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Regulation of oxidative stress and somatostatin, cholecystokinin, apelin gene expressions by ghrelin in stomach of newborn diabetic rats

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ABSTRACT

The aim of the study was to determine whether ghrelin treatment has a protective effect on gene expression and biochemical changes in the stomach of newborn streptozotocin (STZ) induced diabetic rats. In this study, four groups of Wistar rats were used: control, ghrelin control, diabetic and diabetic + ghrelin. The rats were sacrificed after four weeks of treatment for diabetes. The gene expressions of: somatostatin, cholecystokinin, apelin and the altered active caspase-3, active caspase-8, proliferating cell nuclear antigen, were investigated in the pyloric region of the stomach and antioxidant parameters were measured in all the stomach. Although ghrelin treatment to diabetic rats lowered the stomach lipid peroxidation levels, the stomach glutathione levels were increased. Exogenous ghrelin caused an increased activities of stomach catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase in diabetic rats. Numbers of somatostatin, cholecystokinin and proliferating cell nuclear antigen immunoreactive cells decreased in the diabetic + ghrelin group compared to the diabetic group. Apelin mRNA expressions were remarkably less in the diabetic + ghrelin rats than in diabetic rats. The results may indicate that ghrelin treatment has a protective effect to some extent on the diabetic rats. This protection is possibly accomplished through the antioxidant activity of ghrelin observed in type 2 diabetes. Consequently exogenous ghrelin may be a candidate for therapeutic treatment of diabetes.

Introduction

Diabetes mellitus (DM) is a syndrome resulting from a variable interaction of enviromental and hereditary factors, and characterized by abnormal insulin secretion. DM causes elevated blood glucose levels and a variety of organ complications. Type 2 diabetes is shown increasingly as a disease of insulin defects frequently among DM types (Masiello, 2006).

Gastrointestinal (GI) functions are regulated by growth factors and nutrition (Vallejo-Cremades et al., 2004). Ghrelin is the endogenous ligand of the growth hormone secretagogue receptor that plays a role in regulating food intake and energy homeostasis and stimulates growth hormone release (Murdolo et al., 2003). Exogenous ghrelin causes the regeneration of GI mucosa and has gastroprotective effects (Fahim et al., 2011). The ghrelin hormone in the GI tract and hypothalamus is secreted predominantly in the stomach (Kamegai et al., 2004; Korbonits et al., 2004). Ghrelin plays an important role in insulin and glucose metabolisms (Delhanty and van der Lely, 2011). Turk et al. (2012) suggested that administration of ghrelin increases insulin and inhibits diabetic effects in type 2 diabetic newborn rats. The gastric total ghrelin-levels are decreased in rats with streptozotocin (STZ)-induced diabetes (Masaoka et al., 2003). According to Shimada et al. (2003) ghrelin secretion from the stomach is regulated by gastric somatostatin (SS). SS plays a role in glucose homeostasis and regulatory functions on GI physiology and nutrient intake (Li et al., 2010). Apelin (AP) is an important stomach peptide that has a physiological role in the GI tract (Wang et al., 2004). Cholecystokinin (CCK) regulates gastric acid secretion, gallbladder emptying, and cell growth in the pancreas and stomach (Rehfeld, 2011). It is known that AP stimulates CCK secretion from a murine enteroendocrine cell line (Wang et al., 2004). On the other

Abbreviations: AEC, 3-amino-9-ethyl-carbazole; AP, apelin; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; BSA, bovine serum albumin; CAT, catalase; CCK, cholecystokinin; DM, Diabetes mellitus; GI, gastrointestinal; GSH, glutathione; GP_x, glutathione peroxidase; GR, glutathione reductase; i.p., intraperitoneally; LPO, lipid peroxidation; NBT, 4-nitro blue tetrazolium chloride; PC12, pheochromocytoma; PCNA, proliferating cell nuclear antigen; PBS, phosphate buffered saline; ROS, reactive oxygen; s.c., subcutaneously; SS, somatostatin; SSC, saline sodium citrate; SOD, superoxide dismutase; STZ, streptozotocin.

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hand, CCK is inhibited by SS in the STZ-induced rat pancreas (Park et al., 2002).

Oxidative stress is a major cause of GI damage and diabetes is associated with an increased production of reactive oxygen species (ROS) (Bhor et al., 2004; Suzuki et al., 2011). Ghrelin has antioxidant effects on systemic oxidative stress and shows gastroprotective effects (Suzuki et al., 2011). The antioxidant activity of ghrelin has been observed *in vivo* and *in vitro* (El Eter et al., 2007). According to Yang et al. (2007), ghrelin has antiapoptotic effects that may inhibit apoptosis signal-regulating kinase 1 mediated apoptosis of rat pheochromocytoma (PC12) cells. Caspase-3 and caspase-8 are known to have a role in the death signal in the apoptotic pathway (Elmore, 2007). Proliferating cell nuclear antigen (PCNA) is an important protein in DNA replication. Cox (1997) reported that PCNA has some roles in DNA repair and possibly cell-cycle control.

In the present study, we aimed to investigate the effects of ghrelin treatment on SS, CCK, AP gene expression and immunoreactivities of PCNA, active caspase-3, active caspase-8 in the pyloric region of the stomach. In addition, biochemical changes were investigated in stomach tissue in STZ-diabetic rats and controls.

Materials and methods

Animals

Wistar type albino newborn rats were obtained from the Institute of Experimental Medicine (Istanbul, Turkey). They were maintained on a 12/12 h light–dark cycle under controlled temperature and humidity. The animals were fed standard rat chow and given water *ad libitum*. All protocols used in this study were approved by the Istanbul University Local Ethics Committee on animal research.

Experimental design

The male and female newborn rats were divided into four groups following their birth. (I) Control group ($n = 13$) received a single dose of saline given intraperitoneally (i.p.) for four weeks. (II) Ghrelin group ($n = 13$) were injected subcutaneously (s.c.) ghrelin (AnaSpec, Fremont, CA, USA; cat no: 24160) 100 μg/kg/day dissolved in saline beginning from the third day for four weeks. (III) Diabetic group ($n = 9$) were administered i.p. a single dose of 100 mg/kg STZ (Sigma–Aldrich, St. Louis, MO, USA; cat no: S0130) to the newborn rats on the second day after the birth. The blood glucose levels were determined 48 h after STZ injection. The animals with blood glucose levels greater than 200 mg/dL were accepted for inclusion as diabetic. (IV) Diabetic + ghrelin group ($n = 7$) was given s.c. ghrelin (100 μg/kg/day) to diabetic animals for four weeks.

Tissue preparation

At the end of four weeks, the stomach tissues were obtained from animals sacrificed under ether anesthesia after an overnight fast. Tissue samples were immediately washed with saline and frozen until needed for study. The pyloric tissues were fixed in 10% neutral formalin for immunohistochemistry and *in situ* hybridization studies. Frozen stomach tissues were stored at −80 °C for biochemical studies.

Biochemical assay

In this study, biochemical investigations were made on the stomach tissue from all groups. Stomach tissues were homogenized in cold 0.9% NaCl with glass equipment and made up to 10% homogenate. The homogenates were centrifuged, the clear supernatants were used for protein, lipid peroxidation (LPO) and glutathione (GSH) levels and antioxidant enzyme analysis. GSH levels were determined according to Beutler's method using Ellman's reagent (Beutler, 1975). LPO levels in stomach homogenates were estimated by Ledwozyw's method (Ledwozyw et al., 1986). Catalase (CAT) activity was assayed in stomach tissues by the method of Aebi (Aebi, 1984). Superoxide dismutase (SOD) activity was assayed by Mylroie's method (Mylorie et al., 1986). Glutathione reductase (GR) activity was determined by Beutler's method (Beutler, 1971). Glutathione peroxidase (GP_x) activity was determined with Paglia and Valentine's method modified by Wendel (1981). The protein contents in the supernatants were estimated by the method of Lowry using bovine serum albumin (BSA) as standard (Lowry et al., 1951).

Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick paraffin sections. They were mounted on poly-L-lysine-coated glass slides. For antigen retrieval, slides were kept in citrate buffer (10 mM) in microwave oven for CCK, AP, active caspase-3 and active caspase-8 antibodies, however citrate buffer was not used for SS and PCNA antibodies. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Histostain Plus Broad Spectrum Kit for streptavidin–biotin–peroxidase technique (Zymed, San Francisco, CA, USA cat no: 85-9043) was performed. After rinsing with 0.01% Triton-X-100 enriched 50 mM Tris, sections were covered with blocking serum to block non-specific binding sites. They were incubated with CCK antibody (Novacastra, Newcastle-on-Tyne, UK; cat no: 404605) overnight at 4 °C with 1:250 dilution, SS antibody (Zymed, Camarillo, CA, USA; cat no: 18-0078) for 60 min at room temperature with 1:100 dilution, AP (Phoenix, Burlingame, CA, USA; cat no: H-057-28) for 1 h at room temperature with 1:300 dilution, caspase-3 (Millipore, USA; cat no: AB3623) overnight at 4 °C with 1:50 dilution, caspase-8 (Lab vision, Fremont, CA, USA; cat no: RB-1200-PABX) overnight at 4 °C with 1:25 dilution and PCNA antibody (Neomarkers, Fremont, CA, USA; cat no: MS-106-P) for 30 min at room temperature with 1:50 dilution. After that, the biotinylated secondary antibody and the streptavidin-peroxidase conjugate were applied respectively. The enzyme activity was developed using 3-amino-9-ethyl-carbazole (AEC) and then the sections were counterstained with Mayer's hematoxylin. Finally slides were mounted on glycerol gelatine and stored 4 °C. Negative control sections were prepared by substituting all antibodies with phosphate buffer saline (PBS). Negative controls had no signal.

In situ hybridization

Five to seven micrometer thick sections were cut from the paraffin block. Deparaffinized and hydrated sections were treated with 0.02% pepsin for 15 min at 37 °C, postfixed in 4% paraformaldehyde for 30 min, washed three times for 5 min in 0.2% glycine. After treatment with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, the sections were incubated in hybridization buffer for 90 min at 37 °C. Hybridization was carried out in a humidified chamber at 37 °C overnight, using digoxigenin labeled probes concentrations of 1200 ng/ml for CCK and SS, 1700 ng/ml for AP (Table 1) followed by post-hybridization washing with 1 × SSC, 1.5 × SSC, 2 × SSC and 0.2 × SSC at 37 and 55 °C. After washing in blocking solution (Tris–HCl, NaCl and BSA) for 1 h, the sections were incubated with 1:1000 anti-digoxigenin antibody (Roche, Mannheim, Germany cat no: 1 093 274) for 16–18 h at 4 °C then detected using the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/4-nitro blue tetrazolium chloride (NBT) substrate system (BCIP: Roche, Mannheim, Germany cat no: 10 760 994 001, NBT: Roche, Mannheim, Germany cat no: 11 087 479 001) for 11–14 h in 37 °C and mounted in glycerol gelatine, stored at 4 °C. As negative