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Original Contribution

Inhibition of Hydrogen peroxide signaling by 4-hydroxynonenal due to differential regulation of Akt1 and Akt2 contributes to decreases in cell survival and proliferation in hepatocellular carcinoma cells

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ABSTRACT

Dysregulation of cell signaling by electrophiles such as 4-hydroxynonenal (4-HNE) is a key component in the pathogenesis of chronic inflammatory liver disease. Another consequence of inflammation is the perpetuation of oxidative damage by the production of reactive oxidative species such as hydrogen peroxide. Previously, we have demonstrated Akt2 as a direct target of 4-HNE in hepatocellular carcinoma cells. In the present study, we used the hepatocellular carcinoma cell line HepG2 as model to understand the combinatorial effects of 4-HNE and hydrogen peroxide. We demonstrate that 4-HNE inhibits hydrogen peroxide-mediated phosphorylation of Akt1 but not Akt2. Pretreatment of HepG2 cells with 4-HNE prevented hydrogen peroxide stimulation of Akt-dependent phosphorylation of downstream targets and intracellular Akt activity compared with untreated control cells. Using biotin hydrazide capture, it was confirmed that 4-HNE treatment resulted in carbonylation of Akt1, which was not observed in untreated control cells. Using a synthetic GSK3α/β peptide as a substrate, treatment of recombinant human myristoylated Akt1 (rAkt1) with 20 or 40 μM 4-HNE inhibited rAkt1 activity by 29 and 60%, respectively. We further demonstrate that 4-HNE activates Erk via a PI3 kinase and PP2A-dependent mechanism leading to increased Jnk phosphorylation. At higher concentrations, 4-HNE decreased both cell survival and proliferation as evidenced by MTT assays and EdU incorporation as well as decreased expression of cyclin D1 and β-catenin, an effect only moderately increased by the addition of hydrogen peroxide. The ability of 4-HNE to exert combinatorial effects on Erk, Jnk, and Akt-dependent cell survival pathways provides additional insight into the mechanisms of cellular damage associated with chronic inflammation.

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Introduction

Oxidative stress has been implicated in a wide range of chronic inflammatory diseases in the liver including hepatitis C, primary biliary cirrhosis, and alcoholic liver disease (ALD) [1–4]. Under conditions of chronic inflammation, reactive species such as hydrogen peroxide (H_2O_2) and 4-hydroxy-2-nonenal (4-HNE) are produced within the cell. 4-HNE is a primary marker for measuring increased oxidative stress in cells and is increased in ALD [3]. In experimental models of steatohepatitis and liver fibrosis, influx of proinflammatory cells stimulates production of reactive oxidative species (ROS) and formation of lipid peroxides such as 4-HNE, leading to increased cell death via either apoptosis or necrosis [5–7]. 4-HNE is a potent electrophile that will react with nucleophilic amino acids such as Cys, Lys, and His [8]. A number of signaling proteins have been identified to be modified by 4-HNE within cells, including the lipid phosphatase PTEN and protein kinases such as Akt2 and LKB1 [9–11]. In addition, using RKO cells treated with 100 μM 4-HNE, Codreanu et al. identified over 1500 proteins using biotin hydrazide capture followed by LC/MS proteomic analysis [12].

Under conditions of enhanced oxidative stress, a major cellular response is the activation of the Akt pathway. Hydrogen peroxide has been shown to induce activation of Akt by several mechanisms including inactivation of PTEN and activation of the PI3K pathway [13,14]. Although H_2O_2 is a known activator of Akt, very little is known concerning the specific isoform of Akt activated. In a recent study, knockdown of Akt1 led to increased resistance to

Abbreviations: ALD, alcoholic liver disease; Erk, extracellular-signal-related kinase; JNK, c-Jun-N-terminal kinase; GSK3β, glycogen synthase kinase 3 beta; 4-HNE, 4-hydroxy-2-nonenal; MDM2, ubiquitin ligase murine double minute oncogene 2; NASH, nonalcoholic steatohepatitis; PDK-1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PtdIns (3,4,5) P_3, phosphatidylinositol 3,4,5 trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; ROS, reactive oxidative species

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low micromolar concentrations of H_2O_2 in human lens epithelial cells via upregulation of Akt2 [15]. In other studies, using mouse embryonic fibroblasts, a deficiency in both Akt1 and Akt2 led to increased resistance to H_2O_2-mediated apoptosis at concentrations up to 1 mM [16]. Combined, these observations suggest cell type specific regulation of H_2O_2 resistance via different Akt isoforms.

Previously, 4-HNE has been shown to decrease cellular proliferation in several cell types including prostate and breast cancer cells [17,18]. One of the major proteins involved in cellular proliferation is the protein kinase Akt. Protein kinases such as Akt regulate the cell cycle and proliferation via phosphorylation of multiple proteins including glycogen synthase kinases 3β (inactivation leading to increased stability of cyclin D1) and ubiquitin ligase mouse double minute 2 (MDM2) (inhibition of p53 degradation) [19,20]. 4-HNE has been shown to inhibit insulin signaling via direct modification of Akt2, leading to a decrease in phosphorylation of both GSK3β and MDM2, both downstream targets of Akt [11]. It is well known that under oxidative stress and inflammatory conditions, 4-HNE is not the only reactive intermediate produced. Therefore, examining combinatorial effects of different reactive species may provide greater insight into the pathogenesis of inflammation. In this study, we report that preincubation of HepG2 cells with 4-HNE inhibits H_2O_2-mediated activation of the Akt pathway in leading to decreased cell proliferation and decreased expression of cyclin D1.

Materials and methods

Treatment of HepG2 cells

HepG2 cells were maintained at 50–80% confluence in RPMI supplemented with 10% fetal bovine serum, 100 mM Hepes, 100 IU/ml penicillin, 100 g/ml streptomycin. Cells were plated into 6-well plates at a density of 1×10^6 cells per well. The following day, the cells were washed twice in serum-free RPMI and treated with indicated concentrations of 4-HNE in serum-free media. Where indicated, Ly294002 (Calbiochem/EMD Biosciences, Philadelphia, PA) (50 μM/30 min), okadaic acid (Calbiochem) (100 nM/30 min), U0126 (Calbiochem) (10 μM/30 min), or H_2O_2 (1.0 mM/5 min) (Sigma Aldrich, St. Louis, MO) was added as described.

Western blotting

Following treatment, cells were lysed for 5 min in 50 mM Hepes, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA (pH 7.7), plus protease and phosphatase inhibitors Sigma (St. Louis, MO), followed by sonication for 3×10 s. For each gel, 10 μg of whole cell lysates was loaded per well on 7% SDS PAGE gels, electroblotted to PVDF, blocked in Tris-buffered saline with 1% Tween (TBST) and 5% nonfat dry milk for 1 h, and incubated overnight in primary antibody. The rabbit polyclonal 4-HNE antibody was used at 1:1000 as previously described [21]. The following secondary antibodies were used at 1:5000 dilution in TBST/5% NFDM: HRP-conjugated goat polyclonal anti-rabbit (Jackson Immunoresearch laboratories, West Grove, PA), HRP conjugated donkey polyclonal anti-mouse (Jackson). Blots were subsequently processed and developed using chemiluminescence as previously described [9]. All Western blots were quantified using NIH freeware Image J.

Primary antibodies

The following primary antibodies were used at 1:1000 dilution in TBST/5% BSA: rabbit polyclonal, pAkt (Ser^{473}), pAkt (Thr^{308}), pAkt (Thr^{450}), pMDM2 (Ser^{166}), pGSK3β (Ser^{9}), GSK3β (Nos. 4060, 4691, 3521, 9323, 9315; Cell Signaling, Danvers, MA); mouse monoclonal, actin 1:5000 (Sigma No. A5441), Akt2 (1:1000 No. 5329 Cell Signaling), Akt1 (1:1000 No. 2967 Cell Signaling), total MDM2 (ABCAM AB-10567).

Preparation of recombinant activated Akt1

The N-terminal myristoylation sequence from Src tyrosine kinase was added to full-length Akt1 by PCR from Flag Ha-tagged Akt1 PCDNA3 (Addgene No. 9021) using the following oligonucleotides: -5′ (5′-ccc aag gac ccc agc cag cgc atg agc gac gtg gct att gtg-3′ sense), 5′-cat atg cat ggg gag cag caa gag caa gcc caa gga ccc cag c-3′) for the N-terminus and (5′-ggt acc tca ggc cgt gcc gct ggc-3′ antisense). All oligonucleotides were purchased from IDT (Coralville, IA.). Following amplification, each fragment was TOPO-cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), transformed into TOP-10 cells, and grown overnight on LB-ampicillin plates (100 μg/mL) according to the manufacturer's instructions. Colonies were picked and placed into 3-mL LB cultures for 16 h with 100 μg/mL of ampicillin. DNA was subsequently purified using Qiagen minipreps (Qiagen, Valencia, CA) and sequences were verified at the University of Colorado Cancer Center core facility. myrAkt1 pCR2.1 was digested for 1 h with Nde1/Kpn1 (New England Biolabs, Ipswich, MA), and ligated overnight into pACGHLTA ΔGST using T4 DNA ligase (New England Biolabs). MyrAkt1 pACGHLTA ΔGST was subsequently cotransfected with linearized Baculogold DNA (BD-Pharmingen, San Diego, CA) into SF-9 cells using Lipofectamine by the UC Denver Cancer Core Protein Center. Virus was amplified for 5 days, viral titer determined, and SF-9 cells were infected for 48 h. Recombinant protein was purified as previously described [11].

Akt activity assays

Akt activity was determined by immunoprecipitation of p-Ser^{473} Akt from 150 mg of total protein lysates and in vitro phosphorylation of a GST-GSK3α/β fusion protein (1 μg/assay). Detection of phosphorylation was via Western blotting using anti-phospho GSK3α/β polyclonal antibodies as previously described [11].

Determination of intracellular carbonylation of Akt1

Akt1 protein carbonylation was determined using 150 μg of total cellular protein as previously described [9].

Assessment of cellular proliferation by EdU incorporation

Cell proliferation was assessed using a Click-iT EdU incorporation kit (Invitrogen/Molecular Probes) and confocal microscopy. In these studies, the HepG2 cells were seeded at 0.5×10^6 cells in triplicate per well onto coverslips in 12-well plates and allowed to attach for 16 h. Medium was removed by aspiration, and the HepG2 cells were either pretreated with 4-HNE (12.5–100 μM) or untreated and then treated with in H_2O_2 (1 mM) for 5 min in serum-free medium for 24 h. At 20 h, 10 μM ethynyl-2-deoxyuridine was added and the cells were allowed to incubate for an addition 4 h. The medium was removed, cells were fixed for 30 min in 3.7% paraformaldehyde, and coverslips were processed according to the manufacturer's instructions. Confocal images were taken with a 40× oil immersion objective on a Nikon Eclipse TE2000-E instrument, with identical instrument laser and contrast settings used within each group. Images were acquired using EZ-C1 software (Nikon, Melville, NY). Cell proliferation was determined by a comparison of the total number of cells with total nuclei with EdU incorporation counting a minimum of 10 fields with 100 nuclei per condition. EdU was visualized using Alexa Flour 488 (excitation 485 nm/emission 565 nm); nuclei were stained with Hoechst 33342 (excitation 355 nm/emission 465 nm).