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Induction of xanthine oxidase activity, endoplasmic reticulum stress and caspase activation by sodium metabisulfite in rat liver and their attenuation by Ghrelin

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ABSTRACT

Sodium metabisulfite is used as a preservative in many food preparations but can oxidize to sulfite radicals initiating molecular oxidation. Ghrelin is a peptide hormone primarily produced in the stomach and has anti-inflammatory and anti-oxidant effects on gastrointestinal and cardiovascular systems. This study was performed to elucidate the effect of ghrelin on sulfite-induced endoplasmic reticulum (ER) stress and caspase activation in rat peripheral organs. Xanthine oxidase (XO), xanthine dehydrogenase (XDH) enzyme activities, ER stress markers [phosphorylated PKR-like ER kinase (pPERK); C/EBP-homologous protein (CHOP)], caspase-3, -8, -9 activities, nuclear factor kappa-B (NF-κB) levels were determined in liver, heart and kidney of rats treated with sodium metabisulfite and/or ghrelin for 5 weeks. Sodium metabisulfite treatment significantly elevated XO activity, induced expression of GRP78, CHOP and increased caspase-3, -8 and -9 activities in liver but had no significant effect in heart and kidney. Ghrelin treatment decreased XO activity to baseline levels and attenuated ER stress and caspase activation in liver tissue of sodium metabisulfite treated rats. In conclusion, metabolism of sodium metabisulfite in liver tissue increased XO activity, induced ER stress and caused caspase activation which was attenuated by ghrelin treatment. Ghrelin's hepatoprotective effect could be through modulation of XO activity.

1. Introduction

Sodium metabisulfite ($Na_2S_2O_5$) is commonly used as preservative in foods, beverages and drugs (Gunnison and Jacobsen, 1987) to control enzymatic and non-enzymatic browning and for antimicrobial function (Taylor et al., 1986). When ingested, $Na_2S_2O_5$ reacts with water leading to the generation of bisulfite (HSO_3^-), sulfur dioxide (SO_2) and sulfite ($SO^2{}_3^-$) (Gunnison, 1981). Thus, $Na_2S_2O_5$ is termed as a "sulfating agent" because it releases SO_2. Ingested $Na_2S_2O_5$ is absorbed in the gastrointestinal tract and is distributed to all tissues via systemic circulation (Gunnison and Benton, 1971). Hepatic oxidation of exogenous sulfite is diffusion limited (Gunnison and Palmes, 1976), thus the liver metabolizes a constant fraction of sulfite it receives, but a limited amount will pass through the organ and enter the systemic circulation (Gunnison and Jacobsen, 1987). Studies have shown that sulfite oxidation can cause oxidative damage in organs such as liver and kidney (Elmas et al., 2005).

Ghrelin is an acylated peptide that stimulates the release of growth hormone (GH) from the anterior pituitary via binding to the GH secretagogue receptor (GHS-R) (Kukol, 2008). Circulating ghrelin is produced primarily in the stomach by X/A-like cells of the fundic glands, while the remainder originates in X/A-like cells of the small intestine (Date et al., 2000). Growth hormone secretagogue receptors are present in tissues other than the hypothalamus and pituitary, which indicates that ghrelin has other effects in addition to stimulating the release of growth hormone (Leite-Moreira and Soares, 2007). Indeed, besides the stimulation of GH release, ghrelin has also been described to have a potent orexigenic effect (Leite-Moreira and Soares, 2007), as well as a beneficial effect on gastrointestinal (El Eter et al., 2007), cardiovascular (Kui et al., 2009), reproductive (Kheradmand et al., 2009) and coagulation systems (Arıcı and Cetin, 2011). Recent studies have revealed that ghrelin may be an antioxidant and anti-inflammatory agent in many organs such as the

Abbreviations: ADI, acceptable daily intake; Apaf-1, apoptotic protease activating factor-1; ATF4, activating transcription factor 4; CHOP, C/EBP-homologous protein; eIF2α, eukaryotic initiation factor 2 alpha; ER, endoplasmic reticulum; G, ghrelin; GH, growth hormone; GHS-R, GH secretagogue receptor; H_2O_2, hydrogen peroxide; HSO_3^-, bisulfate; ip, intraperitoneally; $Na_2S_2O_5$, sodium metabisulfite; NF-κB, nuclear factor kappa-B; $O_2^{\bullet-}$, superoxide; pNA, p-nitroaniline; pPERK, phosphorylated PKR-like ER kinase; SO_2, sulfur dioxide; $SO^2{}_3^-$, sulfite; SOX, sulfite oxidase; UPR, unfolded protein response; WHO, World Health Organization; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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rat ovary (Kheradmand et al., 2010) and brain of rats (Obay et al., 2008).

Although previous studies have shown that $Na_2S_2O_5$ and its derivatives caused oxidative damage and apoptosis in rat organs such as liver (Bai and Meng, 2005b), lung (Bai and Meng, 2005a), brain (Kencebay et al., 2013) and stomach (Ercan et al., 2013), its effect on endoplasmic reticulum (ER) stress has not been investigated in rat peripheral organs such as liver, kidney and heart. Protein folding, maturation, trafficking, lipid synthesis and intracellular calcium homeostasis are some of the major functions of the ER (Schröder and Kaufman, 2005). Endoplasmic reticulum stress results in accumulation of unfolded proteins in the ER lumen leading to disturbed protein homeostasis. Cells activate an adaptive mechanism known as the unfolded protein response (UPR) to eliminate toxic protein components which relieves ER stress and restores protein homeostasis (Schröder and Kaufman, 2005). However, extended periods of ER stress results in failed UPR response and activates the apoptotic cascade (Schröder and Kaufman, 2005). Fundamental mechanisms resulting in the switch of UPR from a prosurvival to a proapoptic stimulus are still not clearly understood.

We have recently shown that ghrelin inhibits sodium metabisulfite induced oxidative stress and apoptosis in rat gastric mucosa (Ercan et al., 2013); however, the effect of ghrelin on $Na_2S_2O_5$ induced injury on liver, kidney and heart has not been clarified. Therefore, this study was designed to investigate whether $Na_2S_2O_5$ causes ER stress in liver, heart or kidney and whether ghrelin attenuates tissue injury induced by $Na_2S_2O_5$.

2. Materials and methods

2.1. Preparation of animals

All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Medical School. Male Wistar rats weighing 350–450 g were housed in stainless steel cages and given food and water ad libitum. Animals were maintained at 12 h light–dark cycles and a constant temperature of 23 ± 1 °C at all times. Rats were randomly divided into four experimental groups which included control (n = 8); rats treated with sodium metabisulfite ($Na_2S_2O_5$) (n = 10); rats treated with ghrelin (n = 10); rats treated with $Na_2S_2O_5$ + ghrelin (n = 10). Control group received 1 ml/kg/day distilled water via gavage and 1 ml/kg/day saline via intraperitoneal injection as vehicle for 35 days. Sodium metabisulfite treated animals were given freshly prepared $Na_2S_2O_5$ (100 mg/kg/day) solution via gastric gavage for 5 weeks as previously described (Derin et al., 2009). We have shown that $Na_2S_2O_5$ is efficiently absorbed at the given dose when administered via intragastric gavage and significantly increases plasma S-sulfonate levels (Kencebay et al., 2013; Ozturk et al., 2011). Rat Ghrelin (GenScript, NJ, USA) was dissolved in distilled water (1 mg/ml) and stored at –20 °C until the time of preparation for administration. Immediately before administration, ghrelin was diluted with 0.9% physiologic saline to a final concentration of 0.1 mg/ml. Ghrelin was given intraperitoneally (ip) at a dose of 20 μg/kg for 35 days as previously described (Ercan et al., 2013). We have previously shown that the given dose and duration of ghrelin has protective effects against oxidative tissue injury and apoptosis in rats (Ercan et al., 2013).

2.2. Xanthine oxidase/xanthine dehydrogenase (XO/XDH) enzyme activity measurements

All tissues were weighed and homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4). Homogenates were centrifuged (23,000 *g* for 40 min at 4 °C) and supernatants were stored at –80 °C. XDH and XO activity was determined with a fluorimetric assay as previously described (Dogan et al., 2012). This assay is based on the conversion of pterin to the fluorescent product isoxanthopterin and is performed with and without methylene blue to determine XDH and XO activity, respectively. One unit of enzyme activity is defined as 1 μmol isoxanthopterin formation per minute and was calculated on the basis of the increase in fluorescence with the addition of 0.1 μmol isoxanthopterin internal standard.

2.3. Western blot analysis

Tissues were homogenized in ice-cold homogenizing buffer (50 mM K_2HPO_4, 80 mM leupeptin; Sigma–Aldrich, Steinheim, Germany), 2.1 mM Pefabloc SC (SERVA, Heidelberg, Germany), 1 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich), 1 mg/ml aprotinin (SERVA; pH 7.4). Homogenates were centrifuged (23,000 *g*, 40 min, 4 °C) and supernatants were stored at –80 °C until analyzed. Proteins were denatured at 100 °C in sample buffer (BioRad Laboratories Inc. USA) and separated on 12% mini-protean TGX precast electrophoresis gels (BioRad Laboratories Inc. USA). Resolved proteins were transferred to nitrocellulose membranes and incubated with monoclonal or rabbit polyclonal primary antibodies to the proteins of interest. Primary antibody incubations were for 1 hour at room temperature with either anti-78 kDa glucose-regulated protein (GRP 78) (1:1000, # GTX62592 GeneTex, Irvine, CA, USA); anti-C/EBP-homologous protein (CHOP/GADD 153) (1:200, # sc-575, Santa Cruz Biotechnology Inc. USA); phospho-NF-κB p65 (Ser536) (1:1000, #3031 Cell Signaling Technology, Danvers, MA, USA) and anti-actin (1:1000, #AANO1, Cytoskeleton Inc. Denver, CO, USA). Horseradish peroxidase–conjugated goat anti-rabbit IgG (1:10,000 dilution; Zymed Laboratories, San Francisco, CA) was used as a secondary antibody, and immunoreactive proteins were visualized by chemiluminescence via ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). All Western blots were quantified by densitometric analysis using NIH ImageJ 1.44p software.

2.4. Caspase activity

Caspase-3, -8 and -9 activities in tissues were measured via colorimetric assay kits (EMD Millipore Corp. MA, USA). Tissues were homogenized in lysis buffer supplied with the kit and incubated on ice for 10 min. After centrifugation for 20 min at 10,000 *g*, supernatants were transferred to clean tubes and assayed for caspase activity based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrates. Absorbance values of known amounts of pNA standards were plotted at 405 nm. Free pNA in the samples were calculated from their corresponding absorbance values via the standard curve. One unit of enzyme activity is defined as the amount of enzyme that cleaved 1 nmol of the colorimetric substrate per hour at 37 °C.

2.5. Nuclear factor Kappa-B immunoassay

An enzyme linked-immuno-sorbent assay (ELISA) kit (Invitrogen cat#KHO0371, MD, USA) was used to measure NF-κB protein levels in tissue samples. Tissues were homogenized in cell extraction buffer (Invitrogen cat#FNN0011) and incubated on ice for 30 min. After centrifugation for 30 min at 10,000 *g*, supernatants were transferred to clean tubes and assayed for NF-κB. Absorbance values of known NF-κBp65 standards were plotted at 450 nm. NF-κB protein levels in the samples were calculated from their corresponding absorbance values via the standard curve.

2.6. Protein measurements

Protein concentrations were measured at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce Chemical).

2.7. Statistical analysis

Statistical analysis was performed using SigmaStat statistical software version 2.0. Statistical analysis for each measurement is described in the figure and table legends.

3. Results

3.1. XO and XDH enzyme activity

Biochemical results of XO and XDH activity in liver, heart and kidney tissues are given in Table 1. XO activity was significantly increased while XDH/XO ratio was significantly decreased in the liver of $Na_2S_2O_5$ treated rats when compared to control, ghrelin and $Na_2S_2O_5$ + ghrelin treated groups. Treatment of ghrelin significantly decreased liver XO levels and increased XDH/XO ratio when compared to $Na_2S_2O_5$ treated rats. No significant difference was observed in XO and XDH activities measured in the heart and kidney of all experimental groups.

3.2. Induction of ER stress markers

$Na_2S_2O_5$ treatment caused a significant increase in ER stress in the liver as shown by increased expression of glucose-regulated protein 78 (GRP78) and C/EBP-homologous protein (CHOP) when compared to control (C), ghrelin (G) and $Na_2S_2O_5$ + ghrelin treated groups (Fig. 1). Treatment with ghrelin significantly decreased the expression of the analyzed ER stress markers in the liver when compared to $Na_2S_2O_5$ treated rats. No significant difference was observed