Preserved initiatory and potentiatory effect of α -ketoisocaproate on insulin release in islets of glucose intolerant rats

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Summary Insulin responses to glucose and non-glucose secretagogues were studied in short-term cultured pancreatic islets and perfused pancreata of the glucose intolerant F₁ hybrid rats of spontaneously diabetic Goto-Kakizaki and control Wistar rats. After culture at 5.5 mmol/l glucose, hybrid islet responses to 11.1, 16.7 and 27.0 mmol/l glucose were between 60 and 40 % of control islet responses. A combination of 1 mmol/l isobutylmethylxanthine and 16.7 mmol/l glucose induced a pronounced insulin release, which was of similar magnitude in hybrid and control rat islets. This response was not further augmented by addition of glibenclamide and arginine. The slope of potentiation of arginine (10 mmol/l)-stimulated insulin secretion by glucose (5.5–16.7 mmol/l) was greatly impaired in hybrid islets. In contrast to glucose, α -ketoisocaproate (KIC), which is metabolized in Krebs cycle, dose-dependently stimulated insulin secretion to similar levels in hybrid and control islets, cultured at 5.5 mmol/l glucose. Also in hybrid islets depolarized by potassium chloride (30 mmol/l) and with ade-

nosine triphosphate-sensitive K⁺-channels kept open by diazoxide, insulin responses to glucose were greatly impaired but intact to KIC. Furthermore, KIC potentiated normally the insulin response to arginine in hybrid islets. In the isolated perfused pancreas, KIC induced similar insulin responses in hybrid rats and control rats. The potentiating effect by 5.5 mmol/l glucose on the KIC-stimulated insulin responses was, however, greatly reduced in isolated islets and absent in the perfused pancreata of hybrid rats. Taken together, these findings suggest an intact capacity for insulin release, although the initiating and potentiating effect by glucose on insulin release are defective in the Goto-Kakizaki-hybrid rats. An abnormal beta-cell glucose metabolism proximal to the Krebs cycle is likely to account for the impairment of insulin release. [Diabetologia (1998) 41: 1368–1373]

Keywords Insulin, arginine, α -ketoisocaproate, glibenclamide, isobutylmethylxanthine, isolated pancreatic islets, perfused rat pancreas, Goto-Kakizaki rat.

Glucose-stimulated insulin secretion is severely impaired in the Goto-Kakizaki (GK) rat, which is an animal model of hereditary non-obese Type II (non-insulin-dependent) diabetes mellitus [1–3]. F₁

hybrids of GK and healthy Wistar rats show nearly normal fasting blood glucose concentrations but impaired glucose tolerance and decreased insulin response to glucose, in the presence of a normal pancreatic insulin content [4]. The diabetic state is thus milder in F₁ hybrid rats than in GK rats, as indicated by the intermediate glucose tolerance and insulin responses to glucose in the perfused pancreas compared with those representative of GK and control rats [4].

The impaired insulin response to glucose in F₁ hybrid rats is most likely an inherited defect which is not related to intrauterine hyperglycaemia and glucose toxicity [4, 5]. The mechanism behind this defect

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Abbreviations: ATP, Adenosine triphosphate; cAMP, cyclic adenosine monophosphate; GK rat, Goto-Kakizaki rat; IBMX, isobutylmethylxanthine; KIC, α -ketoisocaproate; KRb, Krebs-Ringer bicarbonate buffer solution.

is not clear. To elucidate this issue, we have studied the maximal capacity to secrete insulin in isolated islets from 2–3 month old F₁ hybrid and control Wistar rats. Furthermore, we have compared islet insulin responses to glucose and α -ketoisocaproate (KIC), a substrate entering the Krebs cycle directly without involvement of anaerobic glucose metabolism. The effect of KIC was also evaluated in islets depolarized by 30 mmol/l potassium chloride and with adenosine triphosphate (ATP)-sensitive K⁺-channels kept open by diazoxide. Finally, the potentiatory effects of glucose and KIC on arginine-induced insulin secretion were investigated.

Material and methods

Animals. Female, 2–3 months old F₁ hybrid rats were bred in crosses between female Wistar rats and male GK rats, as described previously [4]. Age-matched female Wistar rats served as controls. The GK rats were bred within our colony, while the nondiabetic Wistar rats were obtained from B&K Universal (Sollentuna, Sweden). The animals were killed by decapitation after determining body weights and fed blood glucose concentrations (Reflolux S, Boehringer-Mannheim, Mannheim, Germany).

Isolated pancreatic islets. Isolation of islets was done aseptically by collagenase (Boehringer-Mannheim, Mannheim, Germany) digestion of the pancreata. Before incubations, islets were maintained for 24 h in tissue culture medium RPMI 1640 (SVA, Uppsala, Sweden), containing 5.5 mmol/l glucose, 10% heat-inactivated calf serum and antibiotics, to allow the islets to recover at normoglycaemic conditions after the isolation procedure. The cultured islets were preincubated for 30 min in Krebs-Ringer bicarbonate buffer solution (KRb), pH 7.4, gassed with 95% O₂/5% CO₂ and supplemented with 10 mmol/l N-2-hydroxyethylpiperazine-N-2 ethanesulphonic acid HEPES, 2 g/l bovine plasma albumin (Fraction V, Sigma Co.) and 5.5 mmol/l glucose. Thereafter, batches of three islets were incubated for 60 min in KRb as above but with additions of glucose and other insulinogogues, as given in the Tables. After incubations, aliquots of the medium were immediately taken for radioimmunoassay of insulin [6]. In some experiments, islets were collected after incubations for measurement of islet DNA by a fluorometric procedure [7]. These islets were washed two times in saline before sonication in batches of ten islets in 0.1 ml distilled water.

Isolated, perfused pancreas. Rats were anaesthetized with sodium thiopental (100 mg/kg, i.p.) and each pancreas was dissected free from adjacent tissues [8] and removed to a perfusion chamber. The perfusion medium was directed into the isolated pancreas through a cannula in the aorta by a non-recycling perfusion system with a flow rate of 2.8 ml/min. The basal perfusion medium consisted of KRb, supplemented with 20 g/l bovine plasma albumin. Following an equilibration period with a glucose-free medium (not shown in the Figures), 10 or 20 mmol/l α -ketoisocaproate (KIC) was added in the presence or absence of 5.5 mmol/l glucose during the test period. In another series of experiments, perfusions were done on pancreata from overnight fasted rats. Samples of the perfusate were collected in prechilled tubes, at intervals shown in the Figures, for radioimmunoassay of insulin.

Table 1. Insulin secretory capacity of hybrid and control rat islets

Additions to the medium (mmol/l)	Hybrid islets	Control islets
Glucose (5.5)	3.5 ± 0.5	5.2 ± 1.1
Glucose (16.7)	14.4 ± 1.5 ^a	23.3 ± 1.5
Glucose (16.7) + IBMX (1)	86.4 ± 9.7	90.2 ± 9.6
Glucose (16.7) + IBMX (1) + glibenclamide (2 · 10 ⁻³) + arginine (10)	107.8 ± 6.9	97.5 ± 7.9

Insulin secretion (μ U/islet per h) from islets cultured overnight at 5.5 mmol/l glucose. Mean ± SEM of islets from 5 hybrid and 5 control rats.

^a $p < 0.01$ vs control islets

Expression of results and statistical analysis. Results are given as means ± SEM. In batch incubations, the mean secretion of 3–4 batches of islets from each rat was regarded as a single observation. In the perfusion experiments, integrated insulin responses were calculated as areas under the curve, using the hormone concentration at start of the test period as basal value. Significance of differences between mean values was assessed by use of the Mann-Whitney rank sum test, or Student's *t*-test for paired or unpaired data, and applying Bonferroni's correction for multiple testing, as appropriate.

Results

Characterization of rats. Hybrid rats ($n = 37$) had similar body weights as control rats ($n = 38$) (227 ± 6 vs 219 ± 5 g) but somewhat higher fed blood glucose concentrations (6.9 ± 0.2 vs 5.5 ± 0.2 mmol/l, $p < 0.001$). The DNA content was not different between islets from hybrid and control rats (30.6 ± 3.1 ng/islet, $n = 8$, vs 29.1 ± 3.3 ng/islet, $n = 8$).

Insulin secretory capacity. After culture at 5.5 mmol/l glucose, the insulin responses to 16.7 mmol/l relative to 5.5 mmol/l glucose were increased in both hybrid and control rat islets (Table 1). The insulin response to 16.7 mmol/l glucose was, however, 37–48% less in hybrid than in control islets ($p < 0.01$) (Table 1, Fig. 1). Addition of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 1 mmol/l), to 16.7 mmol/l glucose elicited pronounced and similar insulin responses in hybrid and control islets and these responses were not further enhanced by the simultaneous stimulation with glucose, IBMX, glibenclamide (2 μ mol/l) and arginine (10 mmol/l) (Table 1).

Potentiating effect by glucose on arginine-induced insulin release. Insulin release was lower in hybrid than in control rat islets at glucose concentrations from 5.5 to 16.7 mmol/l (Fig. 1). The insulin responses to arginine in combination with 5.5, 11.1 and 16.7 mmol/l glucose were also lower in hybrid rat is-

Fig. 1 A, B. Insulin responses to glucose and arginine in hybrid rat islets (**A**) and control rat islets (**B**). Before incubations, islets were cultured for 24 h at 5.5 mmol/l glucose. Results ($\mu\text{U insulin} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$) are shown of incubations at different concentrations of glucose alone ($\blacktriangle$) and in the presence of 10 mmol/l arginine ($\bullet$). Mean $\pm$ SEM of 5–7 experiments in each group

lets, amounting to 31–46% of the control responses ($p < 0.05$, or less).

Comparison of effects of glucose and KIC on insulin secretion. In the next series of experiments, we compared effects of glucose and KIC on insulin responses. Similarly to experiments presented in Fig. 1, the glucose effect was decreased in hybrid islets, also at 27 mmol/l glucose, but KIC – in the absence of glucose –, dose-dependently stimulated insulin secretion to a similar extent in hybrid and control islets (Table 2). In the presence of a depolarizing concentration of KCl (30 mmol/l) and diazoxide (0.25 mmol/l), to keep the ATP sensitive K^+ -channels open, insulin responses to 5.5, 11.1 and 16.7 mmol/l glucose were again much lower in hybrid than in control islets, whereas the insulin responses to 5, 10 and 20 mmol/l KIC were similar (Table 3).

Notably, the addition of 5.5 mmol/l glucose to 10 mmol/l KIC increased insulin release from 8.5 ± 1.6 to $31.6 \pm 4.6 \mu\text{U/islet per h}$ ($n = 6$) in hybrid islets and from 10.8 ± 1.6 to $73.8 \pm 7.8 \mu\text{U/islet per h}$ ($n = 7$), the difference being significant ($p < 0.01$). The almost preserved KIC effect but its impaired potentiation by glucose was also evident in perfusions of isolated pancreata from F_1 hybrid rats fed ad libitum. In these experiments, following a preperfusion period in the absence of glucose, 10 mmol/l KIC alone induced similar insulin responses ($7\,870 \pm 1\,710$ vs $11\,120 \pm 1\,340 \mu\text{U/15 min}$) in hybrid and control pancreata, respectively (Fig. 2). The combination of 10 mmol/l KIC and 5.5 mmol/l glucose resulted in an enhanced insulin response in control pancreata ($16\,600 \pm 1\,420 \mu\text{U/15 min}$; $p < 0.02$ vs experiments

Table 2. Effects of glucose and KIC on insulin secretion from hybrid and control islets

Additions to the medium (mmol/l)	Hybrid islets	Control islets
Glucose (3.3)	2.2 ± 0.4	3.0 ± 0.4
Glucose (5.5)	3.3 ± 0.4	5.1 ± 1.0
Glucose (11.1)	5.8 ± 0.6^a	9.6 ± 1.7
Glucose (16.7)	11.9 ± 1.3^b	24.8 ± 3.0
Glucose (27.0)	13.3 ± 1.5^c	29.3 ± 3.2
KIC (5)	5.0 ± 0.7	6.9 ± 0.8
KIC (10)	9.5 ± 1.6	12.8 ± 1.6
KIC (20)	28.4 ± 4.2	30.6 ± 4.8

Insulin secretion ($\mu\text{U/islet per h}$) from islets cultured overnight at 5.5 mmol/l glucose. Islets were incubated at different concentrations of glucose or KIC (α -ketoisocaproate); no glucose was added with KIC. Mean $\pm$ SEM of 6–7 rats of each kind.

^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs control islets

Table 3. Effects of glucose and KIC on insulin secretion from hybrid and control islets depolarized by KCl (30 mmol/l) and at 0.25 mmol/l diazoxide

Additions to the medium (mmol/l)	Hybrid islets	Control islets
Glucose (3.3)	8.7 ± 1.5	12.2 ± 1.7
Glucose (5.5)	10.6 ± 1.5^a	22.4 ± 2.8
Glucose (11.1)	26.6 ± 3.5^b	80.0 ± 11.4
Glucose (16.7)	41.8 ± 5.3^b	118.6 ± 15.2
KIC (5)	20.8 ± 2.2	26.2 ± 3.1
KIC (10)	53.3 ± 6.0	51.5 ± 6.7
KIC (20)	98.7 ± 8.7	116.2 ± 18.9

Insulin secretion ($\mu\text{U/islet per h}$) from islets cultured overnight at 5.5 mmol/l glucose. Islets were incubated at different concentrations of glucose or KIC (α -ketoisocaproate); no glucose was added with KIC. Mean $\pm$ SEM of experiments in 6–7 rats of each kind.

^a $p < 0.01$, ^b $p < 0.001$ vs control islets

with KIC only) but not in hybrid pancreata ($7\,140 \pm 2\,430 \mu\text{U/15 min}$) (Fig. 2). When pancreata from rats fasted overnight were perfused with the medium containing no glucose but 20 mmol/l KIC, the insulin release was fully preserved in hybrids as compared with controls ($26\,660 \pm 1\,075$, $n = 4$, vs $26\,548 \pm 1\,123 \mu\text{U/20 min}$, $n = 4$).

Potentiation by KIC of arginine-induced insulin release. The potentiating effect of KIC on arginine-stimulated insulin secretion was studied in isolated islets cultured at 5.5 mmol/l glucose. Addition of 5, 10, or 20 mmol/l KIC to 10 mmol/l arginine induced a pronounced and similar potentiation of arginine-stimulated insulin release in hybrid and control islets (Fig. 3).

Fig. 2 A, B. Insulin responses to 10 mmol/l ketoisocaproate (KIC) in the absence (**A**) and presence (**B**) of 5.5 mmol/l glucose in the perfused isolated pancreas of hybrid ($n = 4$; ■) and control ($n = 4$; □) rat. In both series of perfusions, the glands were preperfused for 30 min with a buffer containing no glucose. Mean $\pm$ SEM

Discussion

We have confirmed that isolated pancreatic islets of GK hybrid rats exhibit an impaired insulin response to glucose [4, 5]. This defect was more pronounced when insulin responses of hybrid and control rats were compared in vivo or in the perfused pancreas [4]. Similar observations were made in comparisons of isolated islet and perfused pancreas preparations of GK rats [3] and neonatally streptozotocin-injected rats (nSTZ) [9–11], another rat model of Type II diabetes. The reason for this discrepancy between different preparations is not known.

Our previous studies suggest that the impaired insulin release in hybrid rats is not due to lack of beta-cells. Firstly, the pancreatic content of insulin was normal in hybrid pancreata [4]. Secondly, since a normal beta-cell density was shown in young GK rats [12], it is not plausible that the beta-cell density would be other than normal in islets of young hybrid rats. The present study extends these observations and shows that islet DNA content is no lower in hybrid than in control rat islets. Moreover, despite the greatly impaired glucose-stimulated insulin release, a normal capacity to release insulin is maintained in hybrid rat islets. Thus, the addition of the phosphodiesterase inhibitor, IBMX, to 16.7 mmol/l glucose induced insulin responses which were high and equal in hybrid and control islets, indicating that the enhanced generation of cyclic adenosine monophosphate (cAMP) can compensate for the defective

secretory response to glucose per se. This finding correlates with our recent finding in the perfused pancreas and isolated islets of GK rats, that generation of cAMP by forskolin restores the insulin response to 16.7 mmol/l glucose and, furthermore, elicits a pronounced insulin release also at 3.3 mmol/l glucose [13]. It has been shown that cAMP induces protein phosphorylation in beta cells, especially through activation of protein kinase A [14, 15]. Thus, in such a manner cAMP-induced activation of protein kinase A could interact with other proteins of importance for the exocytotic process [16].

The addition of arginine, which induces insulin secretion by depolarization of the beta cell due to intracellular accumulation of the cationic amino acid [17] and glibenclamide which directly closes the ATP-sensitive K^+ channels by binding to the sulphonylurea receptor [18], did not enhance the insulin response to glucose plus IBMX. In a previous study in healthy man, pretreatment with aminophylline, another phosphodiesterase inhibitor, potentiated the insulinogenic potency of arginine [19]. It is likely that the high concentration of IBMX used in the present study increased the beta-cell cAMP levels by far above those induced by aminophylline dose in the in vivo study. This would at least partly explain the lack of a further augmentation by arginine of the insulin response to glucose plus IBMX. Arginine has been shown to induce a normal or even exaggerated insulin response in the perfused pancreas of GK as well as hybrid rats [2, 4, 5]. In these studies, however, it was not possible to rule out a potentiating effect of glucose on arginine induced insulin release by previous exposure to the hexose in GK and hybrid rats.

Recently, it has been shown that glucose also stimulates insulin release from islets depolarized by a high potassium concentration and with the ATP-sensitive K^+ -channels kept open by diazoxide [20]. These data were interpreted as indicating that glucose metabo-

Fig. 3 A, B. Potentiation by KIC of arginine-induced insulin release from hybrid rat islets (**A**) and control rat islets (**B**). Before incubations, islets were cultured for 24 h at 5.5 mmol/l glucose. Results ($\mu\text{U insulin} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$) are shown of incubations at different concentrations of KIC alone ($\blacktriangle$) and in the presence of 10 mmol/l arginine ($\bullet$). Mean $\pm$ SEM of 6 experiments in each group

lism directly enhances exocytosis of insulin. The present study shows that this important pathway, underlying the insulinotropic action of glucose, is impaired in hybrid islets. In this context our recent finding, that glucose-induced insulin release is also greatly inhibited under depolarized conditions in both isolated islets and perfused pancreas of GK rats, is noteworthy [13].

By comparing effects of glucose and KIC in islets from hybrid and control rats, we investigated whether substrates entering directly into the Krebs cycle, and thereby circumventing the glycolytic pathway, exert a normal or defective insulin response in hybrid rats. Since KIC, unlike glucose, induced a comparable insulin secretion in islets of hybrid and normal rats, it is likely that the defect responsible for impaired transduction of the glucose signal in hybrid rat islets resides in the glycolytic pathway. This hypothesis is further supported by findings in the isolated perfused pancreas, where KIC-induced insulin release was preserved in the hybrid rats, whereas the addition of glucose further enhanced the response in control but not in hybrid rats. In islets of GK rats of our colony, the oxidation of glucose has been shown to be enhanced [21]. Moreover, the oxidation rates of leucin, the amino acid precursor of KIC, and acetate were increased by 40% in GK relative to control rat islets, and the capacity of GK rat islets mitochondria to generate ATP was nearly normal [21]. Hence, there is no reason to anticipate that the oxidative metabolism of glucose, but not of KIC, is decreased in hybrid rat islets.

The preserved potentiatory effect of KIC on arginine-stimulated insulin release in hybrid rats also supports a notion of normal islet metabolism of this substrate. The finding of a similar insulin response to

KIC in hybrid and control islets, which were depolarized with KCl and with the ATP sensitive K^+ -channels kept open by diazoxide, shows that KIC – presumably after being metabolized – directly stimulates exocytosis of insulin and that this pathway is intact in hybrid islets. This finding under depolarized conditions is especially important because KIC has been shown, in the inside-out configuration of patch clamp, to close ATP sensitive K^+ -channels of normal beta cells (O. Larsson, personal communication) which implies that the amino acid could induce depolarization of beta-cell membrane and subsequent release of insulin without its metabolism.

In contrast to hybrid rats, insulin release induced by KIC was decreased in GK rats from our colony [22]. Similarly, insulin responses to KIC and leucine were decreased in GK rats from the Paris colony [23, 24]. The ATP-sensitive K^+ -channels in GK rat beta cells have, however, been shown to be appropriately closed by KIC but not by glucose [25]. In this and another study from the same group [25, 26], as well as in a study on perfused islets of GK rats [27], KIC was reported to increase insulin secretion to the same extent in GK and control islets. These contradictory data together with different islet morphology [12, 28–32], insulin content [3, 27, 29, 31–33] and islet glucose metabolism [3, 21, 23, 25, 27, 34–37] findings suggest that there are important differences between available GK rat colonies. It remains to be established whether the impaired stimulus-secretion coupling for KIC in our GK rats is an inherent defect or solely secondary to long-term hyperglycaemia.

We conclude that both initiatory and potentiatory effects of glucose on insulin release is impaired in islets of F_1 GK-hybrid rats and the KIC-induced insulin response is preserved which indicates that the defect responsible for impaired transduction of the glucose signal in F_1 hybrid rats is most likely localized to the glycolytic pathways.

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