Toxicology and Applied Pharmacology 280 (2014) 314–322

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

# Resveratrol attenuates methylglyoxal-induced mitochondrial dysfunction and apoptosis by Sestrin2 induction

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ARTICLE INFO

*Article history:*
Received 2 May 2014
Revised 3 August 2014
Accepted 13 August 2014
Available online 20 August 2014

*Keywords:*
Resveratrol
Methylglyoxal
Sestrin2
Reactive oxygen species
Mitochondrial dysfunction
Oxidative stress

ABSTRACT

Methylglyoxal is found in high levels in the blood and other tissues of diabetic patients and exerts deleterious effects on cells and tissues. Previously, we reported that resveratrol, a polyphenol in grapes, induced the expression of Sestrin2 (SESN2), a novel antioxidant protein, and inhibited hepatic lipogenesis. This study investigated whether resveratrol protects cells from the methylglyoxal-induced toxicity via SESN2 induction. Methylglyoxal significantly induced cell death in HepG2 cells. However, cells pretreated with resveratrol were rescued from methylglyoxal-induced apoptosis. Resveratrol attenuated glutathione (GSH) depletion and ROS production promoted by methylglyoxal. Moreover, mitochondrial damage was observed by methylglyoxal treatment, but resveratrol restored mitochondrial function, as evidenced by the observed lack of mitochondrial permeability transition and increased ADP/ATP ratio. Resveratrol treatment inhibited SESN2 depletion elicited by methylglyoxal. SESN2 overexpression repressed methylglyoxal-induced mitochondrial dysfunction and apoptosis. Likewise, rotenone-induced cytotoxicity was not observed in SESN2 overexpressed cells. Furthermore, siRNA knockdown of SESN2 reduced the ability of resveratrol to prevent methylglyoxal-induced mitochondrial permeability transition. In addition, when mice were exposed to methylglyoxal after infection of Ad-SESN2, the plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and GSH depletion by methylglyoxal in liver was reduced in Ad-SESN2 infected mice. Our results demonstrated that resveratrol is capable of protecting cells from methylglyoxal-induced mitochondrial dysfunction and oxidative stress via SESN2 induction.



## Introduction

Chronic hyperglycemia has been implicated in various metabolic diseases including obesity, diabetes, fatty liver, and atherosclerosis (Aronson and Rayfield, 2002; Gabbay, 1975; Yki-Järvinen, 1992). Aberrantly increased glucose degradation in cells due to hyperglycemia causes structural and functional modification of proteins with metabolites of glucose, and generates advanced glycation end products (AGEs) (Gaens et al., 2013). Methylglyoxal is the most potent glycation agent and a highly reactive dicarbonyl metabolite (Gaens et al., 2013; Shinohara et al., 1998). Plasma levels of methylglyoxal are found to be increased in metabolic diseases (Mukohda et al., 2012; Rabbani et al., 2011; Rabbani and Thornalley, 2011). Moreover, several lines of evidence support pathophysiological roles of AGEs in hyperglycemia, hyperlipidemia, and oxidative stress related diseases (Gaens et al., 2013; Morgan et al., 2013; Rabbani and Thornalley, 2011). AGEs cause deleterious effects on cells by increasing the production of free radicals and related reactive intermediates. In addition, accumulation of proteins bound with AGEs causes oxidative stress and inflammatory signaling, which contribute to the pathogenesis of metabolic disease (Ramasamy et al., 2011, 2012).

It is well established that metabolic disease is closely related to mitochondrial dysfunction (Ren et al., 2010). The mitochondrial respiratory chain is a major intracellular site of reactive oxygen species (ROS) production during unregulated hyperglycemia (Rolo and Palmeira, 2006; Yu et al., 2006). Excess ROS production under hyperglycemic conditions causes mitochondrial permeability transition, which is indicative of mitochondrial dysfunction. Indeed, mitochondrial dysfunction consequently leads to cellular ATP depletion and finally apoptosis (Kim et al., 2003; Lemasters et al., 1998). Recently, direct evidence that the levels of methylglyoxal and glyoxal within mitochondria are elevated under hyperglycemic conditions was reported (Pun et al., 2014). Therefore, mitochondria are important targets for studying harmful effects in AGE-mediated toxicity.

Resveratrol (3,4′,5-trihydroxystilbene) is a polyphenolic component found in grapes and red wine. Accumulated studies indicate that

*Abbreviations:* AGEs, advanced glycation end products; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GCL, glutamate cysteine ligase; GSH, glutathione; HO-1, heme oxygenase-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NQO, NADPH-quinone oxidoreductase; Nrf2, NF-E2-related factor-2; PRX, peroxiredoxin; RAGE, receptor for advanced glycation end products; Rh123, Rhodamine123; SESN, Sestrin; siRNA, small interfering RNA; SIRT, sirtuin.

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http://dx.doi.org/10.1016/j.taap.2014.08.011
0041-008X/

resveratrol produces many beneficial effects in metabolic diseases and aging (Baur et al., 2006; Howitz et al., 2003; Wood et al., 2004). These biological effects of resveratrol are primarily due to sirtuin (SIRT) or AMP-activated protein kinase (AMPK) activation. SIRT1, as an $NAD^+$-dependent deacetylase for numerous protein targets, regulates cell viability (Vaziri et al., 2001) as well as glucose and lipid metabolism (Hou et al., 2008; Pfluger et al., 2008). Resveratrol also activates AMPK, which increases mitochondrial biogenesis and protects mitochondrial function (Lagouge et al., 2006; Shin et al., 2009). As a natural occurring antioxidant, resveratrol has also been found to increase catalase and superoxide dismutase (SOD) activity and induce NF-E2-related factor-2 (Nrf2)-dependent antioxidant enzymes (e.g., NADPH-quinone oxidoreductase (NQO), heme oxygenase-1 (HO-1), and glutamate cysteine ligase (GCL)) (Chen et al., 2005; Hsieh et al., 2006; Kode et al., 2008; Rubiolo et al., 2008).

In a previous study, we reported that resveratrol induced a novel antioxidant, Sestrin2 (SESN2), which attenuated hepatic lipogenesis (Jin et al., 2013). SESNs regulate cellular hydrogen peroxide by regeneration of peroxiredoxin (PRX) (Sanchis-Gomar, 2013). Three isoforms of SESNs have been identified in humans: SESN1, SESN2, and SESN3. SESN2 is induced during oxidative stress, hypoxia or DNA damage and shows cytoprotective effects on various cells (Ben-Sahra et al., 2013; Budanov and Karin, 2008; Budanov et al., 2002). Recently, Shin et al. reported that SESN2 is transcriptionally regulated by Nrf2, which also regulates antioxidant proteins and phase II detoxifying enzymes (Shin et al., 2012). Moreover, SESN2 is involved in the metabolism of lipid and glucose through modulation of AMPK (J.H. Lee et al., 2012). Nevertheless, the possibility that induction of SESN2 by resveratrol could contribute to its antioxidant activity has never been examined. In addition, relatively little attention has been focused on the role of SESN2 in mitochondrial function in detail.

Because of the importance of mitochondrial function in metabolic disease, this study investigated whether resveratrol inhibits mitochondrial dysfunction induced by methylglyoxal. Finally, we examined the role of SESN2 induction by resveratrol in restoring mitochondrial function and cytoprotection after methylglyoxal treatment.

## Materials and methods

*Materials.* Anti-PARP antibody and rhodamine123 (Rh123) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SESN2 antibody was purchased from Proteinteck (Chicago, IL). Anti-caspase-3 antibody was purchased from Cell Signaling (Danvers, MA). Methylglyoxal, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), resveratrol, rotenone, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), anti-β-actin antibody and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

*Animals and treatment.* The protocols for the animal study were approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6 weeks old) were provided from Oriental Bio (Sungnam, Korea) and acclimatized for 1 week. Mice (N = 4/group) were maintained at 20 ± 2 °C with 12 h light/dark cycles and a relative humidity of 50 ± 5% under filtered, pathogen-free air, with food (Purina, Korea) and water available ad libitum. Adenovirus particles ($1 \times 10^9$ pfu) were suspended in saline and were injected into tail vein. After 48 h, a single dose of methylglyoxal (200 mg/kg body weight) was intraperitoneally injected, and blood and liver samples were collected 12 h after methylglyoxal treatment. Control animals received saline only.

*Adenovirus preparation.* Murine SESN2 ORF was amplified by attB-fused specific primers (forward: 5′-GGGGACA AGTTTGTACAAAAAAGCAGGCT TCATGATCGTAGCGGACTCCGA-3′ and reverse: 5′-GGGGACCACTTTGT ACAAGAAAGCTGGGTCGGTCATGTAGCGGGTGATGG-3′) and inserted into pDONRTM221 entry plasmid by BP recombination reaction (Invitrogen, Carlsbad, CA) to generate an adenoviral SESN2 construct. To construct the recombinant adenovirus, pAD/CMV/V5-DEST gateway plasmid was used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The DNA sequences of recombinant adenovirus were analyzed by ABI7700 DNA cycle sequencer. The purification of recombinant adenovirus for animal study was conducted by $CsCl_2$ density gradient centrifugation. Adenovirus which expresses LacZ was used as an infection control.

*Cell culture.* HepG2 cells, a human hepatocyte-derived cell line, were obtained from ATCC (American Type Culture Collection, Rockville, MD), and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in humidified atmosphere with 5% $CO_2$. For all experiments, cells ($1 \times 10^5$ cells) were plated in 6-well plates for 2–3 days (i.e. 80% confluency) and serum-starved overnight before treatments.

*Establishment of a stable cell line expressing SESN2.* HepG2 cells were transfected with the plasmid pCMV-Tag3A (MOCK) or pCMV-SESN2 in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with geneticin (500 μg/ml). Colonies of geneticin-resistant cells were selected and amplified in culture. SESN2 overexpression was confirmed by immunoblotting.

*MTT assay.* To measure cytotoxicity, cells were plated at a density of $1 \times 10^5$ cells per well in a 48-well plate. After treatment, the MTT assay was performed according to previously published methods (Shin et al., 2009). Briefly, cells were treated with MTT solution (0.1 mg/ml) for 3 h and media was discarded. Produced formazan crystals were dissolved with DMSO. Absorbance was read at 570 nm using a microplate reader (Spectramax, Molecular Device, Sunnyvale, CA). Cell viability was calculated relative to vehicle-treated control [i.e. viability (% control) = 100 × (absorbance of treated sample) / (absorbance of control)].

*Immunoblot analysis.* Immunoblot analysis and preparation of cell lysates were conducted according to previously published procedures (Shin et al., 2009). Briefly, cells were centrifuged at 3000 *g* for 3 min and lysed with RIPA lysis buffer containing protease inhibitors. Lysates were centrifuged at 10,000 *g* for 10 min to obtain supernatants and were stored at −80 °C until use. Immunoblotting was performed with a GE mini-vertical unit. Membranes were incubated with corresponding primary antibody for overnight at 4 °C and further incubated with HRP-conjugated secondary antibody against mouse or rabbit IgG for 1 h at room temperature. Protein bands of interest were visualized using an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal loading of proteins was verified by immunoblotting for β-actin.

*Determination of glutathione (GSH).* GSH in cells and liver homogenates was determined using a commercially available GSH-400 determination kit (Oxis Research, Burlingame, CA) as previously reported (Shin et al., 2009). Briefly, cell or liver homogenates were suspended in ice-cold MPA working solution and centrifuged at 3000 *g* for 10 min. The upper clear aqueous layer was collected and used for GSH measurement. The GSH-400 method was based on two-step chemical reactions. In the first step, substitution products (thioethers) between 4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate and all mercaptans present in the sample were formed. The second step transformed the substituted product (thioether) obtained with GSH into a thione under the alkaline conditions (30% NaOH). The chromophoric thione had a maximal absorbance wavelength at 400 nm. For standard curve analysis, a range of 0–100 μM GSH dissolved in MPA working solution was used. Results were expressed as nanomole of GSH per milligram of protein or tissue.