

Ghrelin and insulin gene expression changes in streptozotocin-induced diabetic rats after rosiglitazone pretreatment

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The aim of the study was to evaluate the effect of rosiglitazone treatment on islet ghrelin and insulin gene expressions in streptozotocin (STZ)-induced diabetic rats. Animals were divided into four groups. 1. Intact controls. 2. Rosiglitazone-treated controls. 3. STZ-induced diabetes. 4. Rosiglitazone-treated diabetes. Rosiglitazone was given for 7 days at a dose of 20 mg/kg body weight. Ghrelin and insulin gene expressions were investigated by immunohistochemistry and *in situ* hybridization. There was no statistically significant difference in body weight between STZ-induced diabetic rats and rosiglitazone-treated diabetic rats during the experimental period. Furthermore, there were no significant differences in blood glucose levels and insulin immunoreactive cell numbers between STZ-induced diabetic rats and rosiglitazone-treated diabetic rats. There was a tendency towards a reduction of ghrelin gene expression in diabetic animals compared with intact controls. We found, in addition, that ghrelin immunoreactive and ghrelin mRNA expressing cells were frequent in the epithelial lining of the ducts suggesting ductal epithelium might be the source of the regenerating islet ghrelin cells, as is known for other islet cells. The results show that short-term rosiglitazone pretreatment had no significant effect on ghrelin and insulin gene expressions.

Key words: ghrelin, rosiglitazone, immunohistochemistry, *in situ* hybridization.

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Diabetes mellitus is an increasing worldwide health problem. Type 1 diabetes is characterized by an immune-mediated destruction of the beta cells, leading to clinical diabetes when 80-90% of the beta cells have been lost (Skak *et al.* 2004). STZ is an antibiotic that induces diabetes by damaging insulin-secreting cells in the pancreas, and rats with STZ-induced diabetes exhibit reduced body weight, hyperglycemia, and hypoinsulinemia (Masaoka *et al.* 2003). PPAR- γ is a member of the nuclear hormone receptor superfamily (Cuzzocrea *et al.* 2004). Thiazolidinediones (TDZs) including troglitazone, pioglitazone, and rosiglitazone, are PPAR- γ agonists, originally designed for ameliorating insulin resistance, and representing a new class of therapeutic agents for diabetes mellitus (Augstein *et al.* 2003). It was shown that TDZs reduce T-cell-mediated inflammatory processes associated with autoimmune beta cell destruction, thereby preventing and delaying the onset of type 1 diabetes (Augstein *et al.* 2003; Ogawa *et al.* 1999; Takamura *et al.* 1999; Yue *et al.* 2001). Rosiglitazone is one of the PPAR- γ agonists and the most potent member of the thiazolidinedione antidiabetic agents (Yue *et al.* 2001). It was reported that rosiglitazone increased body weight, ameliorated hyperglycemia, and improved the intensity of insulin immunostaining in beta cells (Brand *et al.* 2002). Rosiglitazone treatment did not affect islet hypertrophy at early stages but prevented beta cell atrophy at later stages in diabetic rats (Koh *et al.* 2003). There are some reports indicating that rosiglitazone does not affect insulin release, total insulin content, and insulin mRNA expression (Lupi *et al.* 2004); although other studies have reported a dose-dependent effect of rosiglitazone on insulin release from rat pancreatic islets (Yang *et al.* 2001).

Ghrelin is a 28-amino acid peptide identified in the rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (Kojima *et al.* 1999). It stimulates growth hormone secretion,

food intake, and body weight gain (Lupi *et al.* 2004; Masaoka *et al.* 2003). Ghrelin plays a role in gastric motility, and ghrelin dynamics change depending on gastric disease condition (Abiko *et al.* 2005; Fukuhara *et al.* 2005; Masaoka *et al.* 2005; Mori *et al.* 2006; Nishizawa *et al.* 2006; Suzuki *et al.* 2004a, Suzuki *et al.* 2004b, Suzuki *et al.*, 2006). However, studies about the effect of ghrelin on insulin secretion have been controversial in animal models, and both stimulatory (Adeghate and Ponery 2002; Date *et al.* 2002) and inhibitory (Broglia *et al.* 2001; Egido *et al.* 2002; Reimer *et al.* 2003; Wierup *et al.* 2004) effects have been reported. Moreover, data on the cells expressing ghrelin in pancreas have been inconsistent. It was reported that alpha cells (Date *et al.* 2002) and beta cells (Volante *et al.* 2002) co-express ghrelin while another research group suggests that ghrelin is expressed in a novel endocrine cell type in pancreas (Prado *et al.* 2004; Wierup *et al.* 2002; Wierup *et al.* 2004). The aim of the study was to investigate islet ghrelin and insulin gene expressions in diabetic and rosiglitazone-treated diabetic rats.

Materials and Methods

Animals and tissue preparation

Male Wistar rats, weighing 180-200 g. were used in all experiments and were given standard laboratory chow and water *ad libitum*. The experiments were reviewed and approved by the Institute's Animal Care and Use Committee of the University of Istanbul. Animals were divided into four groups. The first group was the intact control group (n=8). The second group was rosiglitazone-treated control rats (n=7). Rosiglitazone (GlaxoSmithKline, Turkey) was given in distilled water for 7 days at a dose of 20 mg/kg body weight using oral needle gavage. The third group was STZ-induced diabetic animals (n=7). Diabetes was induced by single dose 65 mg/kg streptozotocin (STZ) injection given intraperitoneally. The fourth group was rosiglitazone-treated diabetic rats (n=5). They were pre-treated with rosiglitazone for 7 days before STZ-induced diabetes. Blood glucose levels were measured on day 0, 7, and 14 of the experiment by glucometer (Accu-check, Roche). In addition, all the rats were weighed on the 0, 7, and 14 day of the experiment period. After the STZ injection, when the rats were determined to be diabetic, day two, was set as day 0 of the experiment. Diabetic ani-

mals were selected according to their high blood glucose levels exceeding 250 mg/dL.

All the pancreas was dissected out under the ether anesthesia on day 16 of the experiment and fixed in 4% paraformaldehyde. Paraffin tissue sections (5 µm thickness) were cut and thaw-mounted on slides.

Immunohistochemistry

After deparaffinization, antigen retrieval was applied to the sections in citrate buffer, pH 6.0 twice for 7 min in microwave oven. Then, they were rinsed in PBS with Triton X-100 for 15 min. A rabbit ghrelin antibody (1:640) (Phoenix, Belmont, CA; H-031-30) and a guinea-pig insulin antibody (1:1200) (EuroDiagnostica, Malmö, Sweden; B-GP 39-1) were diluted in PBS, pH 7.2, containing 0.25% bovine serum albumin and 0.25% Triton X-100. Sections were incubated with diluted primary antibodies in moisturized chambers overnight at 4°C, followed by rinsing in PBS with Triton X-100 for 15 min. Thereafter secondary antibodies with specificity for rabbit and guinea-pig coupled to either Texas Red (711-075-152, Jackson Immunoresearch, West Grove, PA), or fluorescein isothiocyanate (F-6261, Sigma, St Louis, MO) were applied on the sections. Sections were rinsed in PBS with Triton X-100 for 15 min and then mounted in PBS:glycerol 1:1.

In situ hybridization

The ghrelin probe was complementary to the sequence 172-201 of rat ghrelin cDNA (NM 021669) (Wierup *et al.* 2004). The probe was 3'-end tailed by [³⁵S]-dATP (NEN; Stockholm, Sweden) (Mulder *et al.* 1993). Sections were deparaffinized. Then they were rinsed in PBS with 0.01% Triton X-100 for 2 min. Proteinase K was applied on the sections at 37°C for 30 min. Thereafter fixed in 4% paraformaldehyde for 15 min, washed for 5 min in PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Hybridization was carried out at 37°C overnight, using probe concentration of approximately 1 pmol/mL for ghrelin. The slides were dipped in NTB 2 emulsion (Eastman Kodak; Rochester, NY) and stored in light-sealed boxes at 4°C for 15 days. Then, they were developed in Kodak D-19, fixed in Kodak Polymax, and mounted in glycerol gelatin.

Specificity tests

In immunohistochemistry, rat stomach tissue slides were used as positive control slides for ghrelin and pancreas tissue slides were used as positive control for insulin staining. In negative control sections, primary antibodies were either omitted, or used after preabsorption with antigen in excess (100 microgram peptide per mL diluted anti-serum). In these tests immunoreactive cells were not seen. Stomach tissue slides were also used as positive control for ghrelin *in situ* hybridization. As negative controls for *in situ* hybridization, 100-fold excess unlabeled probe was used (Wierup *et al.* 2002).

Table 1. Body weight (g).

Group	0. Day	7. Day	14. Day
Intact control	199.75	218.25	207.87
	± 11.94	± 9.13*	± 14.70*
Rosiglitazone-treated control	203.57	218.00	197.28
	± 25.06	± 31.12	± 30.98**
STZ-induced diabetic	178.85	180.71	162.14
	± 21.19	± 24.19	± 20.68
Rosiglitazone-treated diabetic	212.00	184.40	137.20
	± 28.79	± 33.26	± 19.85***

Values are mean±SD; * $p<0.05$ of intact control group versus STZ-induced diabetic group; ** $p\leq 0.01$ of rosiglitazone-treated control group versus rosiglitazone-treated diabetic group; *** $p<0.001$ of rosiglitazone-treated diabetic group versus intact control group.

Table 2. Blood glucose levels (mg/dL).

Group	0. Day	7. Day	14. Day
Intact control	92.50	95.37	95.37
	± 11.25	± 6.11	± 9.70
Rosiglitazone-treated control	116.85	120.00	117.71
	± 7.40	± 14.17	± 15.86
STZ-induced diabetic	430.57	458.71	461.42
	± 69.86	± 67.49	± 61.59
Rosiglitazone-treated diabetic	380.20	421.80	435.40
	± 65.03	± 57.26	± 77.46

Values are mean±SD.

Statistical analysis

Ghrelin and insulin immunoreactive cells and ghrelin mRNA(+) cells were counted in all the islets in one section of each animal. The values were expressed as mean number of cells per islet and given as mean±SD. Statistical analysis was carried out using an ANOVA followed by Kruskal Wallis' *post hoc test* and Bonferroni's *post hoc test*. $p<0.05$ was considered significant.

Results

Body weight

In the intact and rosiglitazone-treated control group, there were significant differences in body weights between day 0-7 and 7-14 of the experiment period ($p<0.05$). On the other hand, there were no statistically significant differences in the body weight of STZ-induced diabetic group during the experiment time ($p>0.05$). In the rosiglitazone-treated diabetic group, there were significant differences in body weights at day 0., 7., and 14. of the experimental period ($p<0.05$) (Table 1).

Blood glucose levels

Blood glucose levels were higher in rosiglitazone-treated control group than intact control group ($p<0.01$). Blood glucose levels decreased insignificantly in rosiglitazone-treated diabetic group compared to STZ-induced diabetic rats ($p>0.05$). However, blood glucose levels were still higher in rosiglitazone-treated diabetic group than rosiglitazone-treated control group ($p<0.01$). There was no significant difference in blood glucose levels between day 0-7 and 7-14 of the experiment period in all groups (Table 2).

Immunohistochemistry

Ghrelin immunoreactive cells were only rarely seen in adult rat pancreas. These cells were usually round but some of them had one or two cytoplasmic extensions. Ghrelin immunoreactive cells were seen in the immediate vicinity of glucagon cells in all groups (*data not shown*). There was no significant difference in ghrelin cell numbers per islet between control groups. Ghrelin cell numbers per islet showed a tendency towards a reduction in the rosiglitazone-treated diabetic group compared to STZ-induced diabetic but this decrease was not statistically significant (Figure 1). Moreover, we demonstrated ghrelin immunoreactive cells in quite

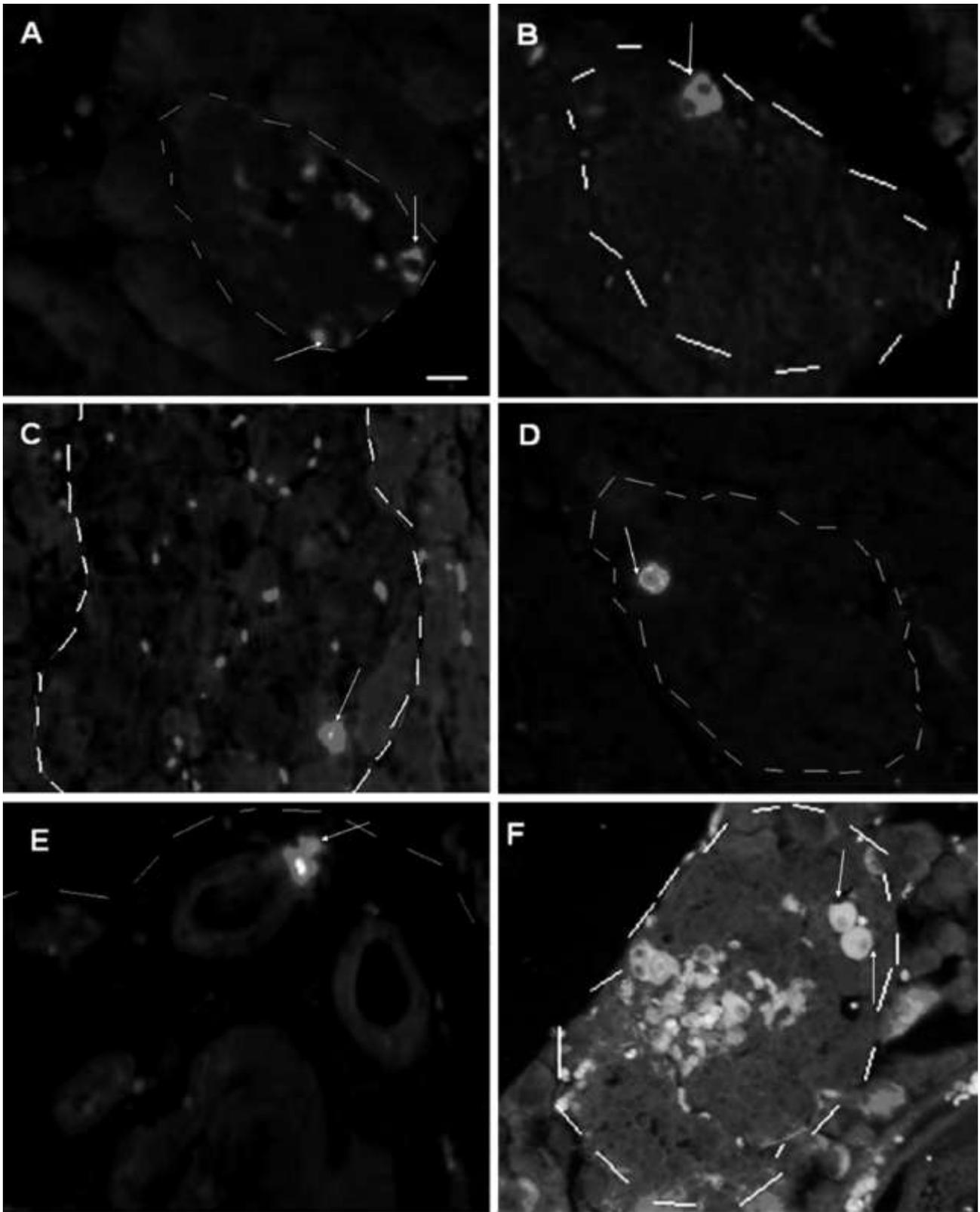


Figure 1. Fluorescence photomicrograph. Ghrelin immunoreactive cells are localized in the periphery of the islet in intact control (A), rosiglitazone-treated control (B), STZ-induced diabetic (C), and rosiglitazone-treated diabetic (D) rats. Ghrelin immunoreactive cell located in the main duct epithelium in rosiglitazone-treated diabetic group (E). Hypertrophic beta cells in the islets in STZ-induced diabetic group (F). Arrows indicate immunoreactive cells. Scale bar= 20 μ m.

Table 3. Immunohistochemistry and *in situ* hybridization results.

Group	Ghrelin peptide	Insulin peptide	Ghrelin mRNA
Intact control	0.82±0.78	28.71±18.53**	0.52±0.44
Rosiglitazone-treated control	0.45±0.51	23.21±10.65*	0.16±0.23
STZ-induced diabetic	0.48±0.51	5.07±5.34	0.35±0.43
Rosiglitazone-treated diabetic	0.24±0.17	1.36±0.99*	0.32±0.30

Values are Mean±SD; ** $p<0.01$ of intact control group versus STZ-induced diabetic group; * $p<0.05$ of rosiglitazone-treated control group versus rosiglitazone-treated diabetic group; * $p<0.05$ of rosiglitazone-treated diabetic group versus intact control group.

high numbers in the epithelium of ducts, most frequent in the main pancreatic duct. Insulin immunoreactive cells were numerous and located in the center of the islet in intact control and rosiglitazone-treated control groups. As expected their number decreased significantly in STZ-induced diabetic and rosiglitazone-treated diabetic groups and they were irregularly distributed in the center of the islet in both groups. Insulin immunoreactive cell numbers per islet were significantly different in rosiglitazone-treated control group compared with rosiglitazone-treated diabetic group ($p<0.05$). However, we detected no significant difference in insulin immunoreactive cell numbers between STZ-induced diabetic rats and rosiglitazone-treated diabetic rats ($p>0.05$). 40% and 10% of the islets had hypertrophic insulin cells in STZ-induced diabetic group and rosiglitazone-treated diabetic group, respectively (Figure 1) (Table 3).

***In situ* hybridization**

Cells with ghrelin mRNA signals were, like ghrelin-immunoreactive ones, rarely seen in adult rat pancreas. We demonstrated that cells labeled for ghrelin mRNA were located at the periphery of the islet in accordance with the immunohistochemistry results. The number of ghrelin mRNA positive cells per islet was slightly higher in intact control group compared to other groups but this difference did not reach statistical significance. We could also demonstrate the presence of many cells labeled for ghrelin mRNA in ductal epithelium by *in situ* hybridization (Figure 2) (Table 3).

Discussion

In this report, we evaluated the effects of rosiglitazone treatment on beta cell destruction and ghrelin gene expression for the first time.

PPAR- γ increases adipogenesis by inducing adipocyte differentiation (Araki *et al.* 1994; Koh *et al.* 2003). Koh *et al.* (2003) reported that rosiglitazone administration increased body weight in Otsuka Long–Evans Tokushima Fatty (OLETF) rats treated for 28 weeks. However, it was reported that rosiglitazone treatment decreased body weight in Zucker diabetic fatty (ZDF) rats up to two weeks. On the other hand, after 4 weeks of rosiglitazone treatment, body weight was significantly higher in ZDF rats (Finewood *et al.* 2001). In this study, there was no significant difference in body weight between STZ-induced diabetic rats and rosiglitazone-treated diabetic rats on day 0.7., and 14. of the experiment. This might be a consequence of the short-term treatment with rosiglitazone before the onset STZ-induced diabetes mellitus.

The thiazolidinediones, which are PPAR- γ agonists, are insulin sensitizing agents that control glycemia primarily by enhancing insulin sensitivity in peripheral tissues (Shibata *et al.* 2000). Recently, it was reported that long-term pioglitazone treatment decreased hyperglycemia and improved glucose-induced insulin secretion in diabetic db/db mice (Ishida *et al.* 2004). Troglitazone treatment prevented hyperglycemia when it was administered for four weeks in mice rendered diabetic by multiple low-dose STZ (Ogawa *et al.* 1999). Moreover, in prevention and early-intervention settings, rosiglitazone normalized glycemic control by improvement of insulin sensitivity in ZDF rats (Brand *et al.* 2002). In the present study, however, we found insignificant decrease in blood glucose levels in rosiglitazone-treated diabetic rats compared to STZ-induced diabetic rats. Our results suggest that it might be due to the β cell mass insufficiency to permit correction of hyperglycemia in rosiglitazone-treated diabetic animals.

Previous studies showed that pioglitazone and troglitazone prevented the loss of β cell mass and enhanced β cell staining by attenuating the autoimmune process culminating in type 1 diabetes (Brand *et al.* 2002; Ogawa *et al.* 1999; Takamura *et al.* 1999). It was also recently reported that rosiglitazone reduced acute inflammation (Cuzzocrea *et al.* 2004). Rosiglitazone treatment did not affect islet hypertrophy at early stages but prevented β cell

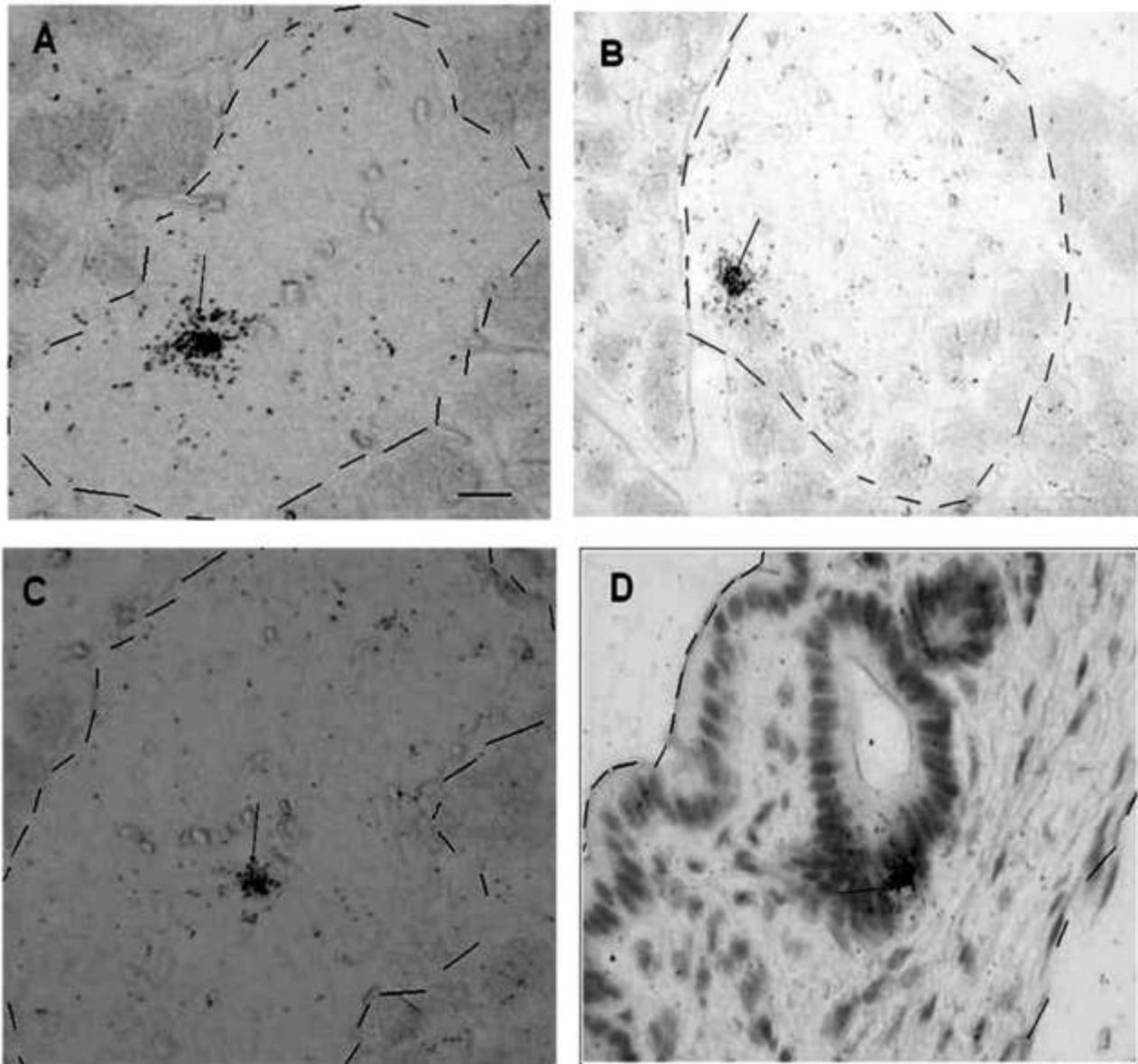


Figure 2. *In situ* hybridization autoradiographs of intact control (A), STZ-induced diabetic (B), and rosiglitazone-treated diabetic (C) rats for ghrelin mRNA. Cell labeled for ghrelin mRNA is seen in the duct epithelium (D). Counterstain Haematoxylin. Arrows indicate cells labeled for ghrelin mRNA. Scale bar= 20 μ m.

atrophy at later stages in diabetes-prone rats (Koh *et al.* 2003). We revealed that β cell hypertrophy ameliorated in rosiglitazone-treated diabetic rats compared to STZ-induced diabetic rats due to the mild effects of rosiglitazone on beta cell insulin sensitizing function. On the other hand, we detected no significant difference in insulin immunoreactive cell numbers between STZ-induced diabetic rats and rosiglitazone-treated diabetic rats. It was reported that rosiglitazone treatment did not affect insulin release and insulin gene expression in human cell culture (Lupi *et al.* 2004), in accordance with our

findings. Insulin immunoreactive cell number decreased significantly in STZ-induced diabetic and rosiglitazone-treated diabetic groups compared with controls, and located irregularly in the center of the islet in both groups. We conclude that short-term rosiglitazone pretreatment did not prevent beta cell destruction in diabetic rats.

Even though ghrelin is mainly expressed in stomach (Bolkent *et al.* 2006; Kojima *et al.* 1999), it is also expressed in pancreas (Wierup *et al.* 2004), including the islets (Kageyama *et al.* 2005), it may be speculated that it could have direct effect on

insulin secretion. In relation to meals, a reciprocal secretion pattern of ghrelin to that of insulin was observed, suggesting that beta cells might be a target for ghrelin (Cummings *et al.* 2001). Although studies about the effect of ghrelin on insulin secretion have been inconsistent; both stimulatory (Adeghate *et al.* 2002; Date *et al.* 2002), and inhibitory effects (Dezaki *et al.* 2004; Wierup *et al.* 2004) have been reported so far, the notion of inhibitory effects have been much more validated by recent studies. In our study, ghrelin cell numbers per islet were decreased insignificantly in STZ-induced diabetic, rosiglitazone-treated control and rosiglitazone-treated diabetic groups compared to intact control, and neither were the numbers of cells expressing ghrelin mRNA per islet significantly higher in intact control group than other groups. The tendency towards a reduction of ghrelin gene expression in diabetic rats compared to intact control might be part of an adaptation to the hyperglycemic conditions. It was reported that rosiglitazone has effects on transcription factors regulating pancreatic hormone secretion (Richardson *et al.* 2006) suggesting that it might also have role in the regulation of ghrelin gene expression via transcription factors which leads to a tendency towards a reduction of ghrelin gene expression in rosiglitazone treated control rats. We conclude that short-term rosiglitazone pretreatment has no significant effects on ghrelin expression. Thus, the way islet ghrelin is regulated has yet to be elucidated.

Date *et al.* (2002) reported that ghrelin peptide was expressed in glucagon producing α cells, while Volante *et al.* (2002) reported the colocalization of insulin and ghrelin in β cells. On the other hand, we observed that ghrelin immunoreactive cells were only rarely seen in adult rat pancreas. Ghrelin immunoreactive cells were located in the immediate vicinity of glucagon cells in all groups and these cells did not colocalized with any other known islet hormone, corroborating other recent observations in rat (Wierup *et al.* 2002) and mouse (Prado *et al.* 2004). These cells were usually round but some of them had one or two cytoplasmic extensions. Although a classical endocrine role for ghrelin as a peptide that is secreted into capillary network is evident, local paracrine activities of ghrelin might play an additional role. Moreover, we demonstrated for the first time that ghrelin immunoreactive and ghrelin mRNA expressing cells were frequent in the epithelial lining of ducts, the major ones in particu-

lar. This finding is in accordance with the notion that ductal epithelium might be a source for regenerating islet cells (Bonner-Weir *et al.* 2004). However, the role of ghrelin in this process is as yet unknown. Due to the fact that we applied rosiglitazone treatment only for one week before STZ-induced diabetes, the long term effects of rosiglitazone treatment on ghrelin expression/regulation need to be established.

In conclusion, we have demonstrated that short-term rosiglitazone pretreatment neither prevented beta cell destruction nor altered ghrelin gene expression in diabetic rats, markedly. We also demonstrated for the first time that ghrelin immunoreactive cells are frequent in ducts where they could be a local source of the peptide of importance for e.g. islet regeneration.

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