blocked once again for 1 h in TBST 5% NFD. Following blocking, membranes were incubated overnight with mouse anti-PTEN (Cell Signaling) and subsequently processed. The rest of the sample was treated with 10mM sodium borohydride in 100mM NaOH for 30 min at 37°C. Samples were boiled in 5X SDS loading buffer and subjected to SDS-PAGE. Gels were stained for 15 min with Coomassie Blue R250 and destained overnight in 10% acetic acid, 20% MeOH. From the gel, each band was excised and washed briefly in a solution of 50% acetonitrile/20 mM ammonium bicarbonate, then placed in a 5 mM dithiothreitol reducing solution and incubated at 70°C for 30 minutes. A solution of 20 mM iodoacetamide was then added to alkylate cysteine residues and samples were incubated in the dark at room temperature for 45 minutes. Three 15 minute washes were then performed using distilled water, 50% acetonitrile, and 100% acetonitrile, respectively. The samples were dried in a vacuum concentrator then underwent incubation periods of 30 minutes at 4°C and overnight at room temperature in 5 ng/µl sequencing grade trypsin (Promega, Madison, WI). Two serial extractions of tryptic peptides were performed using 50% acetonitrile/1% formic acid. Samples were then concentrated to volumes of ~20 µl to be used for LC-MS/MS analysis.

A nano-flow HPLC system (Agilent 1200, Palo Alto, CA) coupled with LTQ Orbitrap Velos Hybrid FT mass spectrometer and nanospray ion source (Thermo Fisher; San Jose, CA) was used for LC-MS/MS analysis. An 8 µl volume of samples was run on a ZORBAX 300SB-C18 trap column (5 µm i.d. × 5 mm, Agilent Technologies, Santa Clara, CA) to remove salts and contaminating analytes and reverse phase separation of peptides was performed on a C18 column (100 µm i.d. × 17 cm length) packed in-house using a 4 µm 80 Å pore size matrix (Synergi, Phenomenex, Torrance, CA). Mobile phase A was composed of 99.9% HPLC grade water/0.1% formic acid and mobile phase B of 99.9% acetonitrile/0.1% formic acid. Peptides were eluted from the column using a linear gradient with mobile phase B increasing from 2–90% over 60 minutes. The positive ion mode was used for data acquisition of MS of ions m/z 300–2000 and MS2 scans were performed for the most intense ions. All data was acquired using Thermo Xcalibur software (version 2.1.0.1140, Thermo Fisher; San Jose, CA).

MS/MS Data Analysis

An in-house script was used to convert raw spectral data files into mascot generic file (mgf) format. The mgf files were searched against the human Swissprot database Mascot (version 2.2, Matrix Science Inc., London, UK). The MS/MS tolerance was set at ± 0.6 Da for the searches and the peptide tolerance was set at ± 15 ppm. The search allowed for the missed cleavage of one tryptic site as well as one carbon-13. The carbamidomethylation of cysteine was searched as a fixed modification. Variable modifications included acylation of the protein N-terminus, pyroglutamic acid formation of the peptide N-terminus, oxidation of methionine, 4-HNE modification of cysteine, histidine and lysine (mass 156.22), and reduced 4-HNE modification of cysteine, histidine and lysine (mass 158.24). Spectral images were obtained from the raw data files using Xcalibur and Mascot was used to assist peak assignment.

Computational-based molecular modeling

All simulations were performed using Discovery Studio software (Version 3.1; Accelrys Inc., San Diego, CA; <http://www.accelrys.com>). The crystallographic coordinates of the 2.1 Å human activated PTEN crystal structure (PDB code: 1D5R) were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>) [25]. The 4-HNE modification of residues Cys⁷¹, Cys¹³⁶, Lys¹⁴⁷, Lys²²³, Cys²⁵⁰, Lys²⁵⁴, Lys³¹³, Lys³²⁷ and Lys³⁴⁴ of PTEN were modified with Michael adducts of 4-HNE and the resulting structure typed with the CHARMM force field and subjected to minimization (10,000 iterations) using the conjugate gradient method to a convergence of 0.001 kcal/mol using the Generalized Born implicit solvent model [26, 27].

Statistical Analysis

Western blots were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>) followed by statistical analysis via a paired Students t-test and Prism 4.0 for Windows (GraphPad Software, San Diego, CA). All data are expressed as mean $\pm$ standard error and *p* values <0.05 were considered significant.

Results

Effects of chronic ethanol administration on basic physical and biochemical parameters

As presented in Table 1, chronic ethanol led to a significant decrease in total weight gain compared to the control animals. Surprisingly, following 9 weeks of a high fat diet, the mean overall liver weight was significantly lower in the ethanol fed animals compared to control fed mice. Chronic ethanol administration resulted in a liver to body weight ratio of 4.5% compared to 4.0% in control animals.

Histochemical analysis of mouse liver sections

Liver sections from ethanol and control pair-fed mice were stained with hematoxylin and eosin. Both groups exhibited lipid accumulation. A closer examination (Figure 1 “zoom”) revealed an increase and redistribution of lipid primarily to zone 2 with some lipid vesicles around the periportal region following ethanol treatment. Periportal (zone 1) accumulation of lipid was evident in control-fed animals.

Chronic ethanol consumption is known to induce accumulation of reactive aldehyde modified proteins within hepatocytes [28]. Liver sections from control and ethanol pair-fed mice were examined for accumulation of the reactive aldehydes 4-HNE, acrolein and MDA. As demonstrated in Figure 1B, immunohistochemical analysis of 4-HNE, acrolein and malondialdehyde demonstrates a low level of panlobular staining in control animals.

Following chronic ethanol treatment, there is a substantial increase in periportal to midzonal staining of 4-HNE, acrolein and MDA.

Biochemical Characterization of liver parameters

The effects of chronic ethanol feeding on serum ALT, liver triglycerides and Redox capacity (GST, GSR, GPX and GSH/GSSG ratio) were examined. As presented in Table 1, chronic ethanol led to a significant increase in overall liver damage as indicated by a 1.72-fold increase in serum ALT. Compared to their respective controls, liver triglyceride content was 36% higher in the ethanol animals a value that narrowly missed statistical significance ($p=0.054$). Chronic consumption of ethanol is known to reduce levels of glutathione via reactive aldehyde conjugation and to affect the overall activity of glutathione metabolizing enzymes [29]. Chronic ethanol consumption led to an overall decrease in the total level of GSH and in the ratio of GSH/GSSG (1.5-fold) suggesting that redox balance is impaired in this model of chronic ethanol consumption. To further characterize the effects of ethanol on the overall redox capacity of the liver, activity assays of GST and GSR and GPX were performed. From the data in Table 1, the activities of both GST (1.58-fold) and GSR (1.23-fold) were significantly increased in ethanol fed compared to control diet animals. Glutathione peroxidase significantly decreased by 1.24-fold following ethanol consumption.

Effects of chronic ethanol administration on PTEN and Akt phosphorylation and activity

The PTEN/Akt pathway contributes to regulation of metabolic pathways in the liver and has previously been implicated in the formation of chronic ethanol mediated steatosis [30, 31]. As shown in Figure 2A and 2B, compared to isocaloric controls, Western blot analysis of whole cell lysates indicated a significant increase in both total PTEN and total Akt2 expression in ethanol treated animals while no significant change occurred in total Akt1 expression. Increased phosphorylation of PTEN on Ser³⁸⁰ contributes to downregulation of enzymatic activity [32]. In ethanol treated mice, PTEN phosphorylation was significantly increased compared to control animals. Examination of total Akt phosphorylation indicated a significant increase in pThr³⁰⁸Akt, pSer⁴⁷³Akt and pThr⁴⁵⁰Akt in ethanol treated animals compared to controls.

Both sterol response element binding protein (SREBP) and carbohydrate response element binding protein (ChREBP) are involved in regulation of genes involved in lipogenesis [33–36]. Akt activation contributes to the formation of steatosis by acting on downstream transcription factors including SREBP [37]. ChREBP has been proposed to link gluconeogenesis and lipogenesis via to Akt phosphorylation [38, 39]. In a high fat diet model, overexpression of ChREBP results in increased Akt phosphorylation and steatosis [38]. During lipogenesis, both ChREBP and SREBP translocate from the cytosol to the nucleus. To determine the effects of Akt activation on ChREBP and SREBP, cytosolic fractions were examined for ChREBP and SREBP expression. From Figure 2A and 2B, compared to pair-fed controls, cytosolic localization of both SREBP and ChREBP in the ethanol fed mice are significantly decreased.

The ethanol-induced decrease in cytosolic SREBP suggests that the change in total Akt phosphorylation leads to increased translocation of SREBP. Therefore the direct effects of ethanol on PTEN activity and total Akt, Akt1 and Akt2 activity were assessed. As presented in Figure 2C, PTEN activity decreased by 25% in whole cell liver lysates from ethanol animals compared to pair-fed controls. PTEN activity directly affects Akt activation via the elimination of PtdIns (3,4,5 P)₃ lipids necessary for Akt recruitment to intracellular membranes and subsequent phosphorylation/activation. Given that PTEN activity is decreased, we therefore sought to determine the effects of ethanol on total Akt, Akt1 and Akt2 activity (Figure 2C). In the ethanol treated mice, there was a significant increase in

both total Akt and Akt2 (30%) activity but not Akt1 activity suggesting that decreased PTEN activity is linked to Akt2 activation.

Data from Figure 2A and 2C clearly show that chronic ethanol results in an increase in Akt phosphorylation. To further substantiate these data, immunohistochemical analysis of pSer⁴⁷³Akt was performed using liver sections from control and ethanol fed mice. From Figure 2D, in the pair fed mice, pSer⁴⁷³Akt is predominantly in the nucleus with strong staining of cholangiocytes around the biliary tract. Ethanol consumption however, resulted in a global increase in cytosolic as well as nuclear staining of pSer⁴⁷³Akt.

Chronic ethanol consumption decreases cytosolic SREBP [40]. In Figure 2A, decreased cytoplasmic localization of ChREBP occurs following chronic ethanol consumption. Ethanol-mediated changes in cytoplasmic localization of ChREBP have not been reported. To further characterize the decrease in cytoplasmic localization of ChREBP, localization was examined using immunohistochemistry on liver sections prepared from ethanol or pair-fed mice. From Figure 3A, in ethanol-fed animals, there is a marked clearing of ChREBP in the cytoplasm of hepatocytes in zones 1 and 2 that corresponds with an increase in nuclear ChREBP staining. As shown in the zoom panel, ChREBP staining is primarily cytosolic in the control-fed animals with mild nuclear staining. Ethanol consumption however significantly enhanced ChREBP nuclear staining. To quantify ethanol-induced increases in nuclear ChREBP, total number of ChREBP positive/200X field were counted. As shown in Figure 3B, nuclear localization of ChREBP significantly increased 5-fold ($p < 0.001$) in ethanol-fed mice compared to pair fed control animals, further substantiating chronic ethanol-induced ChREBP translocation to the nucleus.

Effects of chronic ethanol administration on thioredoxin expression

Another key regulator of PTEN activity is the Thioredoxin system. Via their ability to reduce oxidized cysteine residues, thioredoxin's (Trx's) play a key role in mitigation of protein oxidative damage [41]. Thioredoxin has been demonstrated to reduce cysteine residues on PTEN following hydrogen peroxide treatment [17]. In addition, Trx1 has been demonstrated to physically interact with PTEN via the PTEN C2 domain [42]. Recently, relative expression of Trx1 was determined to be decreased following four weeks of ethanol treatment in female C57Bl6J mice [43]. Given the role that Trx's play in PTEN activity, the effects of long term ethanol consumption on Trx expression was examined. As shown in Figure 4A and B, Trx1 expression significantly decreased by 35% following ethanol but no change was evident in TrxR1. In the mitochondrion, a distinct isoform of Trx is expressed (Trx2). Thioredoxin 2 has been demonstrated to contribute to mitochondrial resistance to oxidative stress promoting an increase in resistance to reactive aldehydes and cell survival [44]. Chronic ethanol consumption resulted in a 33% increase in Trx2 expression and a 30% increase in TrxR2 mitochondrial expression.

PTEN is carbonylated in livers of mice chronically treated with ethanol

Both carbonylation and phosphorylation of PTEN are known to decrease enzymatic activity [7, 8, 45]. Previously, we have demonstrated that in HepG2 cells, PTEN is a target of carbonylation which contributes to a decrease in enzymatic activity [8]. To determine if there is an effect of chronic ethanol ingestion on intracellular PTEN carbonylation in mice, a method of biotin hydrazide modification of protein bound reactive aldehydes was employed [7]. From Figure 5A, overall carbonylation of PTEN is significantly increased by 1.5-fold in chronic ethanol treated mice compared to control animals. To provide additional insight into the specific amino acid residue modified by reactive aldehydes, whole cell lysates were first treated with iodoacetic acid (IAA) followed by 2-dimensional SDS-PAGE/Western blotting.

From Figure 5B, compared to control-fed animals, treatment with IAA induced a decrease in the isoelectric point of PTEN in ethanol-fed animals.

In Figure 2A and 2B there is an increase in PTEN phosphorylation in the ethanol-fed animals. Phosphorylation also affects protein isoelectric point. To determine the isoelectric point of differentially phosphorylated species of PTEN, samples were treated as described in Figure 5B followed by Western blotting for PTEN phosphorylation. From Figure 5C, PTEN phosphorylation is increased in the ethanol fed animals but the shift in isoelectric point is not as evident as in Figure 5A. Previously, we reported the PTEN was a target of reactive aldehydes in HepG2 cells [8]. To further substantiate our data, HepG2 cells we treated with 50 μ M 4-HNE followed by IAA and 2D analysis. As shown in Figure 5D, treatment of HepG2 cells with 4-HNE results in a decrease in the isoelectric point of PTEN. Overall, this data demonstrates, for the first time, carbonylation of PTEN occurs in an animal model of ethanol-induced oxidative stress.

Based on the increase in PTEN carbonylation we repeatedly attempted to identify intracellular sites of modification using immunoprecipitations of total PTEN from 4mg of total hepatic protein and a LTQ Orbitrap mass spectrometer. As shown in Supplemental Figure 1A, Western blotting analysis clearly indicates PTEN immunoprecipitation. Immunoprecipitates from control and ethanol-treated mice were run on a standard SDS-PAGE followed by Coomassie staining. As shown in Supplemental Figure 1B, there were no significant differences in the staining intensity between control and ethanol immunoprecipitates. From the gel, 6 unique regions were excised followed by trypsin digestion and LC/MS analysis. Surprisingly, although PTEN was detectable in the Western blot, no detectable peptides from PTEN were identified via LC/MS/MS (Supplemental Figures 1C, D, E, F, G, H). The lack of identification of PTEN from cellular lysates is not surprising. To our knowledge, PTEN has not been identified from animals using proteomic screens. This suggests that although PTEN is identifiable as carbonylated via Western blotting techniques, the total protein levels prepared for analysis are not sufficient for detection by LC/MS.

Characterization of PTEN modification by 4-HNE *in vitro*

We have previously reported that recombinant human PTEN (rhuPTEN) was modified by 4-HNE at a molar ratio of 1:1 and that IC₅₀ of 4-HNE towards PTEN was approximately 1.5 μ M [8]. The locations of the 4-HNE-mediated modifications of PTEN however, were not identified. To identify the residues of PTEN modified in response to treatment with 4-HNE, recombinant protein was incubated with increasing molar ratios of 4-HNE followed by SDS-PAGE, excision and trypsin digestion. Peptides were extracted and analyzed by LC/MS. Concurrently a small sample of treated and untreated protein was run on SDS-PAGE and analyzed by Western blotting for 4-HNE modification. As illustrated in Supplemental Figure 2, treatment of rhuPTEN with 4-HNE led to the formation of multimeric forms of PTEN at approximate molecular weights of 55–60, 120 and 180 kDa via protein crosslinking (Bands 1, 2, 3).

MS Identification of 4-HNE modified rhuPTEN

Previously, rhuPTEN has been demonstrated to be inhibited by 4-HNE at a molar ratio of 1:1, which corresponded to a molar concentration of 1.5 μ M. To identify the amino acids modified by 4-HNE, rhuPTEN was treated *in vitro* with increasing molar concentrations of 4-HNE. 4-HNE modifications were stabilized via sodium borohydride reduction followed by SDS-PAGE purification and trypsin digestion. Analysis of tryptic digests from each band resulted in an average MS peptide sequence coverage of 55–65%. Surprisingly, no peptides containing the active site Cys¹²⁴ were identified. The resulting mass spectra data presented

in Table 2 represents a summary of peptides and amino acids identified from rhuPTEN treated at a 1:1 molar ratio (1 μ M) or from each band (bands 1, 2, 3) excised at a 10:1 molar ratio (10 μ M). From Table 2 and Supplemental Table 1, using Mascot and mass shifts of (Michael addition 156.22, 158.24 reduced, Schiff Base 138.104, 140.12 reduced), Cys⁷¹, Cys¹³⁶, Lys¹⁴⁷, Lys²²³, Cys²⁵⁰, Lys²⁵⁴, Lys³¹³, Lys³²⁷ and Lys³⁴⁴ were adducted by 4-HNE at the 1:1 molar ratio condition with an ions scores greater than 32. Electron Transfer Dissociation (ETD) is an alternative method to Collision Induced Dissociation (CID) to identify post-translational modifications. Recombinant huPTEN was treated with 4-HNE, digested and analyzed via ETD. Not surprisingly, similar modifications were identified using Mascot searches of tryptic peptides isolated from each multimeric band (Supplemental Table 2). From the resulting mass spectra no new amino acids were identified to be modified (Data not Shown). In addition, the percent peptide sequence coverage did not decrease significantly following multimerization. This suggests that other residues may be modified resulting in formation of protein crosslinks. To verify each site of modification, each peptide was subjected to MS/MS fragmentation and the resulting distribution of b/y ions examined. As presented in Figure 6A, 6B and Supplemental Figure 3A–F, the residues that we identified to be 4-HNE adducted were confirmed via b/y ion distributions, with the exception of Cys¹³⁶ which could only be confirmed using b ions. It should be noted that although its role with respect to enzymatic activity is not clear, Cys¹³⁶ has been shown to be mutated in some Cowden's disease patients [46].

Although we have identified amino acids in PTEN modified by 4-HNE, the data shown in Figure 5 does not provide evidence concerning the susceptibility of each modified residue to adduction. Therefore, we performed a time course using rhuPTEN and 10-fold molar excess of 4-HNE. A trypsin/chymotrypsin double digest followed by analysis by LC/MS/MS was performed in an attempt to increase our coverage of the active site. As shown in Supplemental Figure 4A, at the two minute time point, 4-HNE treatment resulted in modification and crosslinking of rhuPTEN. A summary of peptide coverage is presented in Supplemental Figures 4B, C and D. Compared to our previous trypsin digestion, the combination of trypsin and chymotrypsin did not result in an increase in peptide coverage (63%-control, 63% 4-HNE treatment) but active site cysteine was identified in the untreated samples (LSEDDNHVAAIHCK mascot score 39). This peptide was not present in the 4-HNE modified digest. In addition, peptides containing Cys⁷¹ or Lys³²⁷ were not identified under either condition. The fact that a peptide containing the active site Cys¹²⁴ was not identified suggests that Cys¹²⁴ may be post-translationally modified resulting in a different mass.

***In Silico* molecular modeling of 4-HNE modified PTEN**

Computational-based minimization simulations were performed using the crystal structure of human PTEN bound to L(+)-tartrate (Figures 7, 8 and 9) [25]. Each amino acid identified in at the 1:1 molar ratio in table 3 (Cys⁷¹, Cys¹³⁶, Lys¹⁴⁷, Lys²²³, Cys²⁵⁰, Lys²⁵⁴, Lys³¹³, Lys³²⁷ and Lys³⁴⁴) was modified by 4-HNE and subjected to computer based minimization (Supplemental Figure 5). To determine the effects of 4-HNE modification on overall PTEN structure, minimized structures of untreated and 4-HNE treated were overlaid. From Supplemental Figure 6, no significant structural changes were evident. Of all the residues identified, two were determined to be of particular relevance to PTEN catalytic activity and function: Cys⁷¹ (Figure 7A, B, C, D) and Lys³²⁷ (Figure 8A, B). In Figure 7A, the addition of 4-HNE on Cys⁷¹ via a Michael addition leads to a stable 4-HNE adduct adjacent to the active site of PTEN. From Figure 7B, the 4-HNE modification of Cys⁷¹ is not adjacent to Cys¹²⁴ in the tartrate bound state. The only crystal structure of PTEN is in the tartrate-bound state. In addition, Cys⁷¹ is known to form a disulfide with Cys¹²⁴, however it was determined that in the tartrate bound state Cys¹²⁴ was not sufficiently close enough to form

a disulfide bond. Therefore, the structure of unbound PTEN was minimized to allow for the formation of a disulfide and Cys⁷¹ subsequently modified with 4-HNE. In the new unbound state, the side chain of Cys¹²⁴ is flipped away from the active site and located adjacent to Cys⁷¹ (Figure 7C). Thus, 4-HNE modification of Cys⁷¹ in the disulfide form of PTEN has the potential to hinder substrate access to Cys¹²⁴.

The effect of 4-HNE adduction of Lys³²⁷ in the PTEN C2 domain was then examined using minimization studies. From Figure 8A, 4-HNE adduction of Lys³²⁷ creates a hydrophobic surface that perturbs the positively charged surface formed by the calcium binding regions (CBR's 1,2,3) on the C2 domain of PTEN. Another perspective is shown in Figure 8B, 4-HNE modification forms an additional hydrophobic surface laterally along the membrane interacting surface of the PTEN C2 domain. Thioredoxins bind PTEN via interactions with Cys²¹¹ in the PTEN C2 domain, therefore the effects of Lys³²⁷ adduction on the Trx1 binding interface was examined [47]. From Figure 8C, D, adduction of Lys³²⁷ creates a bulky hydrophobic group that alters the Trx1/PTENC2 interface. Therefore 4-HNE modification of Lys³²⁷ has the potential to alter not only membrane association but Trx1 association as well.

Discussion

An important consequence of chronic ethanol ingestion is the development of steatosis and hepatocellular injury. Accompanying these responses is an increased level of oxidative stress and production of lipid aldehydes, foremost of which is 4-HNE [48]. We have previously identified PTEN as an intracellular target of carbonylation by 4-HNE in a hepatic cell culture model [8]. Treatment of HepG2 cells with 4-HNE led to increased carbonylation of PTEN and an inhibition of enzyme activity resulting in an increase in Akt phosphorylation and contributing to increased formation of neutral lipids [8]. Thus, a primary objective of the present study was to determine the effects of increased oxidative stress and carbonylation on PTEN signaling in an *in vivo* murine model of ethanol toxicity. In addition we sought to identify the mechanism of inhibition of PTEN activity by the reactive aldehyde 4-HNE.

A key regulator of the PI3K pathway is the lipid phosphatase PTEN. PTEN has previously been demonstrated to regulate both cell survival (through Akt1) and hepatic lipid accumulation (via Akt2) [22, 49]. In a PTEN hepatospecific deletion model, mice exhibit a 5-fold increase in liver triglycerides. Concurrent deletion of Akt2 ameliorates this effect indicating that Akt2 regulates hepatic lipid accumulation [22]. Increased phosphorylation of the C-terminus of PTEN corresponds to a decrease in activity [32]. However, published reports examining regulation of the PI3K pathway in ethanol models are not consistent. In one report, PI3K activity is increased following ethanol feeding [50]. In another study, using female Long Evans rats, PTEN was identified as a potential contributor in ethanol-mediated toxicity with respect to decreased cell survival [30]. In that second report, the investigators found decreased PI3K activity and PTEN phosphorylation accompanied by increased PTEN expression and activity. The authors concluded that this contributed to a decrease in Akt signaling and the subsequent increase in hepatocyte apoptosis in ethanol treated animals. Interestingly, in another study using male rats, no change in PTEN expression or phosphorylation was evident but Akt phosphorylation of Thr³⁰⁸ decreased and phosphorylation of Ser⁴⁷³ was increased. Phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³ is necessary for full Akt activation [51]. As expected based on Akt phosphorylation, the authors found overall Akt activity decreased in ethanol-fed compared to control animals. In the current study using mice and 9 weeks of ethanol feeding, we find that while there is a significant increase in PTEN expression, PTEN phosphorylation is also significantly increased. Concurrently, ethanol administration further decreases PTEN activity compared

to high fat diet alone. Consistent with our results, a recent study indicated decreased PTEN activity in response to high fat diet-induced hepatosteatosis [18].

Carbonylation of PTEN has not been documented in an animal model. Here, we demonstrate for the first time that a significant increase in carbonylation of PTEN occurs in the ethanol-fed mice. In several studies, alkylation/carbonylation decreases PTEN activity leading to increased Akt activity [8, 52, 53]. None of these studies examined individual isoforms. However results of the present study show a significant increase in Akt2 expression but not Akt1 expression occurs in the ethanol treated animals potentially contributing to the increase in Akt2 activity exhibited in this model. No increase in Akt1 activity was evident. In addition, we observed a significant increase in both pThr³⁰⁸ Akt, and pSer⁴⁷³ Akt. Akt activation has also been shown to be regulated via a growth factor-independent, PI3K-independent mechanism. Phosphorylation of Ser¹²⁴ and Thr⁴⁵⁰ on Akt is thought to prime the protein for further phosphorylation on Thr³⁰⁸ and Ser⁴⁷³ [54]. Compared to pair-fed control animals, we find a significant increase in phosphoThr⁴⁵⁰ suggesting that ethanol may regulate Akt phosphorylation via a yet unknown mechanism. As a result of Akt activation we find decreased cytosolic localization of both SREBP and ChREBP and an increase in nuclear localization of ChREBP which may contribute to the increase in liver triglycerides and steatosis in our model.

In vitro modification of recombinant PTEN by incubation with 4-HNE at a molar ratio of 1:1 resulted in a number of aldehyde-adducted amino acid residues. Foremost amongst these are Cys⁷¹ and Lys³²⁷. Due to the presence of a reactive cysteine within its active site, PTEN is known to be susceptible to regulation by oxidative modification [15, 17, 42]. Regulation occurs via a redox sensitive mechanism involving the active site cysteine (Cys¹²⁴) and an adjacent cysteine (Cys⁷¹). Treatment of PTEN with hydrogen peroxide leads to the formation of a disulfide between these two residues. This has been shown both in cell culture experiments and with recombinant protein *in vitro*. To verify this we used molecular modeling methods with the only existing crystal structure of PTEN (1D5R) to form a disulfide bond between the two residues. We found this required “flipping” the side chain of Cys¹²⁴ nearly 180 degrees to a location adjacent to Cys⁷¹. This suggests that the crystal structure is not indicative of the structure of PTEN upon formation of the disulfide bond. The actual conformation of the active site of PTEN in the unbound state or during disulfide bond formation is not known. It may be that the addition of a synthetic substrate (L(+)-tartrate) prevents the interaction between Cys⁷¹ and Cys¹²⁴. The fact that we find Cys⁷¹ to be modified by 4-HNE is intriguing. Molecular modeling of Cys⁷¹ suggests that in the inactive state, Cys⁷¹ is adjacent to Cys¹²⁴ and will inhibit substrate binding. Although we identified the active site cysteine following trypsin/chymotrypsin cleavage in untreated samples (LSEDDNHVAAIHCK), we did not observe the peptide containing Cys¹²⁴ following 4-HNE treatment. This may be due to several reasons. First, Cys¹²⁴ or Lys¹²⁵ or both may be sites for 4-HNE modification. As shown in Supplemental Figure 3B, 4-HNE results in chemical crosslinking of rhuPTEN. Cross-linking has been demonstrated to occur between 4-HNE modified lysine residues in other proteins such as tubulin [55]. If the active site peptide is crosslinked, a crosslinked peptide may be created that is too large to be detected by LC/MS/MS. Second, there are several lysine residues immediately adjacent to Cys¹²⁴ (HCKAGKGRT) that may also be modified, subsequently leading to altered and unrecognizable cleavage sites and the production of an even larger peptide. Within the active site of PTEN, Cys¹²⁴ has a calculated pKa of approximately 4.5. This produces a nucleophilic center that is sensitive to both peroxidation and nitrosylation [15, 56]. Thus, based on our data, we hypothesize that although not identified, the active site cysteine is modified by 4-HNE.

Membrane association of PTEN is in part regulated by its C2 domain with a preference towards polar membrane lipids such as phosphatidylserine and PtdIns (3,4,5) P₃ [25]. The mechanism of membrane association is primarily via 3 loops on the end of a (β-barrel (CBR's 1,2,3). In addition, there is a helix (Cα2) that results in an additional basic patch that has been identified in other C2 domains to contribute to membrane association [57, 58]. Following treatment of 4-HNE, we identified Lys³²⁷ as a modified residue of PTEN. Based on the crystal structure, Lys³²⁷ is in a position to contribute to membrane association. The addition of 4-HNE creates a hydrophobic surface instead of a cationic surface. Preventing membrane association would thereby inhibit the ability of PTEN to bind to its substrate and be reflected in a decrease in enzymatic activity. Furthermore, adduction of Lys327 may alter the ability of Trx to interact with PTEN further altering activity. Research has suggested that Trx both restores as well as inhibits PTEN activity [15, 17, 41, 42, 47]. Trx1 has also been shown to be a target of reactive aldehydes in other systems [59]. By not reducing oxidized cysteine residues on PTEN, ethanol-dependent decreases in Trx1 expression may contribute to PTEN inhibition. Thus, alteration of the Trx1 binding site combined with overall decreased Trx1 expression/altered glutathione homeostasis reflects an environment of increased oxidative stress contributing to decreased PTEN activity. Overall however, this supports a second plausible mechanism of enzyme inhibition.

In conclusion, we have identified for the first time, an increase in carbonylation of the lipid phosphatase PTEN in an animal model of ethanol-induced hepatic damage. The increased carbonylation of PTEN corresponds to decreased phosphatase activity and an increase in Akt2 activation (Figure 9). Furthermore, we have determined that carbonylation of both Cys⁷¹ near the active site of PTEN and Lys³²⁷ in the lipid binding C2 domain of PTEN contributes to the decrease in PTEN activity *in vitro*. Last, we hypothesize that the observed increased activation of Akt2 may stimulate increased lipid accumulation *via* activation of SREBP and ChREBP during chronic ethanol consumption. Future studies will examine the relative contribution of Akt activation on nuclear transcription factors in relation to lipid accumulation in chronic ethanol treated mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALD	alcoholic liver disease
ALT	alanine aminotransferase
ChREBP	carbohydrate response element binding protein
Cyp2E1	Cytochrome P4502E1
4-HNE	4-hydroxy-2-nonenal
NASH	non-alcoholic steatohepatitis
PDK-1	phosphoinositide-dependent kinase 1
PI3K	phosphatidylinositol 3-kinase
PtdIns (3,4,5) P₃	phosphatidylinositol 3,4,5 tris-phosphate
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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Figure 1. Histopathology of fixed liver tissues isolated from ethanol and control fed mice
 A. Hematoxylin and eosin staining demonstrates a predominantly periportal lipid accumulation in the control and predominantly centrilobular lipid accumulation in ethanol-fed mice (Arrows/Zoom). B. Increased 4-HNE, acrolein and MDA staining in the periportal region (Zones 1–2) in liver sections from ethanol fed mice. (CV, central vein, PT, portal triad). Original Magnification 400X.

Figure 2. Chronic ethanol mediated changes in PTEN and Akt in mouse cytosolic liver fractions (A) Cytosolic fractions from both ethanol fed and control groups were analyzed via Western blotting and probed for total PTEN, phospho Ser³⁸⁰ PTEN, total Akt1, total Akt2, phosphoSer⁴⁷³ Akt, phosphoThr³⁰⁸ Akt and phosphoThr⁴⁵⁰ Akt, cytosolic ChREBP and cytosolic SREBP using rabbit polyclonal antibodies (B) Quantification of the Western blots (actin normalized). (C) Using mouse liver whole cell extracts, 150µg of total protein was examined for PTEN activity using a malachite green phosphate release assay and DiC₈ PtdIns(3,4,5)P₃. Total Akt, Akt1 and Akt2 activities were determined using an *in vitro* Akt kinase activity assay with a synthetic GSK3α peptide substrate and total immunoprecipitated total Akt, Akt1 and Akt2. (D) Increased pSer⁴⁷³Akt cytosolic and nuclear staining in liver sections isolated from ethanol fed mice. Data were analyzed using paired Students t-test (respective ethanol and control group) n=5 (*p<0.05, **p<0.01).

Figure 3. Effects of chronic ethanol consumption on ChREBP nuclear localization

(A) Positive ChREBP nuclear staining was observed in the periportal region (zones 1–2) in liver sections from ethanol fed mice. (B) Digitally enhanced view of ChREBP stained nuclei. (C). Quantification of ChREBP positive nuclei per 400 $\times$ field, N=3 (***) ($p<0.001$) (5 fields counted for each Con/ETOH fed animal). Original Magnification 400 $\times$.

Figure 4. Chronic ethanol induced changes in mouse cytosolic/mitochondrial liver thioredoxins (A) Cytosolic /mitochondrial fractions from pair fed and ethanol fed groups were analyzed via Western blotting and probed for Trx1, TrxR1, Trx2 and TrxR2. (B) Quantification of the Western blots (actin/VDAC normalized). Data were analyzed using paired Students t-test (respective ethanol and control group) n=5 (*p<0.05, **p<0.01).

Figure 5. Effects of chronic ethanol consumption of carbonylation of PTEN

(A) 150 µg of cytosolic protein from ethanol or control fed mice was incubated for 2 h with 2.5 mM biotin hydrazide and purified using streptavidin pulldown. Samples were subsequently analyzed using reducing SDS-PAGE/Western blotting with rabbit polyclonal anti-PTEN. (B) Two-dimensional SDS-PAGE Western blotting of PTEN from whole cell extracts (50mM IAA treatment 30min) isolated from control and ethanol-fed mice. (C) Two-dimensional Western analysis of phosphorylated PTEN from whole cell extracts (50mM IAA 30min) isolated from control and ethanol-fed mice. (D) Two-dimensional Western analysis of PTEN from lysates isolated from HepG2 cells treated with 50µM 4-HNE

(60min) followed by 50mM IAA 30 min. Data were analyzed using paired Students t-test (respective ethanol and control group) n=5 (*p<0.05)

Figure 6. 4-HNE modification of rhuPTEN using LC/MS/MS analysis

(A) 4-HNE modified peptide containing Cys71 (+158), MS/MS analysis was performed and the resulting b/y ion fragmentation confirmed peptide identity (IYNLC*AER). (B) 4-HNE modified peptide containing Lys 327 (+158), MS/MS analysis was performed and the resulting b/y ion fragmentation confirmed peptide identity (NDLDK*ANK).

Figure 7. *In silico* molecular modeling of 4-HNE modified Cys71 on huPTEN [60]
 (A) Ribbon diagram demonstrating location of Cys⁷¹ and Cys¹²⁴ (B) Electron density map demonstrating charge polarity (blue +, red -) and relationship of 4-HNE (light blue stick) modified Cys71(yellow) and Cys¹²⁴ (colored yellow-lower). (C) Electron density map demonstrating relative location of Cys¹²⁴ following movement into the inactive state (4-HNE light blue stick, Cys⁷¹ is colored yellow CPK, Cys¹²⁴ is colored green CPK)

Figure 8. *In silico* molecular modeling of 4-HNE modified Lys327 on the C2 domain of huPTEN (A) Surface electron density map of the C2 of PTEN protein surface with 4-HNE modified Lys³²⁷ (Red- negative charged residues, blue-positively charged residues, 4-HNE-stick, Lys³²⁷-yellow/magenta). Note additional hydrophobic surface formed by 4-HNE addition. (B) Ribbon diagrams demonstrating location of calcium binding regions 1,2,3 and 4-HNE modified Lys³²⁷. (C) Ribbon diagram depicting relative location of 4-HNE-Lys327 and the Trx1 binding site. (D) Surface electron density map depicting top view of relative location of Cys211 (Yellow) and Lys327 (Magenta).

Figure 9. Model of ethanol induced carbonylation in mice

During chronic ethanol consumption, production of reactive aldehydes such as 4-HNE leads to increased carbonylation and inhibition of PTEN. The resulting decrease in PTEN activity corresponds to increased activation of Akt2 and increased steatosis.

Table 1
Physical and Biochemical analysis of serum and liver homogenates from control and ethanol pair-fed mice

Serum ALT, liver triglycerides, GST and GSR and GPX activities were determined as described in Methods. Data were analyzed using paired Students t-test. All values are presented as the mean $\pm$ SEM. Significance was determined by comparison to the control fed animals. n=5 for both groups.

	Control	Ethanol	Fold change	Significance
Change in body weight	12.72 $\pm$ 1.34	4.44 $\pm$ 1.62	-2.86	***
Liver Weight	1.38 $\pm$ 0.04	1.22 $\pm$ 0.06	-1.13	*
liver/body weight	4.00 $\pm$ 0.14	4.51 $\pm$ 0.11	1.12	p=0.1598
ALT (U/L serum)	21.67 $\pm$ 3.96	37.22 $\pm$ 5.08	1.72	*
Liver Triglycerides (mmol/L/mg tissue)	0.23 $\pm$ 0.03	0.31 $\pm$ 0.07	1.36	p=0.0548
GST activity (U/mg protein)	6.12 $\pm$ 0.31	9.69 $\pm$ 0.26	1.58	***
GRX activity (U/mg protein)	1.01 $\pm$ 0.03	1.24 $\pm$ 0.03	1.23	***
GPX activity (U/mg protein)	42.75 $\pm$ 1.23	34.55 $\pm$ 1.33	-1.24	**
GSH (μ Mol/g tissue)	3.35 $\pm$ 0.34	2.21 $\pm$ 0.27	-1.51	*
GSSG (μ Mol/g tissue)	0.13 $\pm$ 0.02	0.13 $\pm$ 0.01	-1.03	p=0.8365
GSH/GSSG	25.67 $\pm$ 2.25	17.13 $\pm$ 1.32	-1.50	*

*
p<0.05

**
p<0.01

p<0.001

Table 2
Mascot results from LC/MS/MS analysis of rhuPTEN: UCntreated and 4-HNE modified (NaBH₄ reduced)

Amino acid number (AA), Experimental (expt), calculated (calc).

StartEnd	Observed	Mr(expt)	Mr (calc)	Ions Score	Sequence	Modification
16 – 41	1023.1533	3066.4381	3066.4321	60	YQEDGFDLDLTYIPNIAMGPAER	Oxidation (M)
42 – 47	368.7033	735.3920	735.3915	50	LEGVYR	
48 – 55	472.7441	943.4736	943.4723	62	NNIDVVVR	
67 – 74	570.3118	1138.6090	1138.6056	33	IYNLCAER	HNE+Delta: H(2) (C)
75 – 80	367.6782	733.3418	733.3395	40	HYDTAK	
131 – 142	517.6209	1549.8409	1549.8360	33	TGVMICAYLLHR	Oxidation (M); HNE+Delta: H(2)(C)
145 – 159	893.4635	1784.9124	1734.9097	104	FLKAQEALDFYGEVR	
145 – 159	643.6920	1943.0542	1943.0404	42	FLKAQEALDFYGEVR	HNE+Delta: H(2)(K)
148 – 159	699.3386	1396.6626	1396.6623	90	AQEALDFYGEVR	
148 – 161	552.2785	1653.8137	1653.8111	38	AQEALDFYGEVRTR	
164 – 172	493.2924	984.5702	934.5716	67	KGVTIPSQR	
165 – 172	429.2441	856.4736	856.4767	41	GVTPSQR	
173 – 183	765.9042	1529.7938	1529.7918	64	RYVYYYSYLLK	
174 – 183	687.8534	1373.6922	1373.6907	52	YVYYYSYLLK	
184 – 197	861.9746	1721.9346	1721.9365	57	NHLDYRPVALLFHK	
222 – 233	654.8484	1307.6822	1307.6834	71	VKIYSSNSGPTTR	HNE+Delta: H(2) (K)
222 – 233	733.9147	1465.8148	1465.8140	45	VKIYSSNSGPTTR	
224 – 233	541.2672	1080.5198	1080.5200	72	IYSSNSGPTTR	
235 – 254	859.7638	2576.2696	2576.2583	49	EDKFMYYFEPQPLPVCGLDK	Oxidation (M); HNE+Delta: H(2) (K)
255 – 260	403.7126	805.4106	805.4123	36	VEFFHK	
255 – 263	588.8116	1175.6086	1175.6087	47	VEFFHKQNK	
268 – 289	901.4262	2701.2568	2701.2523	69	DKMFHFVWNTFFIPGPEETSEK	Oxidation (M)
270 – 289	820.3848	2458.1326	2458.1304	34	MFHFVWNTFFIPGPEETSEK	Oxidation (M)
309 – 322	811.9340	1621.8534	1621.8563	84	ADNDKEYLVLTLTK	
309 – 322	594.3384	1779.9934	1779.9870	51	ADNDKEYLVLTLTK	HNE+Delta: H(2) (K)
323 – 330	538.3031	1074.5916	1074.5921	46	NDLDKANK	HNE+Delta: H(2) (K)
336 – 342	451.7245	901.4344	901.4334	44	YFSPNFK	
343 – 349	449.7740	897.5334	897.5324	39	VKLYFTK	

StartEnd	Observed	Mr(expt)	Mr (calc)	Ions Score	Sequence	Modification
343 –349	528.8394	1055.6642	1055.6631	37	VKL.YFTK	HNE+Delta: H(2) (K)
350 –378	1049.4603	3145.3591	3145.3596	120	TVEEPSNPEASSSTSVTPDVSDNEPDHYR	
379 –403	971.0820	2910.2242	2910.2316	61	YSDTTDSDPENEPFDEDEQHTQITKV	