Regulation of Insulin Secretion in Relation to Nitric Oxide, Carbon Monoxide and Acid alpha-Glucoside Hydrolase Activities

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Recent investigations have demonstrated that under normal physiological conditions the islets of Langerhans have the capacity to produce significant amounts of the gases nitric oxide (NO) (1–8) and carbon monoxide (CO) (9–12). There is now growing evidence that these gaseous molecules serve as novel signaling messengers in the regulation of insulin and glucagon secretion (1–23). The enzymes responsible for these signaling gases are for NO, the neuronal isoform of constitutive NO synthase (ncNOS); and for CO, the constitutive isoform of heme oxygenase (HO-2). Both ncNOS and HO-2 have been found to reside in all four main types of endocrine cells in the islets, i.e. the insulin-producing β-cells and the glucagon-producing α-cells, and in the somatostatin cells and the pancreatic polypeptide cells (10–12, 23, 24). Most data speak in favor of ncNOS-derived NO being a negative modulator of glucose-stimulated insulin secretion, whereas HO-2-derived CO is stimulatory in this regard (1–14, 17–20, 22, 23). It should be recalled that also the inducible forms of NOS (iNOS) and HO (HO-1) have been shown to occur in the β-cells under certain pathophysiological conditions, and iNOS in the β-cell is proposed to be implicated in the development of type 1 insulin-dependent diabetes, by means of its ability to produce large, cytotoxic amounts of NO (5, 6, 8, 11, 12, 24–29). In contrast, CO and bilirubin produced by HO-1 have been suggested to be protective agents in this context (11, 12, 29, 30).

We have previously hypothesized (5, 6, 8, 10–12, 14, 17, 20, 31) that dysregulation of the production of NO and/or CO in the β-cell might be of importance for the development of type 2 non-insulin-dependent diabetes. The mildly diabetic Goto-Kakizaki (GK) rat, an animal model of the nonobese type 2 diabetes (32), has previously been suggested to be equipped with multiple abnormalities in the mechanisms of glucose-stimulated insulin release such as abnormal glucose and glycogen metabolism, deficient activity of FAD-linked glycero-3-phosphate dehydrogenase, increased expression of protein-tyrosine phosphatase, defective K_{ATP} channel activity, and impaired [Ca^{2+}] handling (33–40). However, the results reported from different laboratories are often contradictory and the nature of the defects in glucose-induced stimulus secretion coupling is still unclear (33–38). Thus, in the present investigation, we have attempted to evaluate whether a dysfunction of islet CO production might play a role in the impairment of glucose-stimulated insulin release seen in the GK rat.
Materials and Methods

Chemicals

The RIA kits for insulin determination was obtained from Diagnostica (Falkenberg, Sweden). Collagenase was obtained from Wrothington Biochemicals (Freehold, NJ). BSA was purchased from ICN Biomedicals (High Wycombe, UK). Hemin-HCl and \( \beta \)-reduced nicotinamide-adenine dinucleotide phosphate were from Sigma Chemical (St. Louis, MO). The antisera to HO-1 (OSA 100) and HO-2 (OSA 200) were from StressGen Biotechnol (Victoria, British Columbia, Canada). All other chemicals were from British Drug Houses Ltd. (Poole, UK) or Merck AG (Darmstadt, Germany).

Animals

Young male GK rats of the Stockholm colony and Wistar controls (B&K Universal, Sollentuna, Sweden), weighing 150–200 g, were used. They were fed a standard pellet diet (B&K Universal) ad libitum with free access to water. The animal experiments were approved by the local animal welfare committee (Lund, Sweden).

Immunocytochemistry

Pancreatic glands were dissected, divided into pieces, and processed for immunocytochemical demonstration of HO-2 as previously described (10, 24). Cryostat sections, cut at a thickness of 8 μm, were incubated overnight with rabbit antisera to HO-2 (OSA 200) (1:1000). After rinsing the sections were incubated for 90 min with fluorescein isothiocyanate- or Texas Red-conjugated donkey antirabbit IgG (IgG), rinsed, and mounted. The HO-2 antiserum was diluted with PBS. In control experiments no immunoreactivity could be detected in sections incubated in the absence of HO-2 antisera or with antisera absorbed with excess of the HO-2 immunizing antigen (100 μg/ml). Epi-illumination and appropriate filter settings for Texas Red and fluorescein isothiocyanate immunofluorescence were used in the microscopic examination of the sections.

Western blot analysis

Approximately 150 freshly isolated islets were collected in Hanks’ buffer (100 μl) and sonicated on ice (3 × 10 sec). Homogenate samples, representing 10 μg of the islet protein, were then run on 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes by electrotransfer (10–15 V, 60 min) (semidry transfer cell, Bio-Rad, Richmond, CA). The membranes were blocked in 9 mmol/liter Tris-HCl (pH 7.4) containing 5% nonfat milk powder for 40 min at 37 C. Immunoblotting with rabbit antiserum to HO-1 (OSA 100) (1:500) and HO-2 (OSA 200) (1:1000) was performed for 16 h at room temperature. The membrane was washed twice with excess of the HO-2 immunizing antigen (100 μg/ml) and then incubated with alkaline phosphatase-conjugated goat antirabbit IgG, rinsed, and mounted. The HO-2 antiserum was diluted with PBS. In control experiments no immunoreactivity could be detected in sections incubated in the absence of HO-2 antisera or with antisera absorbed with excess of the HO-2 immunizing antigen (100 μg/ml). Epi-illumination and appropriate filter settings for Texas Red and fluorescein isothiocyanate immunofluorescence were used in the microscopic examination of the sections.

Assay of islet HO activity

Islets were isolated with the aid of collagenase and used for gas chromatographic measurements of the CO production essentially as described by Henningson et al. (9, 10). This procedure was performed either directly after islet isolation, \textit{ex vivo}, or after a 60-min incubation of the islets at low (3.3 mmol/liter) and high (16.7 mmol/liter) glucose, respectively. Approximately 300 islets were then collected in 200 μl ice-cold 0.1 mmol/liter phosphate buffer (pH 7.4) and frozen immediately at −20 C. When assayed, the islets were sonicated on ice, and 30 μl methemalbumin, 100 μl \( \beta \)-reduced nicotinamide-adenine dinucleotide phosphate (4 mg dissolved in 1 ml 0.1 mmol/liter phosphate buffer), and 2 mg hemoglobin were added with phosphate buffer up to a final volume of 1 ml. Hemoglobin was included in the assay mixture to trap the liberated CO. The methemalbumin solution (substrate) was prepared by dissolving 25 mg hemin, 82.5 mg NaCl, and 12.1 mg Tris base in 5 ml 0.1 mmol/liter NaOH, followed by the addition of 5 ml albumin solution (20 g/liter) and 5 ml distilled water. No glucose was included in the assay mixture, the assay time being only 6 min (9, 10). The homogenate was thereby incubated in a water bath at 37 C while protected from light. Aliquots (330 μl) were thus taken after 6 min of incubation, which was terminated by placing the tubes on ice. Appropriate blanks were included. The samples were then injected into reaction tubes containing ferricyanide-citric acid (100 μl). Nitrogen was used as a carrier gas as well as to purge the reaction vessels for 4 min before the samples were injected into them. After a reaction time of 4 min, the liberated CO was brought to a nickel catalyst and allowed to react with H2 to give methane, which was brought further to the detector. CO (99.9%) was used as a standard. The amount of CO produced was calculated from the area under the curve. Protein was measured in samples from the original homogenate as described by Bradford (41).

Hormone secretion

Freshly isolated islets (9, 10) were preincubated for 30 min at 37 C in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mmol/liter HEPES, 0.1% BSA, and 3.3 mmol/liter glucose. All islets isolated from GK and Wistar controls were always carefully matched. Each incubation vial contained 10 islets in 1.0 ml buffer solution and, unless otherwise stated, was gassed with 95% O2/5% CO2, to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium containing 3.3 or 16.7 mmol/liter glucose together with different test agents, and the islets were then incubated for 60 min. Aliquots of the medium were then removed and frozen for subsequent assay of insulin (9, 10, 42). For determination of insulin content, freshly isolated islets were extracted with acid ethanol as previously described (37). Hemin-HCl was dissolved in 0.1 mmol/liter NaOH, followed by titration with 0.1 mmol/liter HCl.

Effects of exogenously administered CO

One hundred milliliters of the incubation buffer was purged with helium and saturated with CO. The control buffer was saturated with helium. The solubility of CO is 2.3 ml/100 ml H2O (1 ml/liter). Islets were then incubated in these media as described above, except that the incubation vials were gassed with air instead of 95% O2/5% CO2.

Statistics

Statistical significance between sets of data were assessed using unpaired Student’s t test with Welch correction when appropriate, or where applicable, ANOVA followed by Tukey’s multiple comparisons test. Results are expressed as means ± SEM.

Results

Basal characteristics of Wistar controls and GK rats

The basal plasma concentrations of glucose and insulin of rats used in the present study are summarized as follows: plasma glucose levels in Wistar rats, 7.4 mmol/liter ± 0.4 vs. GK rats 10.5 ± 0.31 (P < 0.001); and plasma insulin levels in Wistar rats, 139.5 pmol/liter ± 22.4 vs. GK rats 111.3 ± 15.1 (not significant), n = 11–12; i.e. the concentration of glucose was significantly higher in GK rats than in Wistar controls, whereas no difference in insulin levels was found. Islets isolated from six Wistar and six GK rats showed no difference in insulin content; 6.06 ± 0.54 nmol/mg islet protein (Wistar) and 7.44 ± 0.72 nmol/mg islet protein (GK). There was no difference in protein content within islets isolated from Wistar vs. GK rats (data not shown).

Immunocytochemistry

In the islets of Langerhans of both GK and Wistar control rats, diffuse cytoplasmic HO-2 immunoreactivity surrounding dark unlabeled nuclei was observed in most endocrine
No overt difference in the number or distribution of immunolabeled cells or in the intensity of immunofluorescence could be noted between GK and Wistar control rats (Fig. 1).

**HO-derived CO production and Western blots of HO-1 and HO-2 in Wistar control and GK islets.**

Freshly isolated islets from GK rats displayed a prominent impairment of CO production (~50% reduction) compared with the Wistar controls (Fig. 2A). Western blots of HO-2 and HO-1 expression (Fig. 2B) revealed a clear reduction of HO-2 expression in GK islets compared with controls (with densitometric measurement, Wistar, 74.9 ± 3.1 vs. GK 38.9 ± 3.3 density U/mm²; P < 0.001, n = 4). Moreover, we found that GK islets expressed the inducible HO-1, whereas no expression of HO-1 could be detected in Wistar control islets (Fig. 2B).

**HO-derived CO production and insulin release from Wistar and GK islets incubated at low and high glucose.**

Isolated islets were incubated at low (3.3 mmol/liter) and high (16.7 mmol/liter) glucose, and islet CO production and insulin release were recorded. Figure 3A shows that there was a comparatively low CO production at basal glucose (3.3 mmol/liter) in GK islets compared with control islets. The increase in CO production in response to glucose was approximately 500 pmol/min/mg protein above basal levels for Wistar islets but only 200 pmol/min/mg protein above basal levels for GK rat islets. Similarly, the insulin response to glucose was highly impaired in GK islets, whereas basal insulin release at low glucose was of approximately the same magnitude in both types of islets (Fig. 3B).
was found at low glucose in the Wistar control islets. No effect of hemin or CO on insulin release from islets of the GK rat that was comparable to the hemin-induced response. The CO-stimulated increase was 4.5 ng/islet h for Wistar islets in the presence of hemin or CO gas at low and high glucose. Figure 4 shows that at 16.7 mmol/liter glucose, the addition of hemin (100 μmol/liter) induced a similar amplification of the insulin response to glucose in GK vs. Wistar islets. The hemin-stimulated increase of the insulin response (Δ) was 4.6 ± 1.2 ng/islet·h for Wistar islets vs. 3.1 ± 0.5 ng for GK islets (not significant). There was also a very slight stimulation of insulin release from GK islets by hemin at low glucose (Fig. 4). Addition of CO gas (100 μmol/liter) to the incubation media brought about an amplification of the glucose-stimulated insulin response from GK and Wistar islets that was comparable to the hemin-induced response. The CO-stimulated increase was 4.5 ± 1.1 ng/islet·h for Wistar islets vs. 3.2 ± 0.4 ng for GK (not significant). Hence, the potentiation of the glucose-stimulated insulin response in the presence of either hemin or CO gas was of similar magnitude in GK islets as in the Wistar control islets (Fig. 4). No effect of hemin or CO on insulin release was found at low glucose in the Wistar control islets.

### Discussion

The present data show that among the different stimulus secretion coupling abnormalities in glucose-stimulated insulin release previously described in the mildly diabetic GK rat (32–40) is now added a marked impairment of the CO-signaling pathway. Our previous data have shown that glucose is a strong stimulator of islet CO production and that CO in turn stimulates insulin release (9, 10). Now, we find that the well-known impairment of glucose-stimulated insulin response from islets of the GK rat is associated with a marked reduction of their CO production. Moreover, by comparison with the control rats, the CO production of GK islets directly isolated ex vivo was also significantly reduced. Visually, immunocytochemistry showed a similar fluorescence intensity and an equal distribution and number of HO-2 immunolabeled cells in GK vs. Wistar islets, thus revealing no abnormal redistribution or localized deficiencies explaining the reduced CO production in GK islets. However, immunoblotting showed that the decreased CO production was associated with a similarly reduced expression of HO-2 in the GK islets. Because we used the same antibody for immunocytochemistry and immunoblotting, these results illustrate that caution should be exercised in regarding the immunocytochemical data as quantitative. However, the immunocytochemical picture is rewarding in showing the morphological distribution of the HO-2 protein within the islets. Furthermore, we found that the GK islets also displayed a marked expression of HO-1. HO-1 is known to be expressed in response to noxious stimuli such as endotoxin, heavy metals, and also oxidative stress (12, 29, 30). HO-1 activity is then assumed to protect the cells through metabolizing heme to bilirubin, which is known to have strong antioxidant properties (30). Because the GK rat suffers from chronic hyperglycemia, there is reason to believe that their β-cells respond to this glucotoxicity with induction of HO-1. This would be in accordance with recent studies showing expression of HO-1 in freshly isolated islets from obese hyperglycemic mice (ob/ob) and partially pancreatectomized, hyperglycemic rats as well as in normal rat islets cultured for 18 h in high glucose (31, 43, 44).

We have previously shown that pancreatic islets display an unusually high production of HO-2-derived CO (9–12). In fact, islet production of CO by far exceeds that which usually observed in other tissues rich in HO-2 (45, 46). Moreover, both islet production of CO and glucose-stimulated insulin release were dose-dependently suppressed by the HO inhibitor Zn-protoporphyrin but unaffected by protoporphyrin, which lacks inhibitory effects on HO activities (9, 10). Furthermore, we have shown (9, 10) that in addition to glucose, the HO substrate hemin also was a potent stimulator of islet CO production and that adding hemin to isolated islets in concentrations of 1–100 μmol/liter was associated with a dose-dependent increase in glucose-stimulated insulin release. Although isolated islets apparently have a great capacity to increase the glucose-stimulated CO production in the absence of added hemin (9–12), it should be recalled that both hemin and Zn-protoporphyrin are normal plasma constituents (30), implying that physiological interactions with islet HO are conceivable also by means of the circulation, especially because the pancreatic islets are extremely well vascularized (47). Because the islet blood flow reportedly is much higher in the GK rat than in Wistar controls (47), it is less likely that the availability of circulating hemin should be a limiting factor explaining the reduced CO production in the GK islets. In this context, it should be recalled that young GK rats, used in the present study, reportedly showed no difference in the relative volume of the total endocrine pancreas and a preserved β-cell density compared with Wistar controls (48). Notably, comparing the approximate HO activity in isolated islets of different strains of rats, we have found that islet CO production in the GK rat (~200 pmol/min/mg
protein (present study) was much lower than in Wistar rats (~400–600 pmol/min/mg protein) (Ref. 9 and the present study) and Sprague Dawley rats (~700 pmol/min/mg protein) (Salehi, A., H. Mosén, R. Henningsson, and I. Lundquist, unpublished data). In further comparison, it should be emphasized that we have recently shown (31) that islets from the hyperinsulinemic ob/ob mouse displayed a very high CO production (~900 pmol/min/mg protein) that was associated with an impressive insulin response to glucose. Interestingly, this insulin response was profoundly suppressed by the selective HO inhibitor Zn-protoporphyrin (31), again indicating an important role of HO-derived CO in glucose-stimulated insulin release.

Our previous in vitro data in mouse islets (10) showed that islets incubated at high glucose produced more CO than did islets incubated at low, basal glucose. Thus, a glucose-induced stimulation of HO-2 activity apparently operates in mouse pancreatic islets. Similar results were obtained here in isolated rat islets. Notably, the glucose-stimulated CO generation was markedly reduced in the diabetic GK islets compared with the healthy Wistar islets. In fact, in accordance with the reduction of the glucose-stimulated insulin release, the GK islets displayed a 3 times lower CO production in comparison with Wistar islets when calculated per millimole of glucose increase from 3.3 to 16.7 mmol/liter. Due to the very low level of CO production at basal (3.3 mmol/liter) glucose in GK islets the fold increase at 16.7 mmol/liter was somewhat greater in these islets than in Wistar control islets. It is not inconceivable that the very low basal CO production in GK islets might reflect the inability of glucose to properly maintain the HO-2 activity at this low concentration of the sugar. The defective glucose stimulation of the HO-CO pathway in GK islets was further underlined by the observation that the CO generation in freshly ex vivo isolated islets was 50% reduced in the GK rat, and when calculated in relation to the concentration of plasma glucose, the CO production in GK islets was 3 times lower than in Wistar islets. However, the plasma insulin levels were not decreased in relation to the reduced CO production, showing that efficient compensatory mechanisms are operating in the in vivo situation. Hence, our accumulated data suggest, but do not give absolute proof, that an impaired stimulation of HO-derived CO by glucose might emerge as one of several possible defects of glucose-induced stimulus secretion coupling in the diabetic GK islets.

We have previously shown (10) that addition of gaseous CO to incubated islets resulted in significant enhancement of glucose-stimulated insulin secretion, an effect that could be reduced by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ). It should be noted that CO and hemin (9, 10) have negligible effects on insulin release at low glucose. Thus, these data suggested to us that the HO-CO signaling pathway might have a regulatory role in glucose-stimulated insulin release and acts, at least in part, through perturbation of the guanylate cyclase-cyclic GMP system (10). In the present study, we found that the impaired glucose-stimulated insulin response from GK islets could be amplified by the addition of either hemin or gaseous CO to the incubation media. A similar potentiating effect was recorded in Wistar islets, and both hemin and CO almost doubled the insulin responses to 16.7 mmol/liter glucose in both Wistar and GK islets. There was no significant difference between the ∆ insulin responses to hemin and CO in Wistar vs. GK islets. Hence, these results suggest, but do not definitely prove, that the working capacity of the HO enzymes and the transduction targets of the CO molecule in the stimulus secretion coupling are unimpaired in GK islets vs. Wistar islets. Instead, as pointed out above, our present data speak in favor of a major defect in glucose stimulation of the HO-CO pathway in the GK islets. Because there is a clear reduction of the expression of HO-2 in GK islets as compared with control islets, the decrease in glucose-induced CO production might be explained by the inability of glucose to stimulate the activity of HO-2 in the GK rat islets. Whether the reduced expression of HO-2 and the associated impairment of CO production in GK islets is a primary feature of the defective insulin response to glucose in these rats is still to be elucidated. However, it is notable that the reduced HO-2 activity in the islets of the GK rats does not seem to be a consequence of their in vivo hyperglycemia in a short time perspective because it could not be restored after 90 min incubation at low glucose. Moreover, we have very recently found that the expression and activity of islet HO-2 in the obese hyperglycemic mouse (ob/ob) is considerably increased and probably associated with their elevated glucose levels (31). Interestingly, the prominent expression of HO-1 in GK islets is apparently not sufficient to compensate for the decrease in HO-2 activity. The reason for this is presently unclear.

In conclusion, we show here for the first time that the mildly diabetic GK rat displays an impressive impairment of the islet HO-CO signaling pathway for glucose-stimulated insulin release. This impairment was manifested as a marked reduction of islet CO production associated with a reduced HO-2 expression. Instead, a compensatory and/or glucosprotective expression of islet HO-1 was observed in the GK islets. Because the glucose-stimulated insulin release from isolated GK and Wistar islets was equally amplified by addition of hemin or gaseous CO to the incubation media, there was probably no impairment of the mechanisms of action of CO in GK islets. Hence, our data suggest that an important defect in the stimulus secretion coupling of glucose-induced insulin release is to be found in a deficient transduction of glucose signaling to stimulate the HO-CO pathway in these islets.

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