Biochemical Pharmacology 93 (2015) 221–231

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Adiponectin induces apoptosis in hepatocellular carcinoma through differential modulation of thioredoxin proteins

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ARTICLE INFO

Article history:
Received 4 September 2014
Received in revised form 3 December 2014
Accepted 5 December 2014
Available online 13 December 2014

Keywords:
Adiponectin
Apoptosis
Hepatocellular carcinoma
Thioredoxin

ABSTRACT

Adiponectin blocks hepatocellular carcinoma (HCC) progression by inducing cell apoptosis through the modulation of C-Jun N-terminal kinase and mammalian target of rapamycin. However, the precise upstream signaling pathways or molecules remain elusive. In the present study, we analyzed the role of antioxidant protein thioredoxin (Trx) in adiponectin-induced apoptosis in HCC. Adiponectin treatment decreased the viabilities of both HepG2 and Huh7 HCC cells accompanied by increased accumulation of intracellular reactive oxygen species, as evidenced by 2′,7′-dichlorodihydrofluorescein diacetate staining. Pretreatment of these cells with the deoxidant *N*-acetylcysteine blocked the inhibitory effect of adiponectin. Levels of Trx2 protein in both HCC cells were significantly decreased, and the level of Trx1 was significantly inhibited in Huh7 cells while unchanged in HepG2 cells. However, the redox state of Trx1 was altered from reduced to the oxidized form following adiponectin treatment in HepG2 cells. Overexpression of both Trx proteins rescued adiponectin-induced cell apoptosis, whereas mutated Trx proteins were less effective. Further analysis suggested that both ASK1 and JNK signaling are involved in this process. Trx1 and Trx2 proteins also manifested protective effects on HCC cells in response to adiponectin treatment in a xenograft tumor model. Furthermore, high levels of Trx proteins and low adiponectin expression levels were found in primary human HCC samples compared with paracancerous tissues. These results suggest that Trx proteins play important roles in mediating adiponectin-induced HCC cell apoptosis, thus providing new insights into the pathogenesis of HCC and identifying adiponectin and Trx proteins as potential combinational therapeutic targets for the treatment of HCC.

1. Introduction

Adiponectin is one of the major adipokines abundantly expressed in plasma [1]. Plasma adiponectin levels are significantly decreased in various disease states [2] and in obese individuals [3]. Studies have shown that low levels of plasma adiponectin are associated with several common forms of cancer and that adiponectin negatively regulates breast, prostate, and gastrointestinal carcinogenesis [4–7]. The cellular functions of adiponectin are mediated by its specific receptors, AdipoR1 and AdipoR2, which activate various intracellular signaling pathways including 5′-adenosine monophosphate-activated protein kinase (AMPK), nuclear factor-κB, peroxisome proliferator-activated receptor-α, and p38 mitogen-activated protein kinase pathways [8,9]. The regulatory role of adiponectin in the pathogenesis of hepatocellular carcinoma (HCC) has recently attracted much attention [10–12]. Adiponectin modulates C-Jun N-terminal kinase (JNK) and mammalian target of rapamycin (mTOR), as well as Rho kinase/interferon-inducible protein 10/matrix metalloproteinase 9 signaling, inhibiting the progression of HCC [10,11]. However, the precise mechanisms underlying the functions of adiponectin in HCC require further investigation.

Abbreviations: APN, adiponectin; HCC, hepatocellular carcinoma; JNK, C-Jun N-terminal kinase; Trx, thioredoxin; NAC, *N*-acetylcysteine; AMPK, 5′-adenosine monophosphate-activated protein kinase; ROS, reactive oxygen species; ASK1, apoptosis signal-regulating kinase 1; PCNA, proliferating cell nuclear antigen; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; NQDI-1, 7-dihydro-2,7-dioxo-3*H*-naphtho [1,-2,3-de]-quinoline-1-carboxylic acid ethyl ester.

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http://dx.doi.org/10.1016/j.bcp.2014.12.001
0006-2952/

Reactive oxygen species (ROS) represent a large family of reactive molecules containing oxygen, including superoxide anions, hydroxyl radicals, and hydrogen peroxide. With the highly reactive groups in their molecular structure, ROS participate in several fundamental biological processes, including cell growth and senescence, and programmed cell death [13]. ROS can trigger apoptosis either indirectly, through damage to DNA, lipids, and proteins, or directly by activation of multiple signaling pathways [14]. Oxidative stress is classically defined as a redox imbalance caused by excess formation of oxidants or a defect in the functions of antioxidants, and has been shown to be closely related to the carcinogenesis and treatment of HCC [15,16]. Reagents, especially many anti-cancer drugs, exert their lethal effects by triggering oxidative stress in cancer cells. Although adiponectin has been reported to increase or decrease ROS levels to manifest devastating or protective functions in different cell types [17,18], its effect on the redox state in HCC cells is largely unknown.

The thioredoxin (Trx) system maintains the reducing environment in cells and detoxifies ROS. Trx are small, multi-functional proteins that contain a redox-active disulfide/dithiol group within the conserved active-site sequence Cys-Gly-Pro-Cys [19]. Through cysteine-thiol disulfide exchange, Trx proteins facilitate the reduction of proteins that have been modified by ROS. The resultant oxidized Trx is then converted back to the reduced form by thioredoxin reductases [19]. Two isoforms of Trx have been identified in mammalian cells, cytosolic and mitochondrial Trx (Trx1 and Trx2, respectively), which are involved in a variety of diseases, including cancer [20]. Increased expression of Trx1 was suggested to be associated with decreased patient survival in human colorectal cancer [21], and Trx1 and Trx2 cooperatively inhibit cell apoptosis signal-regulating kinase 1 (ASK1)-mediated apoptosis via the reduced thiol in a JNK-dependent or -independent manner [22,23]. By directly binding to and inhibiting the activity of ASK1, Trx inactivates proapoptotic Bcl-2 family proteins including Bax, inhibiting the release of proapoptotic factors such as cytochrome c and cell death [22]. Although Trx proteins have been suggested as potential diagnostic and prognostic markers of HCC [24,25], the mechanism of Trx in the pathogenesis and treatment of HCC is still not fully understood.

The present study examined the possible involvements of ROS and Trx proteins in the inhibitory effect of adiponectin on HCC, and investigated the role of Trx in the modulation of signal transduction pathways involved in adiponectin-mediated apoptosis.

2. Materials and Methods

2.1. Cell culture and chemicals

HepG2 and Huh7 cells were generously provided by Professor Wei An (Capital Medical University, Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 100 μg/mL streptomycin, 100 U/mL penicillin (Thermo Fisher Scientific Inc.) in a 37 °C/5% CO_2 incubator. Cells were treated with different agents in optimal serum-free conditions, unless otherwise stated. For overexpression of Trx1 and Trx2, pcDNA3.1-V5, recombinant wild-type Trx1 and mutant Trx1-C32/35S (active-site cysteine residues replaced with serine residues), recombinant wild-type Trx2 and mutant Trx2-C90/93S (active-site residues replaced by serine residues) were transfected into HepG2 cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Grand Island, NY, USA). Cells were prepared as follows: adiponectin-treated cells were serum-starved for 16 h before exposure to recombinant human adiponectin (R&D Systems Inc., Minneapolis, MN, USA). To inhibit intracellular ROS generation, cell cultures were pretreated with *N*-acetylcysteine (NAC) (R&D systems Inc.) for 30 min. The JNK pathway was blocked by pretreating cell cultures with SP600125 (R&D systems Inc.) for 30 min, while the ASK1 pathway was blocked by pretreatment with NDQI-1 (Sigma, St Louis, MO, USA) for 30 min.

2.2. Western blot and redox western blot analysis

Cells were treated as indicated and lysed in lysis buffer (final concentration, 50 mM Tris [pH 8.0], 50 mM NaCl, 1% Nonidet P-40, 20 nM okadaic acid, 20 μM sodium orthovanadate, phosphatase inhibitor mixture, and protease inhibitor mixture (Thermo Fisher Scientific Inc., Rockford, IL, USA). Tissues were lysed in lysis buffer. Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific Inc.). Total protein extracts (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8 or 12%) and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked for 1 h with TBST (20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) containing 5% skim milk at room temperature, and incubated with the indicated primary antibodies at 4 °C overnight. The membranes were rinsed with TBST and stained with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Reactive bands were detected using enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific Inc.). The following antibodies were used for western blot analyses: anti-Trx1 (#ab86255) and anti-Trx2 (#ab71261) were purchased from Abcam (Cambridge, UK). Anti-ASK1 (H-300) (#sc-7931), p-c-Jun (KM-1) (sc-822), and proliferating cell nuclear antigen (PCNA) (#sc-25280) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-cyclin D1 (#2922), phospho-SAPK/JNK (Thr183/Tyr185) (#4668), SAPK/JNK (Thr183/Tyr185) (#9254), phospho-AMPK (#2535), AMPK (#3532), poly(ADP-ribose) polymerase (#9542), β-actin (#4967), Bcl-2 (#2876), and Bax (#2772) were all from Cell Signaling Biotechnology (CST, Danvers, MA, USA).

The redox status of Trx1 was measured by redox western blotting. Cells were lysed in collection buffer (6 M guanidine HCl, 50 mM Tris, 3 mM EDTA, 0.5% Triton X-100, pH 8.3). For redox western blotting, 50 mM iodoacetic acid was added to the lysis buffer and incubated at 37 °C for 30 min. Excess iodoacetic acid was removed by Sephadex chromatography (MicroSpin G-25 columns, GE Healthcare Biosciences, Pittsburgh, PA, USA). Samples were subjected to native electrophoresis (15%) to separate the reduced and oxidized forms of Trx1. Proteins were electrotransferred to polyvinylidene difluoride membranes and immunodetected with anti-Trx1 antibody (Abcam). Proteins were visualized using an ECL-detection kit. Densitometric analysis of membranes was performed using ImageJ software. The redox potential (Eh) of Trx1 was calculated using the Nernst equation, $Eh = -254 + 30 \times [\log (\text{oxidized Trx1/reduced Trx1})]$.

For western blotting in human HCC samples, 16 cases of HCC were analyzed using anti-Trx1 (#2429, CST) and anti-Trx2 (#ab71261, Abcam) antibodies. These studies were approved by the Institutional Review Board at Capital Medical University.

2.3. Determination of cell viability by ATP production

The effect of adiponectin on cell growth was assessed by measuring cell viability, determined by ATP production. Briefly, cells were seeded onto 96-well plates at a density of 5×10^3 cells/well in a volume of 200 μL. Cells were incubated with adiponectin at 10 μg/mL for 24 h and then analyzed using the CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI, USA) with cells cultured in serum-supplemented medium for 30 min, following the manufacturer's instructions. The amount of light