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Lack of effect of incretin hormones on insulin release from pancreatic islets in the bile duct-ligated rats

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Niwa, Tae, Yuji Nimura, and Ichiro Niki. Lack of effect of incretin hormones on insulin release from pancreatic islets in the bile duct-ligated rats. Am J Physiol Endocrinol Metab 280: E59–E64, 2001.—Hyperglycemia associated with obstructive jaundice seriously affects the prognosis of patients with hepatobiliary diseases. We investigated secretory properties of isolated islets from bile duct-ligated (BDL) rats. Pancreatic islets from BDL rats lost their secretory responses to glucagon-like peptide-1 (GLP-1), although their responses to glucose were normal. Loss of potentiation of insulin release was also observed in glucagon and glucose-dependent insulinotropic peptide (GIP), whereas modulation of the release by forskolin, dibutyryl cAMP, or epinephrine remained unaffected. cAMP production by BDL islets was not increased by these insulinotropic hormones. Serum levels of glucagon, but not GIP, were increased in BDL rats. GLP-1 levels were also elevated, although they did not reach statistical significance. Immunoblotting of trimeric G protein subunits demonstrated that Gs-αL and Gs-αS, but not Gs-α1/2 and Gs-α3/α6, were less expressed in BDL islets. Therefore, unre sponsiveness of the β-cell to cAMP-raising hormones is involved in glucose intolerance under cholestasis. It results from diminished expression of α-subunits of the relevant G protein, Gs, and desensitization of receptors of these hormones. Cholestasis; pancreatic β-cell; glucagon-like peptide-1; trimeric G protein; adenosine 3′,5′-cyclic monophosphate

IMPAIRMENT OF LIVER FUNCTIONS causes abnormal carbohydrate metabolism both in patients with obstructive jaundice and in various experimental animals with bile duct ligation (18). Resultant hyperglycemia is one of the crucial factors in deciding prognosis of patients with liver diseases (15). The relevant mechanisms are not fully understood, although previous reports suggested a few possibilities to explain associated glucose intolerance.

Plasma insulin concentrations have been reported to be decreased in bile duct-ligated (BDL) dogs (13, 17, 25). Some morphological changes of pancreatic islet cells in BDL dogs have been demonstrated (17), and secretory response of the pancreatic β-cell to cholecystokinin showed deterioration in perfused pancreas in situ in BDL dogs (13). Extrapancreatic effects of cholestasis have also been shown: 1) specific binding activities of insulin and glucagon to hepatic membranes were decreased in BDL rats (10); 2) obstructive jaundice decreased glucagon-induced synthesis of cAMP, a second messenger responsible for multiple actions of glucagon, including cell proliferation, bile acid uptake, and gluconeogenesis, in hepatocytes from BDL hamsters (14).

In the present study, we examined secretory characteristics of isolated islets from BDL rat pancreata, and we found that pancreatic islets from BDL rats lost secretory responses to cAMP-increasing hormones, such as glucagon, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP). Here, we suggest that this loss of secretory responses results from selective decrease in expression of α-subunits of the trimeric G protein Gs and receptor downregulation for these hormones in the pancreatic β-cell.

MATERIALS AND METHODS

Materials. Collagenase (type V), leupeptin, IBMX, glucagon, GLP-1-(7–36)amide, epinephrine, forskolin, dibutyryl cAMP (DBcAMP), and bile acids (cholic acid, taurocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid) were purchased from Sigma (St. Louis, MO). Bovine serum albumin (fraction V) was from Chemicon International (Temecula, CA). GIP was from the Peptide Institute (Osaka, Japan). Bilirubin and phenylmethylsulfonyl fluoride (PMFSF) were from Wako (Osaka, Japan). The bilirubin assay kit was from Azwell (Osaka, Japan). The insulin radioimmunoassay kit was purchased from Eiken Chemical (Tokyo, Japan). The enzyme-linked immunoassay (ELISA) kits for cAMP, insulin, “active” GLP-1 (which recognizes the NH2-terminus of GLP-1), and GIP were from Cayman (Ann Arbor, MI). The enzyme-linked immunoassay (ELISA) kits for cAMP, insulin, “active” GLP-1 (which recognizes the NH2-terminus of GLP-1), and GIP were from Cayman (Ann Arbor, MI), Seikagaku Kogyo (Tokyo, Japan), and Linco Research (St. Charles, MO), and Peninsula Laboratories (Belmont, CA), respectively. Anti-G protein antibodies Gs-α and Gα1/2 were from Gramsch (Schwabhausen, Germany), and Gα3/α6 was from Biomol Research Laboratories (Plymouth Meeting, PA). Polyvinylidene difluoride (PVDF) membranes for Western blotting (Immobilon) were from Millipore (Bedford, MA).

Bile duct ligation. Male Wistar rats (body wt 200–250 g) underwent laparotomy under general anesthesia with diethyl ether. The common bile duct was identified and ligated close to the liver to avoid any congestion of pancreatic juice, because there is evidence that ligation of the pancreatic ducts...
affects secretory activity of the rabbit pancreatic β-cell (8) and causes atrophy of rat islet cells (19). Evident jaundice appeared 3–7 days after operation, and loss of body weight and dark-colored urine were also observed. For sham operation, only laparotomy was performed. Total bilirubin levels were 0.18 ± 0.023 (SE), 0.25 ± 0.08, and 6.37 ± 0.57 mg/dl for normal (nonoperated), sham-operated, and BDL rats, respectively, significantly higher in the BDL group than in the other two groups, *P < 0.05, significantly greater than the other 2 groups.

Blood sampling for intraperitoneal glucose tolerance test and hormone assay. After rats fasted overnight, an intraperitoneal glucose tolerance test was carried out. Glucose (2 g/kg body wt) dissolved in saline was injected intraperitoneally. Blood glucose levels were measured by a compact glucose analyzer (MediSafe, Terumo, Tokyo, Japan) at 0 (before), 30, 60, and 120 min after injection. Serum samples for glucagon, GLP-1, and GIP assay were taken by cardiac puncture. Glucagon and GIP were measured by RIA, and active GLP-1 was measured by ELISA.

Islet isolation and insulin release. Pancreatic islets were isolated using collagenase digestion from normal, sham-operated, and BDL rats fed ad libitum. Insulin contents of isolated islets were not affected by the BDL or sham operation [106 ± 8, 85.2 ± 10, and 93 ± 6 ng/islet for nonoperated, sham-operated, and BDL rats, respectively, n = 6, not significant (NS)]. Groups of five size-matched islets were preincubated at 37°C for 1 h in 1 ml of HEPES-buffered Krebs-Ringer solution containing (in mM): 119 NaCl, 4.75 KCl, 5 NaHCO₃, 2.54 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 20 HEPES (pH 7.4 with NaOH), with 5 mg/ml BSA and 3 mM glucose. After preincubation, isolated islets were incubated for 1 h under various conditions. The amount of insulin released in the media was measured by RIA with bovine insulin as a standard.

cAMP assay. After 1 h of the preincubation described above, 10 size-matched islets were incubated for 1 h at 37°C with 0.5 ml HEPES-buffered Krebs-Ringer solution and 5 mg/ml BSA, 1 mM IBMX (an inhibitor of phosphodiesterase), and various substances as indicated. At the end of the incubation, islets were transferred into 500 μl of 5% TCA. Extraction was performed by two freeze-and-thaw cycles in liquid N₂, and homogenization, followed by boiling for 5 min. Islet extracts were spun at 13,000 g for 5 min, and the supernatant was retained and stored at −20°C until assayed. cAMP assay was carried out using an ELISA kit. Without IBMX, cAMP levels in some batches were undetectable under nonstimulated conditions, and changes by the insulinotropic hormones were not detected.

Fig. 1. Blood glucose profiles after ip injection of glucose into normal, sham-operated, and bile duct-ligated (BDL) rats. Glucose (2 g/kg body wt) was administered ip at time 0 to normal (○, n = 5), sham-operated (△, n = 5), and BDL (●, n = 6) rats. Blood glucose levels were followed for the next 120 min, and the difference of blood sugar at each time point after glucose injection was analyzed. *P < 0.05, significantly greater than the other 2 groups.

Fig. 2. Dose-dependent effects for glucose (A) and glucagon-like peptide-1 (GLP-1, B) to increase insulin secretion from isolated pancreatic islets. A: 5 size-matched pancreatic islets from normal (○), sham-operated (△), and BDL (●) rats were preincubated for 1 h with substimulatory (3 mM) glucose, followed by another 1 h of incubation with different concentrations of glucose. For sham-operated and BDL groups, rats were killed 1–3 wk after the operation. Insulin released into the media was measured by RIA. Symbols represent means ± SE of 4–10 observations for normal (○), sham-operated (△), and BDL (●) islets. **P < 0.01, ***P < 0.001, insulin release by GLP-1 vs. glucose alone in each group.
Immunoblot analysis. Approximately 200–400 islets were isolated and homogenized in 20 mM MOPS (pH 7.4 with NaOH), supplemented with 1 mM PMSF and 10 μM leupeptin. The homogenates were centrifuged at 13,000 g for 2 min at 4°C to avoid nuclei and cell debris. The supernatants were denatured by SDS sample buffer and boiled for 5 min. Proteins in the sample were separated by SDS-PAGE and transferred to PVDF membranes in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (pH 9.2) at 200 mA for 1 h. After blocking with PBS containing 1% BSA, immunodetection was performed by a chemiluminescence kit (Amersham ECL, Amersham Pharmacia Biotech, Chalfont, Bucks, UK). Highly sensitive films (X-OMAT, Kodak, Rochester, NY) were then exposed to the transferred membranes and developed. Quantification of each band was carried out densitometrically using a computer program, NIH Image (version 1.59).

Statistical analysis. Statistical analyses for insulin secretion and cAMP contents were carried out by ANOVA. A P value < 0.05 was considered significant. For immunoblotting, significance between two groups was carried out by Student’s t-test.

RESULTS

Glucose tolerance and insulin levels in bile duct-ligated rats. Figure 1 depicts changes in plasma glucose levels in normal, sham-operated, and BDL rats after intraperitoneal injection of glucose (2 g/kg body wt). Fasting plasma glucose levels were similar among the three groups. However, plasma glucose levels at 30 min after glucose injection, but not at 60 or 120 min, were significantly higher in the BDL group than in the other two groups (P < 0.05). Under fasted conditions, the serum insulin level was higher in BDL rats than in the sham-operated ones (201 ± 44 pg/ml, n = 6, and 41 ± 20 pg/ml, n = 4, for BDL and sham-operated mice, respectively, P < 0.05), whereas the blood glucose levels were similar (86 ± 6 mg/dl, n = 6, and 86 ± 4 mg/dl, n = 5, for BDL and sham-operated mice, respectively, NS), suggesting the presence of insulin resistance in the BDL rats. Insulin levels at 30 min after glucose loading were higher in the BDL rats than in the control rats (1,103 ± 248 ng/dl for BDL, n = 6, and 569 ± 154 ng/dl for sham-operated mice, n = 5). However, when expressed as an insulinogenic index (changes of insulin concentrations divided by those in blood glucose concentrations during the 30-min duration), the values were similar (4.05 ± 0.90, n = 6, and 3.68 ± 1.13, n = 5, for BDL and sham-operated mice, respectively, NS). When insulin resistance is taken into consideration, these findings suggest a secretory defect of insulin in the BDL rats.

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Fig. 3. Modulation of insulin release by cAMP-related hormones and pharmacological substances. A: effects of hormones that increase [glucagon, GLP-1, and glucose-dependent insulinotropic peptide (GIP) at 100 nM] and decrease (epinephrine at 1 μM) cAMP levels in the β-cell were investigated in batch incubation. After preincubation, islets were incubated for 1 h with 10 mM glucose (G10) and specified hormones. B: effects of forskolin (5 μM) and dibutyryl cAMP (DBcAMP, 2 mM) were also evaluated. Each bar represents the mean ± SE for 5–15 observations for normal (open bars), sham-operated (hatched bars), and BDL (solid bars) groups. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. values with glucose alone in each group.

Fig. 4. Effects of bilirubin on insulin release by glucose and GLP-1. Batches of 5 islets isolated from normal rats were incubated with 10 mM glucose in the presence (hatched bars) or absence (open bars) of 100 mM GLP-1, with different concentrations (1–10 mg) of bilirubin. Each bar represents mean ± SE for 4 observations.
GLP-1 to potentiate insulin release induced by glucose (3–40 mM) was unchanged among the three groups (924 ± 6 ng/ml for normal, sham-operated, and BDL rats, respectively). However, the levels were significantly higher in BDL rats, 22, and 910 ng/ml for normal and sham-operated rats were 181 ± 6 ng/ml and DBcAMP (2 mM) (Fig. 3B) and inhibited by epinephrine (1 μM) (Fig. 3A). Because such cAMP-raising agents require the copresence of insulin secretagogues to enhance insulin release, we examined the effects of those incretins and pharmacological substances with glucose at 10 mM (180 mg/dl), the half-maximal concentration for insulin release. Insulin release by 10 mM glucose was enhanced by these insulinotropic hormones by 60–70% in the normal and sham groups, but such effects disappeared in the BDL group. In contrast, potentiation of insulin release by forskolin and DBcAMP was retained in islets from the BDL rats. Epinephrine was equally effective in inhibiting glucose-induced insulin release among these three groups. To identify relevant component(s) in the bile, we examined effects of cholic acid, taurocholic acid, deoxycholic acid, ursodeoxycholic acid, and chenodeoxycholic acid and bilirubin on insulin release. None of these bile acids at 100 μM affected insulin release by 10 mM glucose with or without 100 nM GLP-1 (data not shown). Bilirubin, up to 10 mg/dl, also failed to inhibit insulin release by glucose with or without GLP-1 (Fig. 4).

**Serum Levels of Incretins.** Serum glucagon levels in normal and sham-operated rats were 181 ± 3 pg/ml (n = 5) and 180 ± 8 pg/ml (n = 6), respectively. However, the levels were significantly higher in BDL rats (381 ± 43 pg/ml, n = 7, P < 0.01). Serum levels of GLP-1 were 9.3 ± 1.6 pM in normal animals (n = 5). GLP-1 levels in sham-operated rats were similar (12.1 ± 1.7 pM, n = 6). The levels in BDL rats were raised (21.5 ± 7.3 pM, n = 6), although they did not reach statistical significance. The serum GIP levels were similar among the three groups (924 ± 4, 898 ± 22, and 910 ± 13 pg/ml for normal, sham-operated, and BDL rats, respectively; n = 5–8, NS).

**Insulin Release by Glucose and GLP-1 from Pancreatic Islets Isolated from Normal, Sham-Operated, and BDL Rats.** Insulin release from pancreatic islets derived from normal, sham-operated, and BDL rats (1–3 wk after operation) is demonstrated in Fig. 2. Insulin release by glucose (3–40 mM) was unchanged among these groups (Fig. 2A). Figure 2B shows dose dependency for GLP-1 to potentiate insulin release induced by 10 mM glucose. In normal and sham-operated groups, significant enhancement by GLP-1 was seen within a nanomolar range. In contrast to glucose response, potentiation of insulin release by GLP-1 disappeared in isolated islets from the BDL rats.

**Hormonal Regulation and Pharmacological Modification of Insulin Release.** Figure 3 demonstrates glucose-induced insulin release enhanced by glucagon, GLP-1, and GIP at 100 nM (Fig. 3A) and by forskolin (5 μM) and DBcAMP (2 mM) (Fig. 3B) and inhibited by epinephrine (1 μM) (Fig. 3A). Because such cAMP-raising agents require the copresence of insulin secretagogues to enhance insulin release, we examined the effects of those incretins and pharmacological substances with glucose at 10 mM (180 mg/dl), the half-maximal concentration for insulin release. Insulin release by 10 mM glucose was enhanced by these insulinotropic hormones by 60–70% in the normal and sham groups, but such effects disappeared in the BDL group. In contrast, potentiation of insulin release by forskolin and DBcAMP was retained in islets from the BDL rats. Epinephrine was equally effective in inhibiting glucose-induced insulin release among these three groups. To identify relevant component(s) in the bile, we examined effects of cholic acid, taurocholic acid, deoxycholic acid, ursodeoxycholic acid, and chenodeoxycholic acid and bilirubin on insulin release. None of these bile acids at 100 μM affected insulin release by 10 mM glucose with or without 100 nM GLP-1 (data not shown). Bilirubin, up to 10 mg/dl, also failed to inhibit insulin release by glucose with or without GLP-1 (Fig. 4).

**cAMP Production by Pancreatic Islets.** Increases of cAMP contents in pancreatic islets after 1 h of incubation with glucagon, GLP-1, and GIP are demonstrated in Fig. 5. These three hormones stimulated cAMP production in normal islets. Glucagon and GIP were also effective in increasing cAMP contents in the sham-operated group, whereas GLP-1 failed to cause a statistically significant increase. None of these hormones raised the islet cAMP contents in the BDL group.

**Immunoblotting of G Protein Subunits.** Figure 6 shows Western blotting of islet extracts from sham-operated and BDL rats with specific antibodies against trimeric G protein subunits. Immunoblotting with an antibody against Gα demonstrated two specific bands, GαL with an apparent molecular mass of 50 kDa and GαS with 45 kDa (Fig. 6, left). Although not shown in Fig. 6, contents of the two Gα subunits in normal islet
extracts were almost similar to those of sham-operated rats (the mean values for sham-operated islets from 4 independent experiments: 93.0 ± 4.9 and 90.7 ± 8.3% of those for the normal group for GαL and GαS, respectively). In BDL islets, however, both of the two subunits were less well expressed (GαL: 63.4 ± 5.1%, P < 0.005, and GαS: 62.7 ± 5.3%, P < 0.05, respectively). Neither the Gα1/2-specific band nor the Gα3/α-specific band was affected by the BDL operation (Fig. 6, middle and right).

**DISCUSSION**

Trimeric G proteins are distributed in the pancreatic β-cells and are involved in the regulation of insulin release by the action of various hormones and neurotransmitters (6). Insulinotropic hormones such as glucagon, GLP-1, and GIP potentiate insulin release via activation of Gs and resultant cAMP production. In the present study, we found that potentiation of insulin release and stimulation of cAMP production by these hormones were attenuated in the BDL rats.

Impairment of cAMP production has been reported in neonatal streptozotocin-diabetic rats (4), but the underlying mechanism in our cholestatic model seems to be distinct from that in the diabetic model, because insulin release and cAMP production by glucose were not affected in the present study. Moreover, sensitivities to cAMP-producing hormones were retained in the streptozotocin-treated rats. In the present study, the defect is not due to impairment of adenylate cyclase or any part of secretory machinery distal to cAMP production, because insulin secretion by the adenylate cyclase activator forskolin or the membrane-permeable cAMP analog DBcAMP was not deteriorated in the BDL rats.

We found that lowered expression of G protein subunits occurs in the BDL group; immunoblotting of subunits of the trimeric G proteins demonstrated that the α-subunits of Gα, GαL and GαS, were less detected in the BDL islets. It is interesting that loss of Gα-mediated response has been reported in hepatocytes from cholestatic models. Decreased cAMP production by glucagon and reduction of GαS expression have also been demonstrated in hepatocytes from BDL hamsters, although the other Gα subunit was unchanged (3). In contrast, functional loss of Gα in rat hepatocytes has been reported during cholestasis without changes in Gα expression levels (20). In that report, expression of Gα subunits was decreased in BDL hepatocytes, whereas these subunits were not influenced in the present study. Unaffected Gα expression in BDL islets is compatible with results from the present secretion experiments showing that inhibition of insulin release by epinephrine, which suppresses insulin release via G and Gα (11), remained in BDL islets.

Obstructive jaundice has been reported to cause changes in plasma concentrations of incretins; plasma glucagon levels are increased in both patients and animals with bile duct obstruction (9, 12). Indeed, circulating glucagon levels were raised in the BDL rats in the present study. Such was not the case for GLP-1 and GIP, although some stimulation may be required, as suggested for elevated levels of fat-induced GIP in cholestatic human subjects (24). It has been reported that glucagon injection into cholestatic animals resulted in abnormal responses (9). Because the incretin receptors are known to cause downregulation in pancreatic β-cells and other types of cells (7, 16, 22, 23), it is possible that impairment of insulin release under cholestasis is, at least in part, due to desensitization of these receptors. Heterozygous disruption of the GLP-1 receptor gene resulted in a pattern of plasma glucose profiles similar to that for the BDL animals: 1) no difference was observed in fasting glucose levels, and 2) significant increases in the glucose levels appeared only 30 min after glucose loading (21), although the administration route was different, intraperitoneally for BDL rats in the present study vs. orally for the GLP-1 receptor knockout mice.

Several bile acids are known to be increased under cholestasis (14), and some of them are suggested to impair cAMP formation in hamster hepatocytes, human fibroblasts, and endothelial cells (1, 2). We therefore examined effects of various bile acids and bilirubin on insulin secretion by glucose and GLP-1. However, we could not find any effects of bile acids in our attempt. Bilirubin at a high concentration (10 mg/dl) did influence insulin secretion, but inhibition of GLP-1 potentiation did not take place. Biliary component(s) relevant to such alteration in islet functions still remain(s) unidentified, although we cannot eliminate a possibility that these components may exhibit some effects after prolonged exposure.

Impairment of glucose tolerance associated with obstructive jaundice is a serious complication and a decisive factor for prognosis of the patients. Previous publications and the present study demonstrate that attenuated secretory response to Gα-mediated hormones occurs under cholestasis in two major organs for glucose homeostasis, the liver and the pancreatic islets. We also suggest that a variety of systematic disorders may result from disturbance of G protein-mediated signal transduction in patients with obstructive jaundice.

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