Regulation of Rat Pancreatic CCK$_B$ Receptor and Somatostatin Expression by Insulin

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The cholecystokinin B receptor (CCK$_B$R) is localized on pancreatic endocrine somatostatin δ-cells. Pancreatic somatostatin content was increased in diabetic rats. The mechanisms involved in this phenomenon are unknown, and we believe insulin is involved. In this study, four groups of rats were used: controls, streptozotocin-induced diabetic, streptozotocin-induced diabetic with insulin, and streptozotocin-induced diabetic with insulin and its cessation. Rats were killed after 7–28 days of treatment for diabetes, and somatostatin mRNA expression and pancreatic somatostatin content, CCK$_B$R mRNA and protein expression in total pancreas and purified islets, and the cellular localization of somatostatin and CCK$_B$R in islets was measured. Data indicate that diabetes is established after 7 days, is controlled by insulin, and reappears after treatment cessation. Pancreatic somatostatin mRNA expression and somatostatin content were increased during diabetes, normalized during insulin treatment, and reappeared after treatment cessation. Gland and islet CCK$_B$R mRNA and protein almost disappeared during diabetes; CCK$_B$ mRNA reappeared in response to insulin, but the protein did not. Confocal microscopy confirmed data obtained on somatostatin and CCK$_B$R as established biochemically in the course of the treatments. In conclusion, these data strongly suggest that insulin can negatively control pancreatic somatostatin mRNA and hormone content and positively control CCK$_B$R mRNA; the CCK$_B$R protein appears to be delayed. Diabetes 53:1526–1534, 2004

During the 1970s, major changes were observed in the metabolism of pancreatic somatostatin during diabetes development in human and experimental animals; these modifications included increased secretion, tissue contents, and δ-cell population (1–12). On the contrary, it was also reported (13,14) that in diabetic mice mutants (ob/ob and db/db), pancreatic somatostatin content was decreased, along with a reduction in somatostatin cells within the islet. It was later suggested that in streptozotocin (STZ)-induced diabetic rats, regulation of somatostatin gene transcription was targeted to the pancreas and stomach but not to the other somatostatin-producing tissues (15).

The role played by insulin in somatostatin release remains controversial. Indeed, insulin can stimulate somatostatin release from perfused chicken pancreas-duodenum (16); however, data from monolayer cultures of neonatal rat pancreas (17) and isolated dog pancreas (18) clearly show that insulin fails to induce somatostatin release. In anesthetized normal and diabetic dogs, insulin infusion or injection was associated with an immediate reduction of the venous pancreaticoduodenal release of somatostatin (19). All of these differences could be explained by the different models used to study somatostatin release.

Cholecystokinin (CCK), a duodenal hormone released into the bloodstream after meal ingestion, is recognized as the major hormonal factor involved in the regulation of pancreatic exocrine secretion, gallbladder contraction, gastric emptying, and small bowel motility. CCK is also involved in the regulation of the endocrine pancreas; indeed, it can stimulate insulin secretion from an in vitro rat perfused pancreas (20) and in vivo in the rat (21), pig (22), mouse (23), and human (24). In humans, the insulinotropic effect of CCK was attenuated by the specific CCK-A receptor antagonist L-364718 (25). Finally, it was recently observed (26,27) that a defect in the CCK-A receptor gene OLET (Otsuka Long-Evans Tokushima Fatty) rats led to obesity and diabetes.

With regard to pancreatic somatostatin δ-cells, we recently demonstrated (28,29) that these cells specifically bear the CCK$_B$ receptor (CCK$_B$R) as established by RTPCR, Western blotting, and confocal microscopy in rat, mouse, dog, pig, horse, calf, and human. This new discovery may indicate that the CCK$_B$R could be involved in somatostatin metabolism and/or control of δ-cell growth. Therefore, knowing that diabetes causes modifications in the pancreatic δ-cell metabolism and that these cells express the CCK$_B$R, the objectives for this study are to characterize the changes in somatostatin mRNA expression and contents along with those of the CCK$_B$R in normal and diabetic rats and to determine whether insulin treatment can normalize the modifications observed during diabetes development.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats, purchased from Charles River Laboratories (St. Constant, Canada), were housed in a light- and humidity-controlled room and given free access to food and water.

After an overnight fast, rats (200–220 g) were rendered diabetic (group STZ-D, n = 150) by a single intraperitoneal injection of 65 mg/kg body wt STZ (Sigma, St. Louis, MO) dissolved in 0.1 mol/l citrate buffer, pH 4.5. Controls received the same volume of citrate buffer alone (nondiabetic, group ND, n =
40. Animals were studied for 7 (group STZ-D, n = 20), 14 (group STZ-D14, n = 20), 21 (group STZ-D21, n = 20), or 28 (group STZ-D28, n = 20) days after diabetes induction. Seven days after STZ injection, 80 diabetic rats received twice-daily subcutaneous injections of insulin glargine NPH (30 unit/kg, twice daily) (35 cycles; CCK BR: 30 s 94°C, 45 s 60°C, and 45 s 72°C (30 cycles). PCRs samples were electrophoresed on a 1% agarose gel, and DNA was visualized with ethidium bromide. Immunochemistry and image analysis by confocal microscopy. These procedures were extensively described recently (29,39) with regard to the antibodies used, their dilutions, and their specificity.

RESULTS

Body weight, plasma glucose, triglycerides, and pancreatic amylase. As shown in Fig. 1A, body weights of the diabetic animals (STZ-D) exhibited significant early decreases of 18% at day 7, down to 29.6% at day 28 when compared with their respective controls. Treatment of the diabetic animals with insulin (STZ-I) immediately reversed the losses in body weight; a 15% increase was observed after 7 days, up to 28.4% after 3 weeks, thus indicating a return to control values. Cessation of insulin for 21 days caused a new drop of 14.2% in body weight when group STZ-I21/D21 is compared with group STZ-I21.

The STZ-induced diabetic rats (STZ-D) presented severe hyperglycemia after 7 days (182%), up to 280% after 28 days when compared with their respective controls (Fig. 1B). Under insulin, a gradual recovery was observed and glycemia reached control values after 14 days (ND-28 versus STZ-I28). Cessation of insulin resulted in a significant 173% increase in glycemia after 21 days (STZ-I21 versus STZ-I21/D21). Diabetes was also assessed by measurement of plasma triglycerides. As shown in Fig. 1C, triglyceridermia was already increased by 332% 7 days after STZ injection, and these large increases remained until day 28. Insulin restored plasma triglycerides back to control levels within 1 week. Contrary to glycemia, which returned to high values after cessation of the insulin treatment, triglycerides remained at control values (STZ-I21/D21 versus ND-28).

As previously observed (40), diabetes is also associated with a complete loss of pancreatic amylose activity (Fig. 1D) and content (Fig. 1E). A return to control values was observed after insulin treatment, and a new drop occurred upon cessation of the insulin treatment. It is important to notice that losses in activity also corresponded to losses in protein.

Variations in pancreatic somatostatin mRNA and hormone content. As shown in Fig. 2A, somatostatin mRNA exhibited a significant increase of 28% over control values 28 days after diabetes induction (STZ-D versus ND) when RNA from the total pancreas was used. Insulin given for 21 days returned somatostatin mRNA to control values (STZ-I versus ND), whereas its cessation caused a new significant increase of 10% in somatostatin mRNA (STZ-I21 versus STZ-I21/D21). Because pancreatic somatostatin is exclusively located in the 5-cells of the islet (28), we decided to verify if alterations of somatostatin mRNA expression observed in whole pancreases were also present in RNA extracted from purified islets. RNAs were then extracted from pools of five to seven islet preparations as indicated in Fig. 2B. In control rats (ND), a constant expression of somatostatin mRNA can be observed. Diabetes was asso-
associated with a fourfold increment in somatostatin mRNA 7 days after its induction, an elevation that remained for 28 days. Insulin treatment caused a prompt return of somatostatin mRNA to control values after 7 days, which remained throughout treatment. However, cessation of insulin resulted in a new increase in somatostatin mRNA to levels comparable with those in the initial diabetic animals. These variations in somatostatin mRNA were accompanied by comparable changes in total pancreatic somatostatin content, as is shown in Fig. 2C. Indeed, although somatostatin content remained at control values after 14 days of diabetes (data not shown), a significant 97% increase was observed 14 days later. A 21-day insulin treatment resulted in a significant decrease of 36% in somatostatin total contents below control values. Interestingly, cessation of this insulin treatment for a further 21 days resulted in a significant 140% increase in somatostatin content when compared with insulin treatment (STZ-I21/D21 versus STZ-I21) as observed for its mRNA.

**Mode of insulin action: direct or indirect?** To answer this question, we first investigated the effects of diabetes and insulin treatment on IGF-1 receptor protein expression. Secondly, we determined the effects of insulin on somatostatin mRNA expression in RIN-14B cells. As shown in Fig. 3A, the IGF-1 receptor proteins are expressed in control islets, in those of the diabetic rats, and in islets of insulin-treated rats. However, 14 days of diabetes resulted in an important reduction in IGF-1 receptors in the islet, which seems to be accentuated by 14-day insulin treatment. It is important that the IGF-1 receptors are present on the remaining islet cells and can thus still carry the insulin messages. The direct effect of insulin on somatostatin expression is unequivocally demonstrated by the observation that within 8 h insulin can repress somatostatin mRNA expression by 62% in RIN-14B cells, which synthesize somatostatin (Fig. 3B). These data clearly establish the direct inhibitory effect of insulin, probably through the IGF-1 receptor that is also present on these cells, as is shown in Fig. 3A.

**Variations in CCK<sub>B</sub>R mRNA and protein.** Because the CCK<sub>B</sub>R was previously shown (29) to colocalize with somatostatin in pancreatic endocrine δ-cells, we investigated the potential relationship that might exist between alterations in somatostatin expression and the behavior of the CCK<sub>B</sub>R in the course of diabetes development, during insulin treatment, and after its cessation. As shown in Fig. 4A, expression of the CCK<sub>B</sub>R mRNA evaluated from total pancreas RNA was dramatically and significantly reduced by 72% after 28 days of diabetes when compared with controls (STZ-D versus ND). By contrast, insulin given for 21 days significantly enhanced CCK<sub>B</sub>R mRNA by 60% when compared with diabetic animals (STZ-I21 versus STZ-D28) and with control levels (STZ-I21 versus ND). Cessation of insulin resulted in another drop of 40.5% in CCK<sub>B</sub>R mRNA when compared with the insulin-treated rats (STZ-21/D21 versus STZ-I21). As shown in Fig. 4B, the CCK<sub>B</sub>R proteins present in total pancreas membrane proteins followed a pattern of expression comparable with its mRNA except in the diabetic group, which did not experience as dramatic a drop as its mRNA content. The receptor is visualized as an 80-kDa protein.

With RNA isolated from purified islets (Fig. 4C), we can appreciate the constancy over time in CCK<sub>B</sub>R mRNA expression in the control rats (ND). In the diabetic animals, a significant burst of the receptor mRNA was observed after 7 days of the initial STZ injection. Thereafter, a progressive decline was observed over the next 14 days, to a complete disappearance after 28 days. Insulin treatment brought CCK<sub>B</sub>R mRNA back to control values after 7 days of treatment, and after 21 days, we observed an eightfold increase in CCK<sub>B</sub>R mRNA. Cessation of the insulin treatment for 21 days drove CCK<sub>B</sub>R mRNA back to the levels of the 21-day diabetic animals.

With proteins extracted from purified islets (Fig. 4D),
we can detect the CCKBR even if its concentration seems to be less abundant than that in total pancreas (Fig. 4B). CCKBR expression remains quite constant with time in nondiabetic islets (ND), while it decreased dramatically over 7 days of diabetes (STZ-D) to a complete loss of the protein after 21 days of diabetes (STZ-D). As observed in total pancreas membranes (Fig. 4B), insulin failed to replenish the CCKBR protein in the purified islets (Fig. 4D), contrary to its effect on CCKBR mRNA expression (Fig. 4C).

**Estimation of somatostatin, insulin, and CCKBR protein expression by confocal microscopy.** As shown in Fig. 5, under transmission, diabetes dramatically reduced the size of pancreatic islets by at least 10-fold. Insulin treatment increased their size over time but never to that of a control islet, even after 21 days of treatment. The specificity of the CCKBR and somatostatin antibody signals was demonstrated by the loss of immunofluorescence when the peptide antigen (CCKBR) and the hormone somatostatin were incubated in the presence of their respective antibody. In normal islets, colocalization of the CCKBR (green fluorescence) occurred with somatostatin (red fluorescence) as a yellow signal (merged). In the diabetic animals, the loss of β-cells resulted in increased concentration of the CCKBR and somatostatin after a week, followed by a reduction at 14 days and a loss after 28 days of diabetes, a confirmation of the Western blot data presented in Fig 4D. In response to insulin, the CCKBR protein reappeared slightly during the hormonal treatment and remained at a low level thereafter, a behavior totally different from its mRNA content (Fig. 4C). Because the diabetic islets did not recover their normal size during insulin treatment, it is difficult to estimate their somatostatin content. Indeed, the confocal images seem to indicate that the red immunofluorescence is not as bright during insulin treatment as it is in diabetic islets, a sign of reduced somatostatin content, as is observed with total pancreas content evaluation (Fig. 2C). Cessation of insulin did not change the pattern of CCKBR expression but increased somatostatin content when compared with 21 days of insulin treatment. Recovery of somatostatin after insulin cessation corresponds with the increased contents of the hormone observed in Fig. 2C. As shown in Fig. 5, diabetic animals lost their islet insulin early, and their
hormone content did not recover during insulin treatment. The specificity of the insulin antibody is evident from the image obtained with preincubation of the antibody with insulin.

DISCUSSION

In this study, we show the importance of insulin as a regulator of pancreatic δ-cell activity focused on somatostatin and CCKBR metabolism. Our data confirm the initial observations that pancreatic somatostatin mRNA expression (15) and somatostatin content in STZ-induced diabetic animals (1,2,5) and in spontaneously diabetic mice (11,12) were significantly increased, with a return to control levels during insulin treatment (15). However, some spontaneous diabetic mice also exhibited decreased pancreatic somatostatin content (13,14), a finding that remains unexplained. Among our original data, it was demonstrated that 1) the major variations observed during the different treatments on somatostatin mRNA expression in total pancreas samples were identical to those obtained in purified islets, 2) the increases and decreases in somatostatin mRNA were comparable and in synchrony with those of total gland somatostatin content, 3) insulin negatively and directly modulated pancreatic somatostatin mRNA expression and its hormone contents, possibly through the IGF-1 receptor, 4) diabetes resulted in progressive losses in CCKBR mRNA and protein in total pancreas and purified islets, with return to control values in mRNA but not in receptor protein during insulin treatment, and 5) finally, the modifications observed at the biochemical level can be corroborated by our confocal microscopy analysis, which has never been done previously.

The validity of our data on somatostatin and CCKBR variations observed in this study depends on the demonstration that diabetes occurred when induced, that it could be controlled by insulin treatment, and that it reappeared upon cessation of treatment. Our results on body weight decreases, hyperglycemia, and hypertriglyceridemia following STZ injection clearly indicate that diabetes was established early and sustained for 28 days. Furthermore, the observation that all of these parameters were normalized during insulin treatment and became abnormal again after insulin cessation stressed the diabetes status of these animals (41). Finally, the disappearance of pancreatic amylase during diabetes, its return to normal values during insulin treatment, and its loss again after ending insulin treatment support previous data on the effects of diabetes on the pancreas (40), along with the microscopy data showing shrinking of the islets and loss of insulin.

Our data clearly indicate that pancreatic somatostatin mRNA expression is strongly disturbed soon after diabetes induction, and evident more so when RNA samples were extracted from purified islets. These increased contents were also rapidly normalized within a week of insulin treatment.
A- Total pancreas RNA

B- Total pancreas membranes

C- Purified islets RNA

D- Purified islets
treatment and rebounded after insulin cessation. It is quite interesting to observe that the variations observed in the course of this study on somatostatin mRNA were paralleled by similar changes in somatostatin total pancreatic contents. This observation suggests that these modifications in contents reflect changes in somatostatin synthesis more than in somatostatin accumulation due to inhibition of secretion. This last possibility is doubtful because increased somatostatin secretion was previously observed (8) in alloxan-induced diabetic rats. Therefore, if controls occur at the somatostatin mRNA and protein synthesis level, then what factors are responsible? Earlier studies suggested that glucose could be involved; indeed, high glucose stimulated somatostatin release from monolayer cultures of neonatal rat pancreas (17) and from rat isolated islets (42), observations not confirmed in another study (43). Increased somatostatin secretion could trigger somatostatin synthesis, and glucose was shown (44) to regulate pancreatic preprosomatostatin I expression as it increased somatostatin release from rainbow trout Brockmann bodies. In normal and diabetic dogs, however, the intravenous administration of exogenous insulin immediately reduced basal somatostatin release, an effect that seems independent of blood glucose level because it occurred in both normal and hyperglycemic conditions and happened before any change in blood glucose level (19,45). Interestingly, long-term insulin treatment was associated with decreased pancreatic somatostatin content and somatostatin mRNA expression (15 and this study) in conditions of normalized glycemia; these data thus suggest that insulin is involved in the regulation of somatostatin gene transcription. Our data on the presence of IGF-1 receptors on normal and diabetic islets suggest that insulin may operate through this receptor. Its reduction during diabetes can be explained by the major loss in β-cells following STZ administration, confirmed by confocal microscopy (Fig. 5). The presence of the IGF-1 receptor on the RIN-14B cells and the drastic and rapid inhibitory effect of insulin on somatostatin mRNA expression in these cells strongly suggest a direct action of insulin. Recently, it was shown for the first time that the CCK₉Rs were present on the endocrine somatostatin δ-cells in six different species (28,29). In this study, we present for the first time evidence that the CCK₉R mRNA and protein expressions are modulated differently from somatostatin during diabetes, including receptors mea-
sured in total gland and in purified islets. The observations that the receptor protein remained in total pancreas membrane during insulin treatment while disappearing from purified islets strongly suggest that they are not uniquely localized on the δ-cells. Indeed, our most recent data indicate its presence on the rat pancreatic acinar cells (46); this receptor population could also be affected by diabetes. This needs to be verified on purified acinar cells that are free of islets. The loss of islet CCKR protein during diabetes and its failure to reappear during insulin treatment could be explained according to the following two possibilities, which at the moment remain speculative. First, convertase enzymes could be activated during diabetes development and then digest the external NH₂-terminal section of the receptor protein. If so, antibody 9262, which specifically recognizes this part of the protein, would fail to detect the receptor. Second, diabetes would destabilize the CCKR mRNA due to defects in chaperone proteins. This could result in the translation of a CCKR truncated protein. Such modifications in chaperone proteins have been previously observed (47) during diabetes and resulted in disturbed translation processes involving large mRNA.

If somatostatin secretion is stimulated by the CCK agonists gastrin and its analogs (48), accumulation of pancreatic somatostatin content in the diabetic animals (Fig. 2) could be partially explained by the drastic reduction in islet CCKR protein, as is observed in Fig. 4. The observation that somatostatin content continued to be modulated under insulin treatment and its subsequent cessation in the absence of CCKR proteins in islets strongly suggests that this receptor might not be directly involved in somatostatin synthesis and secretion, although this assumption remains to be investigated.

In conclusion, this study clearly demonstrated that the expression of pancreatic somatostatin and the CCKR associated with islet δ-cells are closely regulated by insulin in diabetes. Insulin can at least negatively control somatostatin expression with positive action on CCKR mRNA and somatostatin expression in these somatostatin cells. An insulin-responsive element was also found on the rat somatostatin gene, and studies are underway to determine the effects of its deletion on somatostatin mRNA expression. The physiological importance of our data are the demonstration that insulin can control the expression and synthesis of one of its most potent inhibitors. Although we do not have any evidence yet, it remains possible that high pancreatic somatostatin levels are involved in the course of type 2 diabetes development as insulin becomes less and less efficient.

REFERENCES


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