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Protective effect of carbenoxolone on ER stress-induced cell death in hypothalamic neurons

Jongwan Kim [a, d], Eun Jung Jung [b], Seong-Su Moon [a, c, **], Minchul Seo [a, *]

[a] Institute of Medical Research, Dongguk University College of Medicine, Gyeongju, South Korea
[b] Department of Biochemistry, Dongguk University College of Medicine, Gyeongju, South Korea
[c] Department of Internal Medicine, Dongguk University College of Medicine, Gyeongju, South Korea
[d] Department of Anatomy, Graduate School of Dongguk University College of Medicine, Gyeongju, South Korea

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ABSTRACT

Hypothalamic endoplasmic reticulum (ER) stress is known to be increased in obesity. Induction of ER stress on hypothalamic neurons has been reported to cause hypothalamic neuronal apoptosis and malfunction of energy balance, leading to obesity. Carbenoxolone is an 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) inhibitor that converts inactive glucocorticoid into an active form. In addition to its metabolic effect via enzyme inhibitory action, carbenoxolone has shown anti-apoptotic activity in several studies. In this study, the direct effects of carbenoxolone on ER stress and cell death in hypothalamic neurons were investigated. Carbenoxolone attenuated tunicamycin induced ER stress-mediated molecules such as spliced XBP1, ATF4, ATF6, CHOP, and ROS generation. In vivo study also revealed that carbenoxolone decreased tunicamycin-induced ER stress in the hypothalamus. In conclusion, the results of this study show that carbenoxolone has protective effects against tunicamycin induced-ER stress and apoptosis in hypothalamic neurons, suggesting its direct protective effects against obesity. Further study is warranted to clarify the effects of carbenoxolone on hypothalamic regulation of energy balance in obesity.

1. Introduction

Recently, obesity has become a serious threat to public health worldwide [1]. Obesity is causally linked to debilitating conditions such as type 2 diabetes, dyslipidemia, atherosclerosis, stroke, and cardiovascular disease [2]. Obesity is caused by energy imbalance toward overnutrition. Abnormality in the central nervous system involving regulation of energy intake and expenditure and feeding behavior has been recognized as a possible pathogenetic mechanism of obesity [3,4]. In particular, the hypothalamus acts as the main regulator of energy balance, including nutrient sensing, appetite control, energy expenditure, and carbohydrate and lipid metabolism by coordinating peripheral homeostatic activities [5–7]. In humans, differential hypothalamic function exists between lean and obese subjects [8]. Therefore malfunction of the hypothalamus eventually leads to obesity.

Endoplasmic reticulum (ER), a highly developed intracellular organelle, plays a central role in protein biosynthesis and maturation and Ca^{2+} storage [9,10]. Overload of metabolic stress in the ER induces the unfolded protein response (UPR). However, unresolved ER stress leads to persistent UPR and activation of the cellular apoptotic pathway [11]. Previous studies have demonstrated that ER stress plays a critical role in the development of obesity-related diseases such as type 2 diabetes, fatty liver, and atherosclerosis [12–14].

Hypothalamic ER stress is suggested to cause hypothalamic malfunction and apoptosis. Overnutrition acts to induce central ER stress in the hypothalamus [15–17]. Pharmacologic or genetic induction of ER stress in the hypothalamus causes central leptin and insulin resistance, resulting in food intake increase, glucose intolerance, and hypertension, whereas reduction of ER stress significantly alleviates these metabolic derangements [15,18–20].

Carbenoxolone is a semisynthetic derivate of glycyrrhizic acid, which is the active principal of licorice (*Glycyrrhiza glabra*), an herb medicinal root. Carbenoxolone is currently used for treatment of

* Corresponding author. Department of Internal Medicine, Dongguk University College of Medicine, Gyeongju, South Korea. Tel.: +82 54 770 2413.
** Corresponding author. Institute of Medical Research, Dongguk University College of Medicine, Gyeongju, South Korea. Tel.: +82 54 770 2413.
E-mail addresses: drmoonss@hanmail.net (S.-S. Moon), nansmc@hanmail.net (M. Seo).

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psoriasis, peptic ulcers, and wound healing. Carbenoxolone is an 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) inhibitor that converts inactive glucocorticoid into an active form. Overexpression of 11β-HSD1 is associated with obesity, insulin resistance, and hypertension [21,22]. In addition to its metabolic effect, Carbenoxolone has been reported to have a direct effect on cell viability. Carbenoxolone treatment on HepG2 cells was shown to directly reduce stress induced by reactive oxygen species [23] as well as oxidative stress and apoptosis in H_2O_2-exposed PC12 cells [24]. To the best of our knowledge, there has been no study elucidating the direct effect of carbenoxolone on ER stress and -induced apoptotic cell death in hypothalamic neurons. In this study, we investigated whether or not carbenoxolone alleviates ER stress and consequently reduces activation of the apoptotic pathway in the hypothalamus.

2. Materials and methods

2.1. Culture of primary hypothalamic neurons

All the reagents used for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Time-pregnant rats (Sprague–Dawley) were ordered at 13th day of pregnancy and housed in controlled temperature with a 12/12 h light/dark cycle and access to food and water *ad libitum*. On the 19th day of pregnancy, pregnant rats were euthanized with isofluorane, and the fetuses were collected. Fetal hyopthalamus tissues were dissected from brains, and neuronal cultures were prepared as previously described with slight modifications [25,26]. Briefly, dissected hypothalamus tissues were collected in Hank's balanced salt solution (HBSS), followed by digestion with 0.05% trypsin-EDTA in neurobasal media for 30 min at 37 °C and trituration with fire polished graded Pasteur pipettes. The dissociated cells were counted with a hemocytometer and plated at a density of 3.0 or 6.0×10^4 cells/well onto poly-DL-lysine (PDL) (Sigma–Aldrich, St. Louis, MO, USA) -coated coverslips or 12 well culture plates. Cultures were maintained in defined serum-free neurobasal media supplemented with B27 and incubated at 37 °C under 5% CO_2 and 95% air.

2.2. Immunocytochemistry

At the indicated time, neurons on coverslips were rinsed briefly with Dulbecco's phosphate-buffered saline (D-PBS, Invitrogen) and fixed by a sequential paraformaldehyde/methanol fixation procedure. For immunostaining, fixed neurons on coverslips were incubated with primary antibodies [mouse monoclonal anti-CHOP antibody (1: 200; Cell Signaling Technology); mouse monoclonal anti-ATF-6 antibody (1: 200; Abcam)], followed by secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000; Molecular Probes) and mounted on slides as described [25].

2.3. Image acquisition and analysis

A Leica Research Microscope DM IRE2 equipped with I3 S, N2.1 S, and Y5 filter systems (Leica Microsystems AG, Wetzlar, Germany) was used for phase-contrast and fluorescence microscopy. Images were acquired with a high resolution CoolSNAP™ CCD camera (Photometrics Inc., Germany) under the control of a computer using Leica FW4000 software. The digital images were processed using Adobe Photoshop 7.0 software.

2.4. Western blot analysis

Tissue or cells were lysed in triple-detergent lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations of cell lysates were determined using a Bio-Rad protein assay kit (Bio-Rad). Equal amounts of protein were separated by 8 or 12% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies [mouse monoclonal anti-CHOP antibody (1: 1000; Cell Signaling Technology); mouse monoclonal anti-ATF-6 antibody (1: 500; Abcam); mouse monoclonal anti-ATF-4 (Sigma–Aldrich); monoclonal anti- α-tubulin antibody (1:2000; Sigma–Aldrich)], and HRP-conjugated secondary antibody (1: 10,000; anti-mouse-IgG antibody; Amersham Biosceinces), followed by detection using an ECL detection kit (Amersham Biosciences).

2.5. Reverse transcription-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Reverse transcription was carried out with Superscript II (Invitrogen) and oligo(dT) primers. PCR amplification was carried out at an annealing temperature of 55 °C for 25–30 cycles using the following specific primer set: Xbp1s Forward, TTACGAGAGAAAACTCATGGGC; Xbp1s reverse, GGGTCCAACTTGTCCAGAATGC; β-actin forward, AGGGAAATCGTGCGTGACAT; β-actin reverse, CGGACTCATCGTACTCCTGC. For analysis of PCR products, 10 μl of each PCR product was electrophoresed on a 1–2.5% agarose gel and detected under UV light. β-actin was used as an internal control.

2.6. Assessment of cell viability by MTT assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Primary hypothalamic neuronal cells were seeded in triplicate at a density of 1×10^4 cells per well on a 96-well plate. At the end of treatment, culture media were removed and MTT (0.5 mg/mL) added, followed by incubation at 37 °C for 2 h in a CO_2 incubator. After dissolving the insoluble crystals that formed in DMSO, absorbance was measured at 570 nm using a microplate reader (Anthos Labtec Instruments, Wals, Austria).

2.7. ROS assay

Primary hypothalamic neuronal cells plated in 24-well culture plates were treated with increasing doses of carbenoxolone (0, 0.1, 1, 5, and 10, μg/ml) for 2 h before tunicamycin (5 μg/ml) treatment, followed by incubation for 30 min at 37 °C in media containing 10 μM 5-(and- 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CMH_2DCFDA; Molecular Probes). Cultures were washed twice with PBS and subjected to fluorescence microscopic analysis. DCFDA-positive cells were counted from three randomly chosen microscopic fields. These three measurements were averaged and regarded as a data point.

2.8. Animals

Male C57BL/6J mice, 8–10 weeks old WT, were obtained from Japan SLC (Hamamatsu, Japan). Mice were given access to a standard chow diet and water, unless otherwise indicated. Mice were housed under conditions of controlled temperature (23 °C) and a 12/12 h light/dark cycle with access to food and water *ad libitum*. All procedures followed the Principles of Laboratory Animal Care (NIH, Washington) and were approved by the Institution Animal Care and Use Committee of College of Medicine, Dongguk University.