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Globular adiponectin inhibits ethanol-induced apoptosis in HepG2 cells through heme oxygenase-1 induction

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ABSTRACT

Hepatocellular apoptosis is an essential pathological feature of alcoholic liver disease. Adiponectin, an adipokine predominantly secreted from adipose tissue, has been shown to play beneficial roles in alcoholic liver disease against various inflammatory and pro-apoptotic molecules. However, the effects of adiponectin on ethanol-induced apoptosis in liver cells are largely unknown. Herein, we investigated the role of globular adiponectin (gAcrp) in the prevention of ethanol-induced apoptosis and further tried to decipher the potential mechanisms involved. In the present study, we demonstrated that gAcrp significantly inhibits both ethanol-induced increase in Fas ligand expression and activation of caspase-3 in human hepatoma cell lines (HepG2 cells), suggesting that gAcrp plays a protective role against ethanol-induced apoptosis in liver cells. This protective effect of gAcrp was mediated through adiponectin receptor R1 (adipoR1). Further, globular adiponectin treatment caused induction of heme oxygenase-1 (HO-1) through, at least in part, nuclear factor (erythroid-derived 2)-like 2, (Nrf2) signaling. Treatment with SnPP, a pharmacological inhibitor of HO-1, and knockdown of HO-1 with small interfering RNA (siRNA) restored caspase-3 activity suppressed by gAcrp, indicating a critical role of HO-1 in mediating the protective role of gAcrp in ethanol-induced apoptosis in liver cells. In addition, carbon monoxide, a byproduct obtained from the catabolism of free heme was found to contribute to the anti-apoptotic effect of adiponectin. In conclusion, these data demonstrated that globular adiponectin prevents ethanol-induced apoptosis in HepG2 cells via HO-1 induction and revealed a novel biological response of globular adiponectin in the protection of liver injury from alcohol consumption.

1. Introduction

Hepatocellular apoptosis, an important pathological feature of human alcoholic liver disease (ALD), is induced by ethanol as an early response to liver injury [1–3]. Clinical evidence suggests that prevention of dysregulated apoptosis in the liver constitutes a critical approach for the treatment of ALD [4,5]. Apoptotic pathway is regulated by both the extrinsic pathway initiated by Fas receptor/Fas ligand system and the intrinsic pathway activated by the release of cytochrome *c* from the mitochondria into the cytosol. Both of these pathways ultimately activate caspase-3, the final executioner of apoptotic cell death, and are implicated in ethanol-induced apoptosis in hepatic cells [6]. Emerging evidence suggests that ethanol metabolites and reactive oxygen species (ROS) generated from ethanol metabolism trigger apoptotic process in the liver through induction of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and/or pro-apoptotic molecules such as Fas ligand and free fatty acids [1,5,7,8].

Adiponectin, an adipokine predominantly secreted from adipocytes, possesses an important modulatory role in various pathophysiological conditions. Apart from its well characterized role in glucose and fatty acid metabolism, adiponectin has received special attention in recent years due to its protective role in inflammation, metabolic syndrome, insulin resistance, cancer, osteoporosis, neurodegeneration, etc. [9,10]. Adiponectin acts on target tissues through binding with adiponectin receptor R1 and/or R2 (adipoR1/adipoR2). Binding of adiponectin with adipoR1 or adipoR2 leads to the activation of various subsequent signaling pathways, such as adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α

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(PPAR-α) pathways in the liver thereby ameliorating insulin resistance, hepatic inflammation and fibrosis [11,12]. Recent studies have demonstrated that adiponectin protects the liver injury against chronic ethanol treatment [13,14]. Furthermore, a number of studies have also demonstrated that adiponectin protects liver cells from apoptosis induced by various pathological stimuli owing to its anti-oxidative properties, blockage of activated c-Jun NH_2 terminal kinase or inhibition of CD95/Fas up-regulation in both in vitro and in vivo models [14–16].

Heme oxygenase (HO) is a rate-limiting enzyme in heme catabolism. Among the three mammalian HO isoforms, heme oxygenase-1 (HO-1) is a highly inducible protein that is sensitive to cellular oxidative stress and apoptotic cell death [17]. A growing body of evidence suggest that redox-sensitive transcription factors such as NF-E2 related factor 2 (Nrf2), nuclear factor kappa beta (NFκB) and activating protein (AP-1) are involved in HO-1 gene expression [17–19]. It has been shown that HO-1 induction mediates complex biological functions, and exerts cytoprotective, anti-inflammatory, antioxidant and anti-apoptotic effects in various cell lines in response to various stimuli. These protective effects are mostly attributed to by- products obtained from the catalytic degradation of heme, such as iron, carbon-monoxide and biliverdin [20,21]. Recent studies have revealed that HO-1 mediates adiponectin-induced anti-inflammatory response [22] and also transmits anti-apoptotic effect of adiponectin in the iron-treated rat liver [23]. However, the effects of adiponectin on prevention of ethanol-induced apoptosis in liver have not yet been demonstrated.

Thus, to better understand the mechanisms underlying protective role of adiponectin from alcohol-induced liver injury, herein we investigated whether globular adiponectin (gAcrp) protects human liver cells from ethanol-induced apoptosis and further delineated potential mechanisms implicated. In the present study, we have demonstrated for the first time that gAcrp protects liver cells from ethanol-induced dysregulated apoptosis and identified that HO-1 plays a critical role in the prevention of apoptosis elicited by adiponectin in liver cells.

2. Materials and methods

2.1. Materials

All the cell culture reagents were obtained from Hyclone laboratories (South Logan, Utah, USA). Absolute ethanol was purchased from Merck chemicals (Whitehouse Station, NJ, USA). Recombinant human globular adiponectin (gAcrp) was obtained from Peprotech Inc. (Rocky Hill, NJ, USA). Caspase-3 activity assay kit and cell titer 96 aqueous one solution cell proliferation assay kit (MTS) were purchased from Promega Corporation (Madison, WI, USA). Cleaved caspase and β-actin antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibody detecting heme-oxygenase-1 (HO-1) was purchased from Enzo life sciences (Farmingdale, NY, USA). Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG was obtained from Pierce biotechnology (Rockford, IL, USA). Sn(IV) protoporphyrin IX dichloride (SnPP), an inhibitor of HO-1, was obtained from Frontier Scientific (Logan, Utah, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless mentioned elsewhere.

2.2. Cell cultures

The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (Rockville, MD, USA) and routinely cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin along with 0.1% amphotericin at 37 °C in an incubator with 5% CO_2 at humidified atmosphere.

2.3. MTS assay

For the determination of cell viability, cells were seeded in 96 well plates at the density of 4×10^4 cells per well. After overnight incubation, cells were washed with phosphate-buffered saline (PBS) once and then pretreated with globular adiponectin with the indicated concentrations for 24 h followed by treatment with ethanol (50 mM) for additional 24 h in 100 μl DMEM. Then, 20 μl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution was added and the cells were incubated for 2 h at 37 °C. The resultant cell viability was monitored via a Versamax microplate reader (Sunnyvale, CA, USA) by measuring absorbance at 490 nm.

2.4. Caspase-3 activity assay

Caspase-3 activity was determined using a Caspase-Glo 3/7 assay kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions. Briefly, HepG2 cells were cultured in 35 mm dishes at a density of 1×10^5 cells per well in 2 ml culture media. After overnight incubation at 37 °C, cells were washed once with PBS and treated with the indicated concentrations of globular adiponectin for 24 h followed by treatment with ethanol (50 mM) for the next 24 h. Finally, 100 μl of cell lysates were tested in duplicate by measuring the luminescence from the cleavage of luminogenic substrate Ac-DEVD-pNA with a micro-plate reader (Flurostar Optima, BMG Labtech, Ortenberg, Germany).

2.5. RNA isolation, reverse transcription (RT) and quantitative PCR (qPCR)

To measure the mRNA levels of genes of interest, total RNAs were isolated using Qiagen lysis solution (Qiagen, Maryland, USA) according to the manufacturer's instructions and reverse transcribed for the synthesis of cDNA. Real time-PCR amplification was then carried out with a Roche LightCycler 2.0 using the absolute QPCR SYBR green capillary mix AB gene system (Thermoscientific, Epsom, UK) at 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 45 s. The primer sequences used for amplification of target genes were as follows: GAPDH forward, 5′-ACCACAGTCCATGCCATCAC-3′ and GAPDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′; Fas receptor forward, 5′-CTTTTCGTGAGCTCGTCTCTGA -3′ and Fas receptor reverse, 5′-CTCCCCAGAAGCGTCTTTGA -3′; Fas ligand forward, 5′-CCAGCTTGCCTCCTCTTGAG -3′ and Fas ligand reverse, 5′-TCCTGTAGAGGCTGAGGTGTCA-3′; HO-1 forward, 5′-CTTCTTCACCTTCCCCAACA-3′ and HO-1 reverse, 5′-AGCTCCTGCAACTCCTCAAA-3′. Comparative threshold (C_t) method was used for the determination of target mRNA after normalizing target mRNA C_t values to those for glyceraldehyde-3-phosphate dehydrogenase GAPDH (ΔCt).

2.6. Transient transfection with small interfering RNA (siRNA)

HepG2 cells were seeded in a six well plate at a density of 2×10^5 cells per well in 2 ml culture media. After 24 h incubation, cells were transfected with corresponding siRNA of target gene or scrambled control siRNA in a 2 ml growth medium with Hiperfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The transfection efficiency was assessed by qRT-PCR after 24–48 h of transfection, followed by gAcrp treatment for the indicated time points. The following siRNA duplexes were chemically synthesized by Bioneer (Daejeon, South Korea) and used for the study. HO-1 siRNA forward, CUGCGUUCCUGCUCAACAUtt and HO-1 siRNA reverse, AUGUUGAGCAGGAACGCAGtt; Adipo R1