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Ghrelin inhibits sodium metabisulfite induced oxidative stress and apoptosis in rat gastric mucosa

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ABSTRACT

This study aimed to investigate the effect of ghrelin administration on sulfite induced oxidative and apoptotic changes in rat gastric mucosa. Forty male albino Wistar rats were randomized into control (C), sodium metabisulfite ($Na_2S_2O_5$) treated (S), ghrelin treated (G) and, $Na_2S_2O_5$ + ghrelin treated (SG) groups. Sodium metabisulfite (100 mg/kg/day) was given by gastric gavage and, ghrelin (20 μg/kg/day) was given intraperitoneally for 5 weeks. Plasma-S-sulfonate level was increased in S and SG groups. $Na_2S_2O_5$ administration significantly elevated total oxidant status (TOS) levels while depleting total antioxidant status (TAS) levels in gastric mucosa. Ghrelin significantly decreased gastric TOS levels in the SG group compared with the S group. Additionally, TAS levels were found to be higher in SG group in reference to S group. $Na_2S_2O_5$ administration also markedly increased the number of apoptotic cells, cleaved caspase-3 and PAR expression (PARP activity indicator) and, decreased Ki67 expression (cell proliferation index) in gastric mucosal cells. Ghrelin treatment decreased the number apoptotic cells, cytochrome C release, PAR and, caspase-3 expressions while increasing Ki67 expression in gastric mucosa exposed to $Na_2S_2O_5$. In conclusion, we suggest that ghrelin treatment might ameliorate ingested-$Na_2S_2O_5$ induced gastric mucosal injury stemming from apoptosis and oxidative stress in rats.

1. Introduction

Humans are exposed to sulfites both exogenously and endogenously. Endogenous sulfites are generated as a consequence of the body's normal processing of sulfur containing amino acids (Cooper, 1983; Griffith, 1987). Exogenous sulfites, on the other hand, exist in a number of foods and beverages either naturally or as a product of fermentation (Lester, 1995). Five sulfite salts including sodium metabisulfite ($Na_2S_2O_5$), potassium metabisulfite ($K_2S_2O_5$), sodium bisulfite ($NaHSO_3$), potassium sulfite (K_2SO_3), and sodium sulfite (Na_2SO_3) are commonly used as additives in food, beverage and drug preparations due to their antioxidant and antimicrobial properties (Gunnison and Jacobsen, 1987; Lakamp and Dobesh, 2000; Rodriguez Vieytes et al., 1994). Although accumulating reports have indicated that sulfite compounds cause toxic and adverse effects on mammals, 30–100 mg sulfurdioxide (SO_2) is announced to have no observed adverse effects in humans by the Federation of American Societies for Experimental Biology (FASEB) (Aydin et al., 2005; Nair and Elmore, 2003). The acceptable daily intake of 0–0.7 mg/kg bw was allocated to sulfur dioxide and to sulfur dioxide equivalents (SDEs) arising from $Na_2S_2O_5$ (Til et al., 1972). With this in mind it is important to note that the mean per capita of sulfite intake from food and beverages is estimated as 19 mg SDE per day. This level is reported to be 163 mg/kg SDE in the 99th percentile of the population (Gunnison and Jacobsen, 1987).

Oxidative stress, which may cause irreversible damage in important cellular compartments, stems from the distortion of the equilibrium between production and scavenging of reactive oxygen species (Duygu et al., 2012). Recent studies have shown that SO_2 and its derivatives can cause oxidative stress through the process of sulfite oxidation and DNA damage in organs such as liver, brain, lung, spleen and stomach (Bai and Meng, 2005a,b; Gordon et al., 2004; Meng, 2003; Meng et al., 2004, 2003a,b). Addi-

Abbreviations: $Na_2S_2O_5$, sodium metabisulfite; $K_2S_2O_5$, potassium metabisulfite; $NaHSO_3$, sodium bisulfite; K_2SO_3, potassium sulfite; Na_2SO_3, sodium sulfite; SO_2, sulfurdioxide; SDE, sulfur dioxide equivalents; GHS-R, growth hormone secretagogue receptor; GPCR, G-protein-coupled receptors; TAS, total antioxidant status; TOS, total oxidant status; PARP, poly (ADP-riboz) polymerase; PBS, phosphate buffered saline; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–digoxigenin nick end-labeling; AI, apoptotic index; SO_3^{2}, sulfite; WHO, world health organization; $SO_3^{-\cdot}$, sulfite radical; SOD, superoxide dismutase; GP_x, glutathione peroxidase; CAT, catalase; GSH, glutathione.

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tionally, a variety of gastric lesions and apoptosis are shown to be induced by sodium metabisulfite in the literature (Beems et al., 1982; Ercan et al., 2010). Apoptosis is the programmed cell death which is essential for eliminating damaged or aged cells and maintain tissue integrity (Szabo and Tarnawski, 2000). The specific morphological changes associated with apoptosis are cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and the formation of apoptotic bodies (Bohm and Schild, 2003; Chandra et al., 2000). The majority of morphological and biochemical changes are the result of various enzymatic reactions most of which are catalyzed by caspases playing essential roles during apoptotic cell death (Bohm and Schild, 2003; Liu et al., 2012). The signaling cascades that control caspase-dependent apoptosis can be classified into two pathways: the extrinsic cell death (or death receptor) pathway and the intrinsic (or mitochondrial) death pathway. The mitochondrial death pathway can be induced by a large variety of signals and results in the release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c induces the formation of the apoptosome complex, that recruits and activates procaspase 9. Activated caspase 9 cleaves and activates caspase 3 which in turn activates downstream death events such as DNA single-strand breakage and poly (ADP-ribose) polymerase (PARP) cleavage (Zhao et al., 2012).

Ghrelin is a 28-amino acid acylated peptide esterified with octanoic acid on Ser 3 and is a ligand for the growth hormone secretagogue receptor (GHS-R) (Kui et al., 2009; Kukol, 2008). Circulating ghrelin is primarily produced in the stomach, but smaller amounts are also produced in the pituitary, hypothalamus, duodenum, jejunum, ileum, colon, lung, heart, pancreas, kidney and testis. The biological activity of ghrelin is mediated by G-protein-coupled receptors (GPCRs) (De Vriese and Delporte, 2008). Besides the stimulation of GH release, ghrelin affects several important biological functions including appetite, food intake and energy balance, cardiovascular function, reproduction, and bone growth (Asakawa et al., 2001; Pemberton and Richards, 2008; Wren et al., 2000). Several reports suggest that ghrelin inhibits apoptosis and oxidative damage in various tissues (Granado et al., 2009; Kui et al., 2009; Lau et al., 2009; Obay et al., 2008; Sehirli et al., 2008). More specifically, ghrelin has been shown to protect gastric mucosa against stress and ischemia-reperfusion induced gastric injury (Brzozowski et al., 2004; Konturek et al., 2006) to enhance gastric motility and gastric emptying (Trudel et al., 2002; Tumer et al., 2008). However, the effect of ghrelin on sodium metabisulfite induced gastric injury has not been clarified yet.

Hence, this study was designed to investigate whether ghrelin attenuates oxidative stress and apoptosis induced by $Na_2S_2O_5$ in gastric mucosa together with the mechanisms underlying gastric apoptosis.

2. Materials and methods

2.1. Preparation of animals

Forty healthy male albino Wistar rats, aged three months were used in this study. The animals were obtained from the Laboratory Animal Unit of Akdeniz University, and the study protocol was approved by the Animal Care and Usage Committee of Akdeniz University. The animals were fed standard laboratory diet and water ad libitum. They were housed at 23 ± 1 °C, and a 12:12-h light-dark cycle. Rats were randomly divided into four groups, each consisting of 10 animals: Group 1; control group (C) 2; rats treated with $Na_2S_2O_5$ (S), group 3; rats treated with ghrelin (G), group 4; rats treated with $Na_2S_2O_5$ + ghrelin (SG). 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. Animals in S and SG groups were given by gastric gavage (100 mg/kg/day) a freshly prepared solution of $Na_2S_2O_5$ for 5 weeks (Derin et al., 2009; Hui et al., 1989). Rat ghrelin 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 again with 0.9% physiologic saline to the final concentration of 0.1 mg/ml. Ghrelin was given intraperitoneally (ip) at a dose of 20 μg/kg for 35 days (Dornonville de la Cour et al., 2005; Konturek et al., 2006). The chosen dose of ghrelin has been shown to posses protective effects on various types of gastric injury (Brzozowski et al., 2004; Konturek et al., 2006).

Under urethane anesthesia the abdomens of the animals were opened by a midline incision. Heparinized blood was collected through the abdominal aorta for the determination of plasma-S-sulfonate levels. Additionally, stomachs were removed rapidly and opened at the lesser curvature. One part of the stomach tissue was immediately fixed in 10% neutral buffered formalin for immunohistochemical staining. Other parts of the tissue were used for total antioxidant status (TAS), total oxidant status (TOS) and cytochrome-c assays. Rats were sacrificed by exsanguination.

2.2. Plasma-S-sulfonate analysis

Plasma-S-sulfonate levels were measured by the method of Gunnison and Palmes (Gunnison and Palmes, 1973). One milliliter of plasma was mixed with 0.2 ml of a solution containing 0.027 mM NaOH and 0.125 mM KCN. The mixture was incubated at 35 ± 1 °C for 1 h. Following incubation, the mixture was cooled in ice and transferred to a cellulose dialysis bag and dialyzed at 4 °C against 5 ml of dialysate containing 10 mM glycine–NaOH buffers at pH 10.2 for 4 h. After dialysis, 200 μl of each reagent given below was added to 1.4 ml of dialysate in the following order. 0.15 M HCl, sodium tetrachloromercurate solution [0.18 M HgCl2 (Merck, Darmstadt, Germany) and 0.43 M NaCl (Sigma, Steinheim, Germany) in distilled water], distilled water, pararosaline hydrochloride (PRA) (SigmaAldrich, Steinheim, Germany) and formaldehyde solution [1:200 dilution of 37% formaldehyde (Merck, Germany) in distilled water]. The obtained solution (2.4 ml) was mixed for 20 min at room temperature and the absorbance of the sulfite-PRA-formaldehyde reaction product was measured spectrophotometrically at 560 nm (Shimadzu UV-1601, Kyoto, Japan). The sulfite level in the dialysate was calculated from a standard curve obtained by sulfite standards (27–206 μmol/ml) prepared by dissolving adequate amounts of $Na_2O_5S_2$ (Merck, Darmstadt, Germany), in distilled water. The results were expressed as μmol/ml.

2.3. Measurement of total oxidant status

Total oxidant status was measured by a commercially available TOS assay kit (Cat. #RL0024,Rel Assay Diagnostics, Gaziantep, Turkey). The assay is calibrated with hydrogen peroxide and the results are expressed as mmol /g protein.

2.4. Measurement of total antioxidant status

Total antioxidant status was measured by a commercially available TAS assay kit (Cat. #RL0017,Rel Assay Diagnostics, Gaziantep, Turkey). The assay is calibrated with a stable antioxidant standard solution. The results are expressed as nmol/g protein.

2.5. Determination of proteins

Protein concentrations in all samples were measured spectrophotometrically (Shimadzu RF-5500, Kyoto, Japan) by a protein assay reagent kit (Pierce, Rockford, IL) via a modified Bradford method (Bradford, 1976). Bovine serum albumin was used as internal standard.

2.6. Measurement of cytochrome C release

Cytochrome C released into the cytosol was measured using an enzyme linked immunosorbent assay-based rat cytochrome C assay kit (R&D Systems, Minneapolis, MN, USA). Briefly, 5 g of gastric mucosal tissue was placed in 100 ml ice-cold phosphate-buffered saline, pH 7.4, supplemented with protease inhibitor mixture in a ratio of 100 μl/ml of phosphate-buffered saline. Tissue samples were homogenized and centrifuged at 200 g for 10 min at 4 °C. The supernatants were further centrifuged at 105.000 g for 60 min at 4 °C to get the clear cytosolic supernatant (Maity et al., 2008). The supernatants were used for the measurement of cytochrome C release convenient to the protocol of the kit.

2.7. Immunohistochemical staining

Stomach tissue samples were immediately fixed in 10% neutral buffered formalin. The samples were then embedded in paraffin and sectioned. Sections were deparaffinized and blocked for endogenous peroxidase activity with methanol containing 3% H_2O_2 for 10 min and for nonspecific binding with Ultra V Block (Labvision, Freemont, CA) for 7 min at room temperature. Mouse monoclonal PAR (anti-PAR Mab (10H), Alexis, San Diego, CA), rabbit monoclonal cleaved caspase-3 (Cell Signaling, US), rabbit monoclonal Ki67 (Abcam) primary antibodies were applied in a dilution of 1:500 and incubated overnight at +4 °C in a humidified chamber. The sections were washed in phosphate-buffered saline (PBS), incubated with biotinylated anti-mouse IgG and anti-rabbit IgG (3 mg/mL; Vector, Burlingame, CA) at 1:500 dilution for 1 h at room temperature. Antibodies were detected using a Vectastain Avidin Biotin Complex (Vector) for 30 min at room temperature and anti-