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Obestatin and insulin in pancreas of newborn diabetic rats treated with exogenous ghrelin

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ABSTRACT

The aim of the study was to evaluate the effect of ghrelin treatment on obestatin, insulin gene expression and biochemical parameters in the pancreas of newborn-streptozocin (STZ) diabetic rats. Rats were divided into 4 groups. Group I: control rats treated with physiological saline; group II: control rats treated with 100 μg/kg/day ghrelin; group III: two days after birth rats that received 100 mg/kg STZ injected as a single dose to induce neonatal diabetes; group IV: neonatal-STZ-diabetic rats treated with ghrelin for four weeks. Sections of the pancreas were examined with immunohistochemistry for the expression of obestatin and insulin and *in situ* hybridization for the expression of insulin mRNA. The blood glucose levels were measured. Tissue homogenates were used for protein, glutathione, lipid peroxidation and non-enzymatic glycosylation levels and antioxidant enzyme analysis. There was a significant difference in blood glucose levels in newborn-STZ-diabetic rats compared to ghrelin treated diabetic rats at weeks 1, 2 and 4. In group IV, pancreatic non-enzymatic glycosylation and lipid peroxidation levels were decreased, however, glutathione levels and enzymatic activities were increased. Insulin peptide and mRNA (+) signals in islets of Langerhans and obestatin immunopositive cell numbers showed an increase in group IV compared to group III. These results suggest that administration of ghrelin to newborn rats may prevent effects of diabetes.

Introduction

Diabetes mellitus is a systemic, chronic metabolic disease characterized by hyperglycemia, dyslipidemia, glucosuria and various concomitant clinical and biochemical symptoms, as well as a high risk of early death. Type 2 diabetes is characterized by a progressive worsening in secretion functions of the produced insulin and the development of peripheral resistance to insulin, rather than a deficit in insulin production (Dickson and Rhodes, 2004). Diabetes models generated by streptozotocin (STZ) are widely used in investigating the types of the disease (Portha et al., 1974; Ferrand et al., 1995; Ozturk et al., 2006). In models where STZ is administered to newborn animals, hyperglycemia occurs and type 2 diabetes with disturbed glucose tolerance is observed (Weir et al., 1981; Takada et al., 2007).

Ghrelin is a 28 amino acid endogenous ligand for the growth hormone secretagogue receptor. Ghrelin is synthesized as the 117 amino acid long preproghrelin (Kojima et al., 1999). Ghrelin has been investigated in various tissues such as pancreas and stomach (Lee et al., 2002; Bolkent et al., 2006; Yildirim et al., 2007; Walia et al., 2009). Hayashida et al. (2002) have shown that ghrelin plays an important role in the development of newborn rats. It has been reported that ghrelin levels in the circulation decrease in patients with type 2 diabetes (Pöykkö et al., 2003; Katsuki et al., 2004). Qader et al. (2008) reported that high levels of acyl ghrelin induce insulin release in a dose-dependent manner, whereas low levels suppress the release.

Obestatin is a 23 amino acid peptide that is derived from the posttranslational processing of proghrelin, which was discovered in rat stomach (Zhang et al., 2005). Dun et al. (2006) detected the immunoreactivity of obestatin in the stomach and testicle of rats. Obestatin immunoreactivity has also been reported in human pancreatic cell cultures (Granata et al., 2008). Plasma obestatin levels have been shown to be decreased in patients with type 2 diabetes (Qi et al., 2007), however, the localization and immunoreactivity of the obestatin peptide in diabetes types has not yet been investigated. A positive relationship between obestatin and ghrelin levels has been reported in postnatal pancreas (Chanoine et al., 2006). It has also been reported that obestatin administration together with

Abbreviations: CAT, catalase; GHR, ghrelin; GSH, glutathione; GP_x, glutathione peroxidase; H&E, hematoxylin & eosin; LPO, lipid peroxidation; MDA, malondialdehyde; n2-STZ, newborn streptozotocin; NEG, non-enzymatic glycosylation; ROS, reactive oxygen species; SSC, saline-sodium citrate; STZ, streptozotocin; SOD, superoxide dismutase.

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glucose suppresses insulin release in rats under *in vivo* and *in vitro* conditions (Ren et al., 2008). High dose ghrelin administration for a period of one week is suggested to increase insulin expression in newborn STZ-diabetic rats (Irako et al., 2006). Ferzli et al. (2009) reported that plasma levels of active ghrelin are decreased in patients with type 2 diabetes.

Exogenous ghrelin administration may provide a new treatment model to ameliorate the effects of diabetes. However, the effects of ghrelin treatment have still not been fully clarified. Therefore, in our study we aimed to investigate obestatin and insulin gene expression and biochemical parameters in newborn normal and diabetic rat pancreas following exogenous ghrelin for a period of four weeks.

Materials and methods

Animals and tissue preparation

All animal experimental procedures were approved by the Istanbul University Local Ethics committee on animal research. When the newborn Wistar albino rats were 48 h old following birth, they were included in the experimental groups. There were four groups of neonatal rats. Group I ($n = 13$): the control group (C) consisting of newborn rats given physiological saline intraperitoneally (i.p.) for a period of four weeks. Group II ($n = 13$): the control group (GHR) was treated subcutaneously with 100 μg/kg/day ghrelin (AnaSpec, Fremont, CA, USA, cat no: 24160) dissolved in physiological saline from the third day for a period of four weeks. Group III ($n = 9$): newborn streptozotocin (n2-STZ) diabetic group; 100 mg/kg STZ (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in physiological saline and given as a single intraperitoneal dose on the second day following birth. The rats whose blood glucose levels were 200 mg/dl or more on the second day following STZ injection were considered as diabetic. Group IV ($n = 7$): the n2-STZ-diabetic group which given ghrelin (n2-STZ + GHR) on the second day following birth for a period of four weeks.

Blood glucose levels of the animals in all groups were measured with a glucometer (Accu-check, Roche Diagnostics GmbH, Mannheim, Germany) using the blood obtained from the tail vein on day-0 and at weeks 1, 2, 3 and 4. Body weights of the animals were also measured at the same time intervals. The second day, when the animals in the groups n2-STZ-diabetic and n2-STZ + GHR were defined as diabetic, was determined as day-0 of the experiment.

At the end of four weeks the animals were left for fasting overnight and pancreatic tissue samples were collected under ether anesthesia. The tissue samples were fixed in 10% neutral buffered formalin for 16 h at +4 °C and then embedded in paraffin using routine light microscopy processing methods.

Biochemical assay

In this study, biochemical investigations were made on the pancreatic tissue from all groups. For biochemical analysis, pancreatic tissue samples were washed with physiological saline and kept frozen until the day of analysis. Pancreatic 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), glutathione (GSH), non-enzymatic glycosylation (NEG) levels and antioxidant enzyme analysis. GSH levels were determined according to Beutler's method using Ellman's reagent (Beutler, 1975). LPO levels in pancreas homogenates were estimated by Ledwozyw's method (Ledwozyw et al., 1986). NEG levels were determined by thiobarbituric acid method (Parker et al., 1981). Catalase (CAT) activity was assayed in pancreatic tissues by the method of Aebi (1984). Superoxide dismutase (SOD) activity was assayed by Mylroie's method (Mylroie et al., 1986). Glutathione peroxidase (GP_x) activity was determined by the method described by Paglia and Valentine and modified by Wendel (1981). The protein content in the supernatants was estimated by the method of Lowry using bovine serum albumin as standard (Lowry et al., 1951).

Histopathology

For histological examination, 5 μm sections were dewaxed in toluene and rehydrated through a series of descending alcohols. Hematoxylin and eosin staining (H&E) was applied to pancreas sections of each animal in all groups.

Islet size measurement

Photographs of the pancreatic islets were taken using a Leica DM 2500 light microscope fitted with a Leica DFC280 digital camera system. Photographs of islets of Langerhans in pancreatic tissue sections for all groups were taken using a ×20 microscope objective. Automatic image analysis for calculation of areas of islets of Langerhans were performed with Leica IM50 (version 4.0) morphometric analysis software. Islets of Langerhans were classified as small, medium and large according to their size after calculating the areas. Large islets were >10,000 μm^2, medium islets were >5000–10,000 μm^2, and small islets were <5000 μm^2 (Thyssen et al., 2006).

Immunohistochemistry

For immunohistochemical staining, the sections were incubated with mouse monoclonal insulin (dilution 1:1250; Sigma–Aldrich, St. Louis, MO, USA) and rabbit obestatin (dilution 1:1000; Phoenix Pharmaceuticals, Belmont, CA, USA) antibodies and were stained with the streptavidin–biotin–peroxidase method. Histostain Plus Bulk staining kit was used (Zymed Lab., S. San Francisco, CA, USA). Detection procedures were carried out as described by the manufacturer. The enzyme activity was developed using 3-amino-9-ethyl-carbazole (AEC) substrate kit (Zymed) and then the sections were counterstained with Mayer's hematoxylin. Staining intensity was scored from one to three as weak (+), medium (++) and strong (+++). Negative control sections were prepared by substituting antibodies with phosphate-buffer saline (PBS). Rat stomach sections were used as obestatin positive controls because of their high obestatin expression.

In situ hybridization

Tissue sections were deparaffinized, hydrated and permeabilized with 0.02% pepsin for 30 min at 37 °C. The sections were hybridized with digoxigenin-labelled rat insulin probe cocktail including 180 mer. *In situ* hybridization was performed in hybridization buffer (50% deionized formamide, 10× SSC, 50× Denhart's solution, 10 mg/ml Salmon testes DNA, dextran sulfate) for 18 h at 45 °C. The final concentration of the probe was 900 ng/ml. After hybridization the sections were treated with 1× SSC (3× 30 min, 55 °C). The detection of the digoxigenin labelled hybrid was incubated with alkaline phosphatase conjugated anti-digoxigenin antibody (Roche, Mannheim, Germany). Hybridization signals were revealed by nitroblue–tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Negative control sections were treated with hybridization buffer without the probe.