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AMPK activator-mediated inhibition of endoplasmic reticulum stress ameliorates carrageenan-induced insulin resistance through the suppression of selenoprotein P in HepG2 hepatocytes

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ABSTRACT

Carrageenan (CGN) has been shown to cause inflammation through toll-like receptor 4, which may play an important role in insulin resistance and type 2 diabetes mellitus. Selenoprotein P (SeP) has recently been identified as a novel hepatokine that causes insulin resistance. Here, we report that treatment of HepG2 cells with CGN increased both CCAAT enhancer binding protein homologous protein (CHOP) and SeP expression. Pretreatment with 4-phenylbutyrate (4-PBA), an endoplasmic reticulum stress inhibitor, and PD98059, a c-Jun N-terminal kinase (JNK) inhibitor, reversed CGN-induced SeP expression. Moreover, both 4-PBA and knock-down of SeP improved CGN-induced insulin resistance. In addition, we found that adenosine monophosphate-activated protein kinase (AMPK) activators ameliorated CGN-induced insulin resistance in addition to suppressing CHOP and SeP expression. In conclusion, CGN-induced ER stress increased the expression of SeP through the JNK pathway, while AMPK activators ameliorated CGN-induced insulin resistance via SeP inhibition through the AMPK-mediated alleviation of ER stress in hepatocytes.

1. Introduction

Carrageenan (CGN) is a common food additive used to improve texture and solubility. It is composed of sugar molecules that are either sulfated or unsulfated, and its main application is in dairy and meat products, due to strong interactions between CGN and proteins. However, CGN is known to cause inflammation by binding to toll-like receptor 4 (TLR4), which has an important role in the innate immune response and insulin resistance (Bhattacharyya et al., 2008a,b; Borthakur et al., 2007). Loss-of-function mutations of TLR4 prevent diet-induced obesity and insulin resistance (Tsukumo et al., 2007). Therefore, the pro-inflammatory effects of CGN may have implications for metabolic disorders including obesity, insulin resistance, and type 2 diabetes mellitus (T2DM).

Selenoprotein P (SeP), a 42-kDa glycoprotein, is produced in the liver and secreted into plasma. SeP was recently identified as a hepatokine associated with insulin resistance in humans through serial analysis of gene expression (SAGE) Misu et al., 2010. Purified human SeP administration worsened insulin signaling and glucose metabolism in both liver and skeletal muscle in female C57BL/6J mice (Misu et al., 2010). Furthermore, SeP-deficient mice and small interfering RNA (siRNA)-mediated SeP knockdown hepatocytes showed enhanced insulin signaling and improved glucose tolerance (Misu et al., 2010). Serum SeP levels were also shown to have a positive correlation with fasting plasma glucose and a negative correlation with serum adiponectin levels, an insulin sensitizing adipokine, in patients with T2DM (Misu et al., 2012). In our previous studies, we found that patients with T2DM (Yang et al., 2011) and those with non-alcoholic fatty liver disease (NAFLD) had higher serum SeP levels than healthy controls (Choi et al., 2013). Adenosine monophosphate-activated protein kinase (AMPK) has also been reported to have a crucial role in SeP-mediated insulin resistance (Misu et al., 2010).

AMPK is an evolutionarily conserved sensor of cellular energy status (Hardie et al., 2006) and suppresses the nuclear factor-κB (NFκB)-mediated inflammatory response (Salminen et al., 2011). AMPK has been reported to be activated by salicylate and play an important role in fat utilization and lowering plasma fatty acids (Hawley et al., 2012). Hundal et al. reported that salicylate improves hyperglycemia and insulin resistance (Hundal et al., 2002). Unfortunately, however, salicylate has side-effects such as stomach irritation and increased risk of bleeding. Salsalate, a prodrug of salicylate, is well tolerated and considered relatively safe

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for long-term clinical use (Goldfine et al., 2011). In a recent multi-center prospective clinical trial, salsalate treatment lowered triglyceride levels and improved markers of glycemic control (Goldfine et al., 2010).

In the current study, we investigated (1) the effect of CGN on ER stress; (2) the regulation of SeP expression by CGN-induced ER stress; (3) the possible mechanisms underlying the inhibitory effect of salsalate on ER stress and SeP expression; and (4) the possible mechanism underlying the inhibitory effect of salsalate on CGN-induced insulin resistance in HepG2 cells.

2. Materials and methods

2.1. Cell culture and reagents

Human (15-year-old Caucasian American male) hepatoma HepG2 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. HepG2 cells were cultured for 4 days to achieve 85% confluence before treatment with CGN or other additives. Mouse primary hepatocytes were purchased from ZenBio (Research Triangle Park, NC, USA). Mouse hepatocytes were plated in collagen type-I coated 6-well plates in Hepatocyte Plating Medium (ZenBio). After 8 h of attachment, the medium was changed with Hepatocyte Maintenance Medium (ZenBio). Cells were treated with λ-carrageenan (CGN, Sigma, St. Louis, MO, USA) (1 mg/l, 20 h) in medium with serum, then with CGN (1 mg/l, 4 h) in medium without serum. Salsalate (Sigma) SB203580 (Sigma), PD98059 (Sigma), SP600125 (Sigma), metformin (Sigma), aminoimidazole carboxamide ribonucleotide (AICAR) (Sigma), and compound C (Sigma) were dissolved in dimethyl sulfoxide (DMSO). 4-Phenylbutyrate (4-PBA) was dissolved in distilled water and added to the culture medium. The final concentration of DMSO did not exceed 0.1%, which did not affect cell viability or AMPK phosphorylation. Human insulin (Sigma) was dissolved in distilled water adjusted with NaOH for insulin stimulation experiments.

2.2. Western blot analysis

HepG2 cells were harvested and extracted with lysis buffer (PRO-PREP™; Intron Biotechnology, Seoul, Korea) for 60 min at

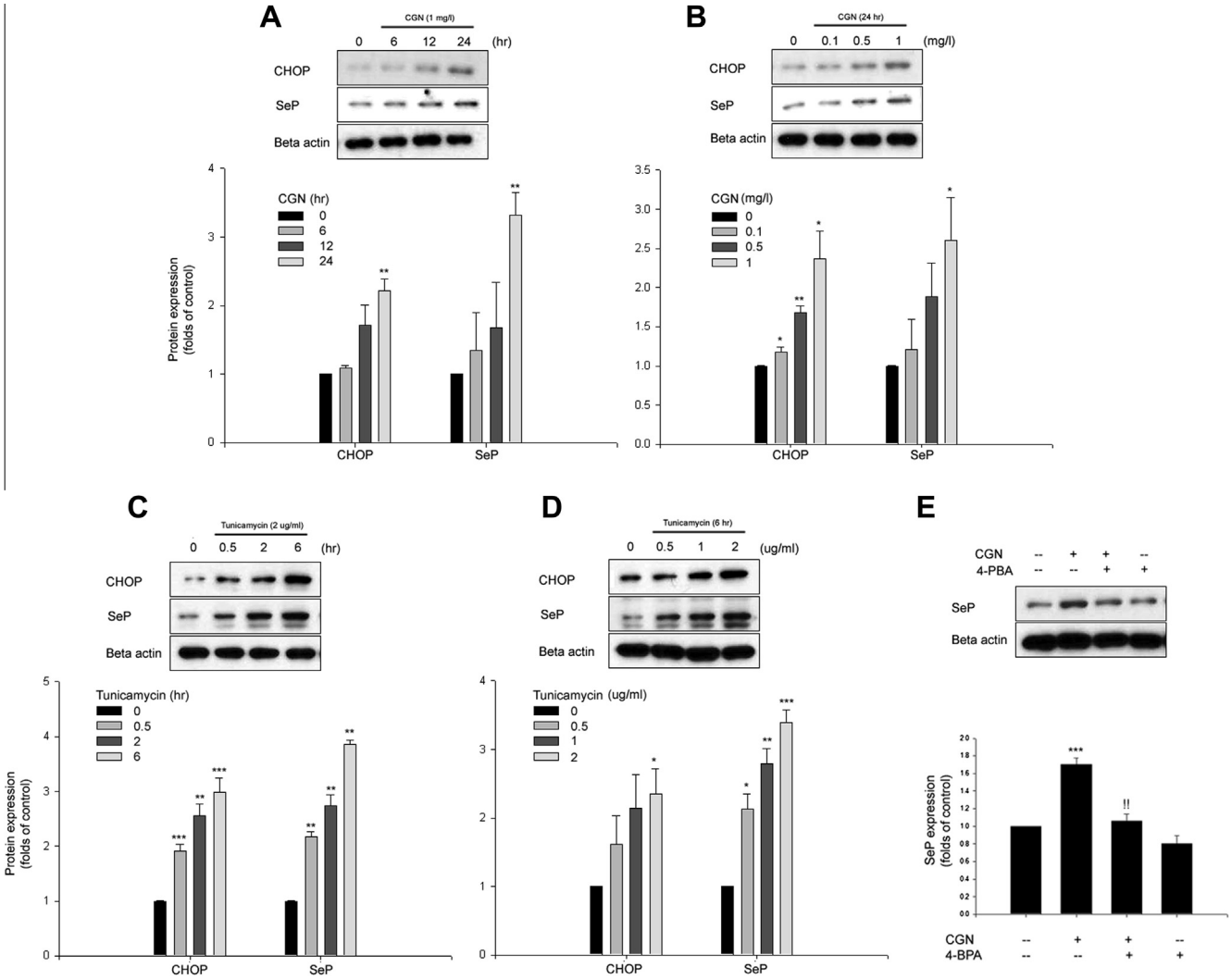

Fig. 1. CGN-induced ER stress increases SeP expression. CGN induced CHOP and SeP expressions in a time- and dose-dependent manner (A and B). Tunicamycin-induced ER stress increased SeP expression. CGN induced CHOP and SeP expression in a time- and dose-dependent manner (C and D). ER stress inhibitor, 4-PBA, prevented CGN-induced SeP expression (E). HepG2 cells were treated with CGN, tunicamycin, and 4-PBA (2 mM, 1 h pretreatment) and harvested at the indicated periods to detect CHOP and SeP expression by Western blot analysis. Data are expressed as means ± SEMs from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ when compared to levels in the corresponding controls.$^{!!}P < 0.005$ when compared to levels in HepG2 cells treated with CGN.