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ORIGINAL ARTICLE

Distribution of serotonin and its effect on insulin and glucagon secretion in normal and diabetic pancreatic tissues in rat

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Abstract

OBJECTIVES: The distribution of serotonin (5-HT) and its effect on insulin and glucagon secretion were investigated to examine whether there are changes in the pattern of distribution and effect of 5-HT after the onset of experimental diabetes. METHODS: The pattern of 5-HT and its effect of insulin and glucagon secretion was examined using immunohistochemical and radioimmunoassay techniques, respectively. RESULTS: 5-HT was demonstrated mainly in the neural elements of the pancreas. 5-HT-containing fine varicose nerve fibers were discerned in the wall of blood vessels and pancreatic ducts. 5-HT-containing nerves were also observed in the periacinar and periinsular regions of normal pancreas. The pattern or intensity of the distribution of serotonergic nerves did not change after the onset of diabetes. The perivascular, periductal, periacinar and periinsular regions of diabetic pancreas all contained 5-HT positive nerves. 5-HT elicited marked increases in insulin secretion from normal pancreas but had an inhibitory effect on insulin secretion from diabetic pancreatic tissues. In contrast, 5-HT inhibited glucagon secretion from normal pancreatic tissue fragments but stimulated glucagon release from diabetic pancreatic tissue fragments. CONCLUSION: 5-HT is well distributed in normal and diabetic pancreatic tissues and has stimulatory effects on insulin secretion from normal pancreas and glucagon secretion from diabetic pancreas. This result indicates that although 5-HT may help in the maintenance of the blood sugar level in normal pancreas by increasing insulin secretion and decreasing glucagon secretion, it may also aggravate the hyperglycemia observed in diabetes mellitus and hence exacerbate the symptoms of hyperglycemia in poorly controlled diabetes mellitus.

Introduction

Neurotransmitters including serotonin (5-HT) have been shown to be present in the nerves innervating the pancreas of many mammalian species [1]. The proximity of 5-HT containing nerves to blood vessels, pancreatic acinar and duct as observed in this study, suggests a physiological role for serotonin in the control of both endocrine and exocrine secretion of the pancreas. It has been shown that serotonin can cause significant contraction of smooth muscle; hence, serotonin is a powerful vasoconstrictor except in human skeletal and heart muscles where it causes vasodilatation [2]. The identification of serotoninergic nerves in the periacinar regions of the pancreas indicates that 5-HT may play a role in the regulation of acinar secretion. It has been demonstrated that 5-HT stimulates protein secretion from lacrimal acinar tissue of both rat and pig [3, 4]. In addition to the demonstration of 5-HT in the pancreas of normal and diabetic pancreatic tissue, serotonin has been shown to be present in blood platelets [5]. Serotonin-containing neurons have also been shown to be present in the spinal cord [6], raphe nuclei of the pons and medulla oblongata [7].

Materials and Methods

Animals and diabetes induction

Wistar rats weighing approximately 250 grams were used throughout this study. Rats were obtained from the United Arab Emirates University breeding colony and the Animal Research Group's guidelines for the care and use of laboratory animals were followed. The rats were divided into two groups, streptozotocin-induced diabetics and age-matched controls. Diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (Sigma, Poole, UK) at 60 mg kg^{-1} prepared in 5 $mMol^{-1}$ citrate buffer pH 4.50. The animals were kept in plastic cages and maintained on standard laboratory animal diet with food and water *ad libitum*. The blood glucose estimations were made by One Touch II ® Glucometer (LifeScan, Johnson and Johnson, USA) for each individual animal. The animals were considered diabetic if the random blood glucose levels were equal to or more than 300 mg/dl. After six weeks from induction of diabetes all the animals from both groups were sacrificed under chloral hydrate general anesthesia by an intraperitoneal injection of 7% chloral hydrate 6-ml kg^{-1} of body weight. A midline abdominal incision was made, and the pancreas was rapidly removed and placed in an ice-cold Krebs solution. Representative fragments were taken from the tail end of the pancreas. Pancreatic fragments were used for both morphological and physiological studies.

Immunohistochemistry

Six rats from each group (control and diabetic) were used for this experiment. The isolated pancreas was trimmed free of adherent fat and connective tissue and cut into small pieces (2 mm^3) and fixed overnight in freshly prepared Zamboni's fixative [8]. The tissues were later dehydrated in graded concentrations of ethanol. The specimens were changed every 2 hours in 70% and 95% and 3 changes in absolute ethanol for 2 h. After dehydration the specimens were cleared in xylene and subsequently embedded in paraffin wax at 55°C. Sections of 6 mm thickness were cut on a microtome (Shandon AS325, USA), and placed in waterbath at 48°C. Thereafter, they were transferred onto pre-washed microscopic slides, which were dried in an oven at 55°C for 30 min to enhance attachment of sections.

The slides were deparaffinized with xylene (twice, 5 min each) and then transferred into absolute ethanol (twice, 5 min each). The sections were then incubated for 30 min in 0.3% hydrogen peroxide solution in methanol to block endogenous peroxidase activity. The sections were hydrated in decreasing concentration of ethanol and brought to Tris buffered saline (TBS). The slides were washed three times (5 min each) in TBS. After washing in TBS, the tissues were marked around with a Dako pen to prevent solutions draining away from the tissue sections. The staining procedure was started by incubating the sections with blocking reagent. After 30 min the blocking reagent was drained off and appropriate dilution of primary antibodies and negative control reagents were applied. The sections were incubated in primary antibodies for 1 h at room temperature. The slides were then washed (3 times, 5 min each) with TBS and incubated for 30 min with prediluted biotinylated anti-rabbit or anti-mouse IgG for 30 min, washed in TBS (3 times, 5 min each) and subsequently incubated in streptavidin peroxidase conjugate for 45 min. After a final wash in TBS (3 times, 5 min each), the peroxidase activity was revealed by incubating the specimens for 3 min in 3,3-diaminobenzidine tetrahydrochloride containing 0.03% hydrogen peroxide in TBS. The slides were later washed for 10 min under running tap water, and counterstained with hematoxylin for 30 seconds. They were then differentiated in acid ethanol and washed for 10 min under running tap water, dehydrated in ascending grades of ethanol, and subsequently cleared in xylene for a longer time to dissolve the Dako pen mark. The tissues were subsequently

mounted in Cytoseal 60 (Stephens Scientific, USA). Slides were examined under Zeiss Axiophot microscope and immunopositive areas of the tissue sections were photographed.

Chemicals and immunological and radiochemical reagents

The antiserum to insulin, glucagon and 5-HT were purchased from Dako (Copenhagen, Denmark).

Immunohistochemical control

No specific immunostaining was observed in pancreatic tissue when primary antisera were omitted.

Estimation of in vitro *pancreatic insulin and glucagon release*

In this experiment, 6 rats from each group were used. The pancreas was removed and placed in ice-cold Krebs buffer (KB). The pancreas was trimmed free of adherent fat and connective tissue and cut into small fragments (1–2 mm^3). The pancreatic fragments were placed in 2 ml glass vials containing 1 ml of KB and pre-incubated for 30 min in a waterbath at 37°C, in order to wash away any enzymes and hormones due to cutting of the tissues. After the pre-incubation period, the KB solution was drained off and the fragments were subsequently incubated for 1 h with different concentrations of either 5-HT (10^{-8}-10^{-4} M). In control experiments, the fragments were incubated in KB solution alone for the same duration of time. During the incubation period, each vial was gassed with 95% oxygen and 5% carbon dioxide every 10 min. At the end of experiment the tissues were removed, blotted, weighed and incubating solutions stored at -20°C for radioimmunoassay.

Radioimmunoassay

Insulin assay.

Insulin was determined by using a modified method of Herbert *et al*. [9]. All test samples and controls were assayed in duplicates, using coated tubes with antibodies to insulin. A volume of 200 μl of either calibrators, controls or test samples was pipetted to previously labeled tubes followed by 1 ml of $^{[125]}$I-Insulin. Each tube was vortexed and incubated for 24 h at room temperature. After the incubation period, the tubes were decanted for 3 min and radioactivity was counted for 1 min using gamma counter (Beckman). Assays were performed by using DPC (DPC, California, USA) radioimmunoassay kits. Results were analyzed by using a Beckman Immunofit EIA/RIA analysis software, version 2.00. Values were expressed as μIU ml^{-1} (100 mg tissue)$^{-1}$.

Glucagon assay.

Effluent glucagon content was determined by double antibody technique of Nishino *et al*. [10]. All test samples and controls were assayed in duplicates. A volume of 200 μl of either calibrator, control or test sample was pipetted into previously labeled tubes. After this, 100μl of glucagon antiserum was added to all tubes except NSB (non specific binding) and T (total count) tubes and vortexed. After vortexing the tubes were covered with parafilm and incubated for 24 h at 4°C. After the first incubation 100μl of $^{[125]}$I-glucagon was added to every tube and vortexed. The samples were incubated for 24 h at 4°C, and then 1 ml of cold precipitating solution was added to all tubes (except T tubes) and centrifuged for 15 min at 100Xg. The tubes were decanted gently and radioactivity was counted for 1 min using a gamma counter (Beckman). All glucagon calibrators were reconstituted 30 min before the assay. Assays were performed by using radioimmunoassay kits. Results were analyzed by using a software, (Beckman Immunofit EIA/RIA version 2.00) and values were expressed in pg ml^{-1} (100 mg $tissue^{-1}$).

Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed using analysis of variance (ANOVA). Values with $P < 0.05$ were accepted as significant.

Results

Distribution of insulin and glucagon in normal and diabetic pancreas

Insulin-positive cells are widely distributed in both the central and peripheral portions of the islet of Langerhans in normal pancreas. Although most of the insulin-positive cells were confined to the islets, solitary insulin-positive cells were scattered throughout the pancreas (Fig. 1a). After the onset of diabetes, the number of insulin-immunoreactive cells was much fewer compared to that of normal pancreas. The pattern of distribution of insulin-positive cells did not differ from that of normal tissue since insulin-positive cells are located both in the peripheral and central parts of the islets of Langerhans (Fig. 1b). Glucagon-immunopositive cells are the second most commonly occurring cells in the endocrine pancreas and were located in the peripheral part of the islet of Langerhans. Solitary glu-

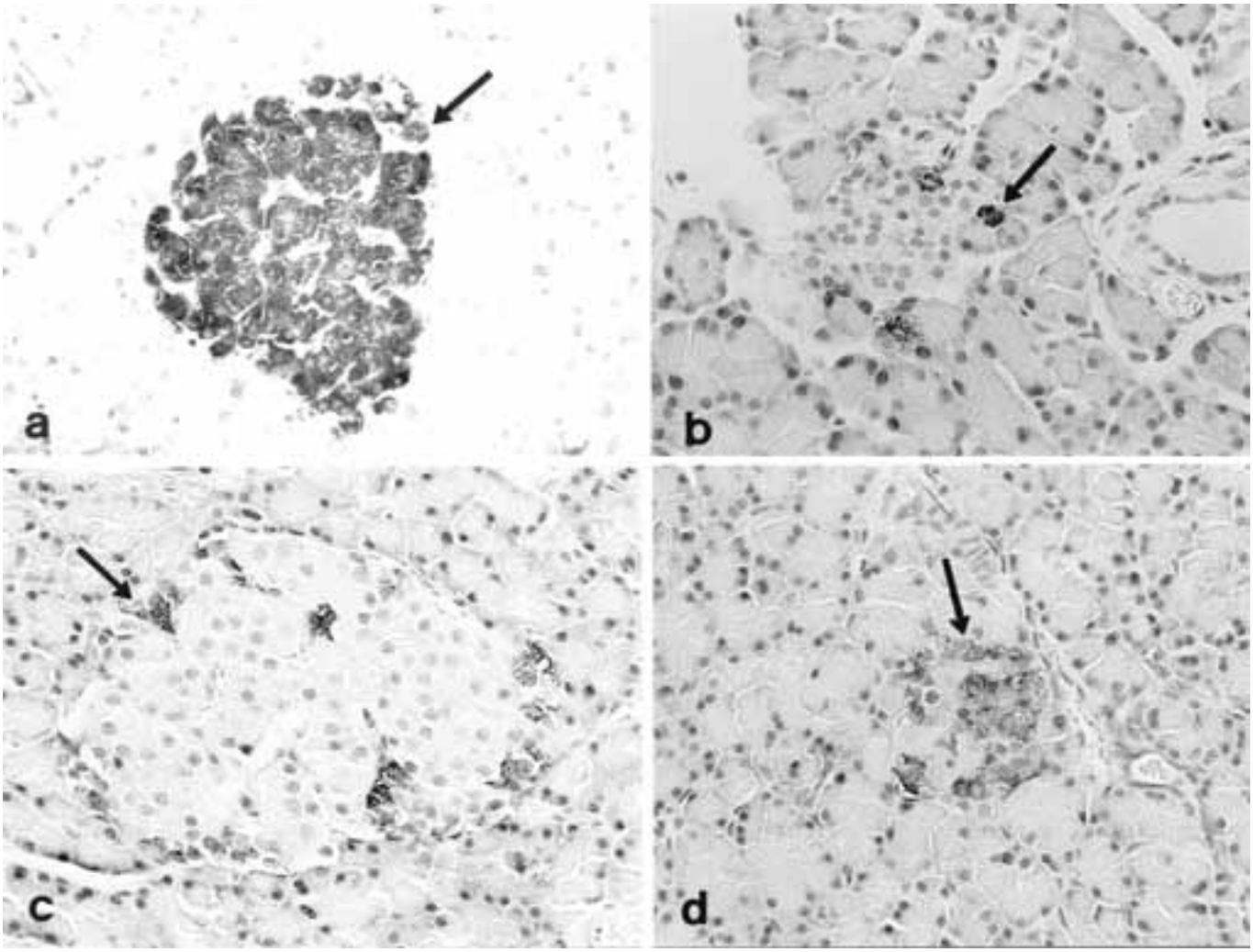

Fig.1. Light micrographs showing (a) insulin immunopositive cells (arrow) in normal pancreas; (b) islet with few insulin immunopositive cells (arrow) in diabetic pancreas; (c) glucagon immunopositive cells (arrow) in normal pancreas; (d) deranged pattern of glucagon immunopositive cells (arrow) in diabetic pancreas. These micrographs are typical of 6 such animals. (X400).

cagon positive cells were also observed scattered throughout the pancreas (Fig. 1c). In diabetic pancreatic tissues, the number of glucagon-immunoreactive cells increased coupled with an abnormal pattern of distribution when compared to normal tissue. In diabetic pancreas these glucagon-containing cells were located both in the peripheral and central regions of the islets (Fig. 1d).

Distribution of serotonin in normal and diabetic pancreas

Serotonin was demonstrated mainly in the neural elements of the pancreas. 5-HT-containing fine varicose nerve fibers were discerned in the wall of blood vessels and pancreatic ducts. 5-HT-containing nerves were also observed in the periacinar and periinsular regions of normal pancreas (Fig. 2a). The pattern or intensity of the distribution of serotonergic nerves did not change after the onset of diabetes. The perivascular, periductal, periacinar and periinsular regions of diabetic pancreas all contained 5-HT positive nerves (Fig. 2b).

Effect of 5-HT on insulin secretion from normal and diabetic pancreatic fragments

Figure 3 shows the effect of 5-HT on insulin secretion in normal and diabetic rat pancreas. The basal insulin response in normal tissue (expressed as mean ± SEM) was 8.5 ± 0.5 μIU ml^{-1} (100 mg tissue)$^{-1}$ (n = 6). After stimulation of normal tissue with either 10^{-8} or 10^{-6} M 5-HT, the insulin measured in the effluent was 79.9 ± 13.4 and 56.2 ± 14.9 μIU ml^{-1} (100 mg tissue)$^{-1}$ (n = 6) respectively. The basal insulin response in diabetic animal was 5.3 ± 0.5 μIU ml^{-1} (100 mg tissue)$^{-1}$. After stimulation of diabetic tissue with either 10^{-8} or 10^{-6} M 5-HT, the insulin measured

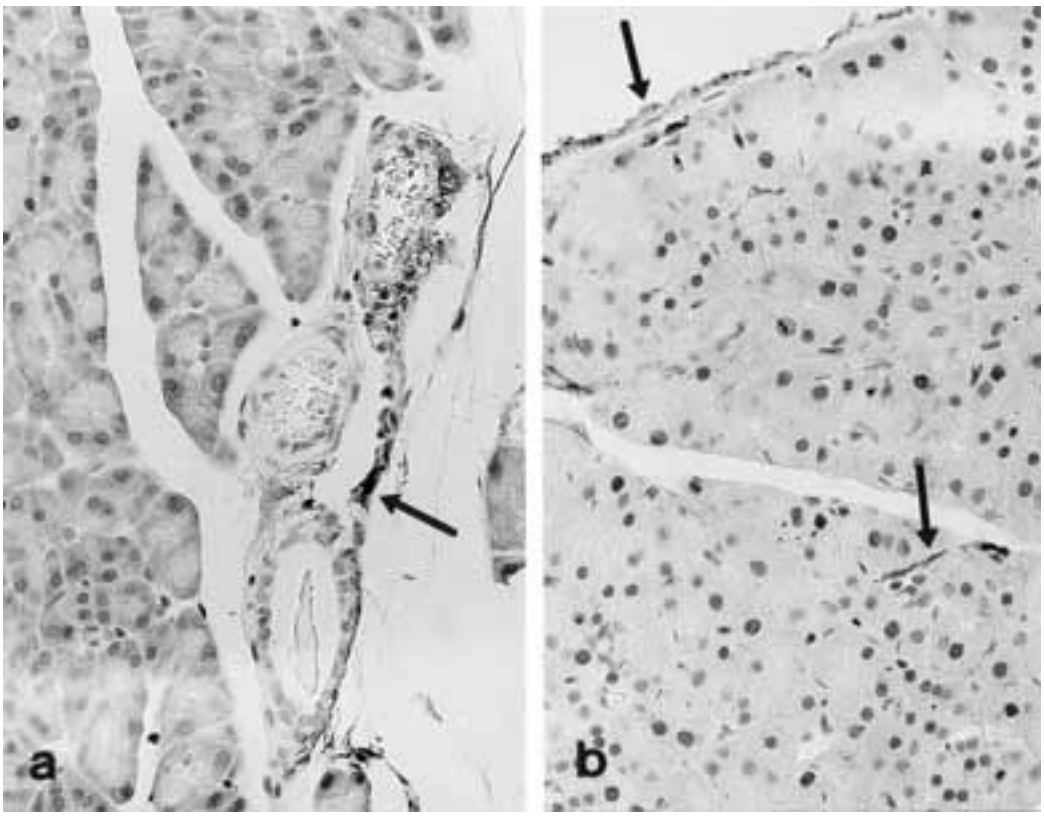

Fig. 2. Light micrographs showing 5-HT immunopositive nerves (arrow) in (a) normal and (b) diabetic pancreas. This micrograph is typical of 6 such animals (X 400).

in the effluent was 2.3 ± 1.3 and 0.7 ± 0.2 μIU ml^{-1} (100 mg tissue)$^{-1}$ (n = 6) respectively. 5-HT elicited marked increases in insulin secretion from normal pancreas.

During diabetes 5-HT had an inhibitory effect on insulin secretion compared to normal pancreas. The results indicate that 5-HT can elicit secretagogue effect on the normal endocrine pancreas to secrete insulin but not in diabetic pancreas.

Effect of 5-HT on glucagon secretion from normal and diabetic pancreatic fragments

The basal glucagon secretion from normal pancreatic tissue (expressed as mean ± SEM) was 67.8 ± 3.7 pg ml^{-1} (100 mg tissue)$^{-1}$ (n = 6). After stimulation of normal tissue with either 10^{-8}, 10^{-6} or 10^{-4} M 5-HT, the glucagon measured in the effluent was 38.7 ± 10.8, 49.1 ± 4.5 and 33.3 ± 19.3 pg ml^{-1} (100 mg tissue)$^{-1}$ (n = 6) respectively. The basal glucagon response in diabetic pancreatic tissue was 133.6 ± 7.7 pg ml^{-1} (100 mg tissue)$^{-1}$ (n = 6). After stimulation of diabetic pancreatic tissue with either 10^{-8}, 10^{-6} or 10^{-4} M 5-HT, the glucagon measured in the effluent was 139.1 ± 33.1, 233.0 ± 11.3 and 48.9 ± 14.8 pg ml^{-1} (100 mg tissue)$^{-1}$ (n = 6) respectively. 5HT caused a significant ($p < 0.05$) decrease in glucagon output from normal pancreatic tissue at all concentrations (Fig. 4). In contrast, 5-HT evoked a marked increase in glucagon output from diabetic pancreatic tissue fragments at all concentrations used (Fig. 4).

Discussion

Distribution of pancreatic hormones

The findings of this study have demonstrated significant changes in the pattern of distribution of insulin- and glucagon-immuno positive cells in the pancreas of diabetic animals when compared to normal controls. The relative distribution of pancreatic islet cells in normal, control animals was similar to the results of previous studies in the rat [11, 12]. The pattern of distribution of these pancreatic endocrine cells in the islets of diabetic rats differed from that of control rats. The number of insulin-positive cells decreased markedly in diabetic rats compared with control animals. The number of insulin positive cells per islet was significantly higher in normal pancreas compared to streptozotocin (STZ)-induced diabetic pancreas. The few surviving insulin-positive cells were found scattered in both the

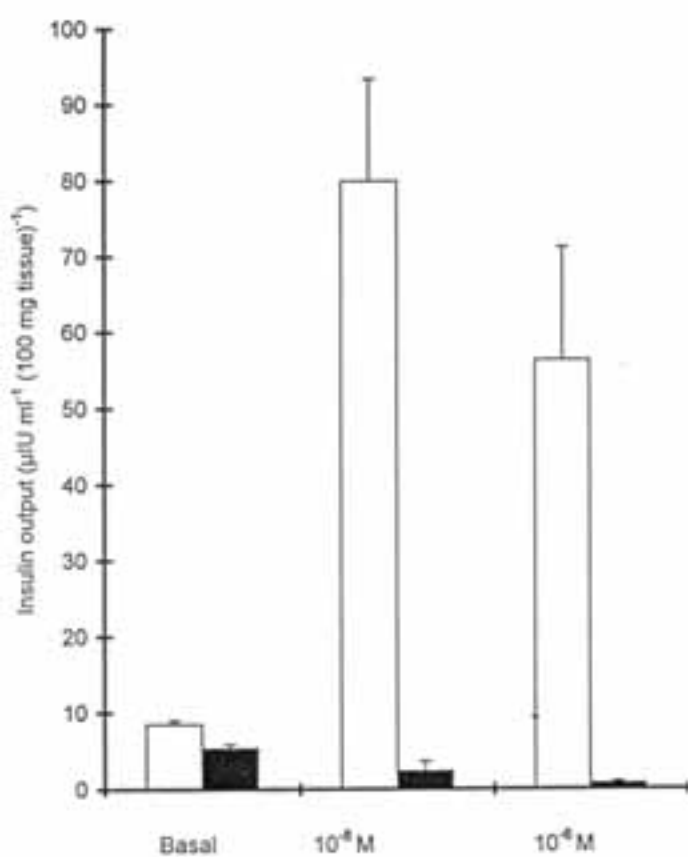

Fig. 3. Histogram showing the effect of 5-HT on insulin secretion from normal (□) and diabetic (■) rat pancreatic fragments. Basal insulin output is also shown for comparison. (Data are mean ± SEM, n=6).

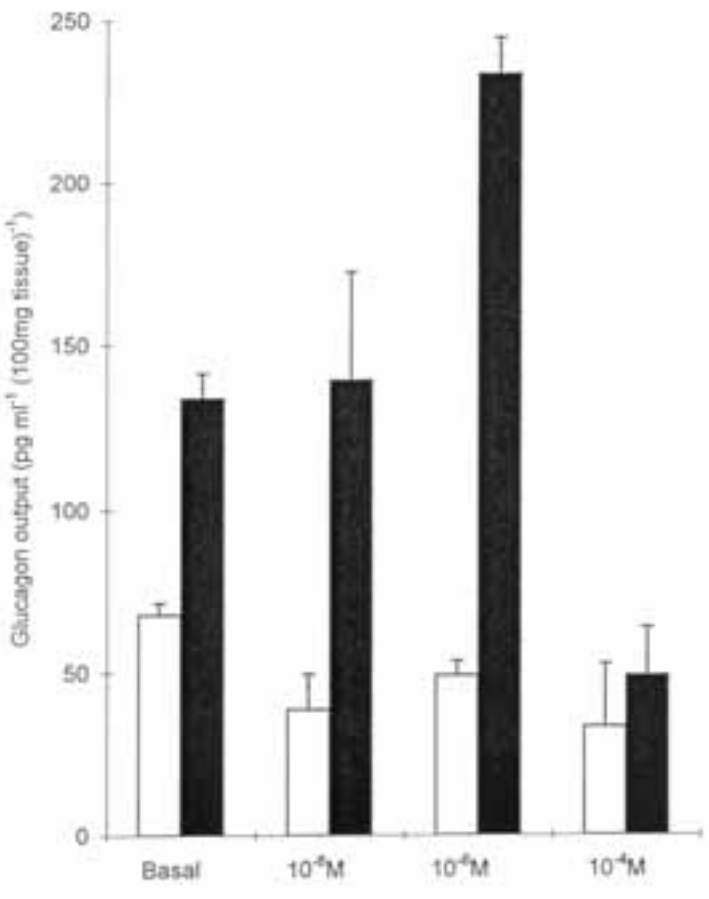

Fig. 4. Histogram showing the effect of 5-HT on glucagon secretion from normal (□) and diabetic (■) rat pancreatic fragments. Basal glucagon output is also shown for comparison. (Data are mean ± SEM, n=6).

peripheral and central parts of the islets of Langerhans. The fact that some insulin-producing cells survived the toxic effect of STZ was in itself an interesting finding. What then could be the reason for this differential damage to pancreatic B-cells? There are two possible explanations for these observations. Firstly, some cells may not be completely exposed to the drug compared to others and secondly, the insulin-positive cells observed may have been derived from cell replication and or regeneration. It has been shown previously that about 10% of pancreatic beta cells are capable of replication [13]. The significant decrease in number of insulin-producing cells in the diabetic pancreas compared to normal explained the gross clinical sign of hyperglycemia observed in these animals.

Immunohistochemical studies also showed that there was a derangement in the number and pattern of distribution of glucagon-producing cells. In normal pancreas, glucagon-positive cells were localized mainly to the peripheral region of the islet of Langerhans. In normal pancreas, the pattern of distribution of glucagon was similar to those reported in the literature [11, 12, 14]. Glucagon-producing cells constitute between 20-28% of the total cell population in the endocrine pancreas. This observation agrees with those of previous studies [11, 12, 14].

After the onset of diabetes, the number and the pattern of distribution of glucagon-positive cells changed significantly. The number of glucagon-positive cells increased significantly in experimental diabetes. This increase in the number of glucagon-containing cells has been reported by others [15]. In contrast to this, some investigators [16] demonstrated that the number of pancreatic alpha cells did not change significantly in STZ-treated rats. The reason for the increase in the number of glucagon-positive cells in STZ-treated rats is unknown. Hypothetically, the increase may arise from the loss of the inhibitory effect of insulin on glucagon or it may be due to a response to the increased blood sugar level in diabetes. This hypothesis may explain why there was a derangement in the pattern of distribution of glucagon-positive cells. This pattern of distribution of glucagon-positive cells was characterized by the localization of glucagon-immunoreactive cells in the central and peripheral portions of the islets Langerhans in diabetic pancreas.

Distribution of 5-HT in normal and diabetic pancreas

5-HT was observed in the neural elements of the pancreas of both normal and STZ-induced diabetic rats. In normal pancreatic tissue, 5-HT immunoreactive nerves were observed in the wall of blood vessels where they innervate the vasculature of the pancreas. In addition to this, 5-HT was also discerned in the periacinar, periductal and interlobular areas of the pancreas. The pattern of distribution of serotonin-containing nerves supports the finding of previous [1] results on the serotonergic innervation of the pancreas. Moreover, using radioautography, Koevary and collaborators [17, 18] were able to show serotonergic nerves in the pancreas of adult and fetal rats. The proximity of 5-HT containing nerves to blood vessels, pancreatic acinar and duct as observed in this study suggests a physiological role for serotonin in the control of both endocrine and exocrine secretion of the pancreas. It was logical, therefore, to attribute a vasomodulator and a secretagogue role for serotonin in the pancreas. In fact it has been shown that serotonin can cause significant contraction of smooth muscle; hence, serotonin is a powerful vasoconstrictor except in human skeletal and heart muscles where it causes vasodilatation [2]. The identification of serotoninergic nerves in the periacinar regions of the pancreas indicates that 5-HT may play a role in the regulation of acinar secretion. It has been demonstrated that 5-HT stimulates protein secretion from lacrimal acinar tissue of both rat and pig [3, 4]. In addition to the demonstration of 5-HT in the pancreas of normal and diabetic pancreatic tissue, serotonin has been shown to be present in blood platelets [5]. Serotonin-containing neurons have also been shown to be present in the spinal cord [6], raphe nuclei of the pons and medulla oblongata [7].

Effects of 5HT on insulin secretion from normal and diabetic pancreatic tissues

In the immunohistochemical part of this study, it was also shown that nerves innervating both normal and diabetic pancreas contain 5-HT. It is apparent therefore that serotonin would have a functional role on pancreatic secretion. In normal pancreatic tissue fragments, serotonin elicited marked increases in insulin secretion. After the onset of diabetes, serotonin had only a small stimulatory effect on insulin secretion. Our result on the effect of serotonin on normal pancreatic tissue segments differed from those reported by Feldman [19, 20]. Feldman [19] reported that serotonin inhibited insulin secretion *in vitro* and that the administration of serotonin-receptor antagonists to individuals with type-II diabetes resulted in increased insulin secretion [20]. In a recent study it was shown that activation of serotonin-1 receptor inhibits insulin secretion in humans [21].

It has been shown that serotonin can induce marked increases in protein secretion from rat lacrimal acinar glands [4]. It is therefore likely that serotonin will have a secretagogue effect on endocrine tissues as well. The fact that serotonin elicits only a small stimulatory action on insulin secretion from diabetic pancreatic fragments indicates that insulin secretion may have been impaired after the onset of diabetes.

Effects of 5HT on glucagon secretion from normal and diabetic pancreatic fragments

5-HT evoked a marked decrease in glucagon output from normal pancreatic tissue at all concentrations used. In contrast, 5-HT caused a significant increase in glucagon output from diabetic pancreatic tissue only at a concentration of 10^{-6} M. All other concentrations (e.g. 10^{-8} and 10^{-4} M) had an inhibitory effect on glucagon output from diabetic pancreatic tissues. The effect of 5-HT on glucagon secretion is in contrast to that on insulin secretion because 5-HT stimulates insulin secretion from normal pancreas. In a recent study it was shown that activation of serotonin-1 receptor inhibits glucagon secretion in humans [21].

Conclusion

In conclusion serotonin is widely distributed in both the normal and diabetic rat pancreas and have potent secretagogue effect on insulin secretion from normal pancreas and glucagon secretion from diabetic pancreas. This result indicates that although 5-HT may help in the maintenance of blood sugar level in normal pancreas by increasing insulin secretion and decreasing glucagon secretion in normal pancreas, it may also aggravate the hyperglycemia observed in diabetes mellitus and hence exacerbate the symptoms of hyperglycemia in poorly controlled diabetes mellitus.

Acknowledgments

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