Hypothalamic Mediation of Reduced GH Secretion in Diabetic Rats: Evidence for Reduced Cholinergic Inhibition of Somatostatin Release

Ikram Ismail, Mary Lewis, John R. Peters and Maurice F. Scanlon
Section of Endocrinology, Metabolism and Diabetes, Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales, UK.

Key words: GH, somatostatin, acetyl choline, diabetes.

Abstract
The Goto-Kakizaki (GK) rat is a new model of diabetes mellitus and in this study we have characterized the diabetic and growth hormone (GH) secretory status of male GK rats at 6 and 16 weeks of age. We have also investigated the role of endogenous somatostatin (SS) and cholinergic manipulation on the GH responses to GH-releasing hormone (GHRH). GK rats were non-obese with significant fasting hyperglycaemia, hyperinsulinaemia and absent insulin responses to IV glucose. The GH response to GHRH was reduced at 16 weeks compared with normal, age-matched Wistar rats but no differences were observed at 6 weeks. Pretreatment of older rats (16 weeks) with anti-somatostatin antibodies (SS-Ab) significantly increased GH responses to GHRH in both normal and GK groups. Cholinergic augmentation with pyridostigmine (PD) reversed the blunted GH responses to GHRH in older GK rats but had no effect in the normal or young (6 weeks) GK rats. These results indicate that SS release mediates the blunted GH response to GHRH in GK rats and that reduced hypothalamic cholinergic signalling to the somatostatinergic neurone may mediate the increase in SS release. This view is supported by the results from in vitro studies in which cholinergic muscarinic blockade with pirenzepine (PIR) caused dose-related stimulation of SS release from normal rat hypothalami but was without effect on GK rat hypothalami. The cause of this alteration in hypothalamic function is, at present, unknown.

Growth hormone (GH) regulatory mechanisms are profoundly altered in diabetes mellitus in both animals and humans but fundamental differences exist between species. Diabetic humans show increased spontaneous (1-4) and stimulated (5-7) GH secretion which may well be due, in part, to decreased release of hypothalamic somatostatin (SS) consequent upon increased cholinergic activity (7-10). In contrast diabetes in rats, whether occurring spontaneously (BB W) or induced by streptozotocin (STZ), results in depressed pulsatile GH secretion and blunted GH responses to various secretagogues (11-15). Any role of cholinergic mechanisms is presently unclear. The Goto-Kakizaki (GK) rat is a new spontaneous model of nonobese diabetes, produced by selective inbreeding of Wistar rats using glucose intolerance as a selection index. It provides a novel genetic model of diabetes that has not been manipulated with drugs that may directly interfere with GH secretion. In adult life, these rats show mild basal hyperglycaemia and hyperinsulinaemia, decreased pancreatic insulin stores, carbohydrate intolerance and a reduced insulin response to either oral (16) or intravenous (17) glucose challenge. In this study, we have demonstrated reduced GH responses to GH-releasing hormone (GHRH) in GK rats. We hypothesized that increased hypothalamic SS release mediates the reduced GH responses to GHRH as in the BB W diabetic rat (14) and that this may be secondary to reduced hypothalamic cholinergic activity. To test these hypotheses, we measured the GH responses to GHRH in GK and normal rats after pretreatment with anti-SS (SS-Ab) or pyridostigmine (PD). We also undertook in vitro studies to compare the levels of cholinergic inhibition of SS release in hypothalami from normal and GK rats. If the hypotheses are true we would expect to see restoration of GH responsiveness to GHRH in vivo following treatment with anti-SS and PD. We would also expect to see reduced SS release following cholinergic blockade in diabetic hypothalami in vitro.

Results
Older GK rats weighed significantly less than older normal rats. Fasting GK rats were hyperglycemic compared with normal rats at both 6 and 16 weeks and fasting plasma glucose levels were significantly higher at 16 weeks compared with 6 weeks in both normal and GK rats (Table 1). Fasting insulin levels were signi-
sificantly higher in GK rats at 16 weeks compared with normal rats but not at 6 weeks. No difference was noted in basal plasma GH levels between GK and normal rats either at 6 or 16 weeks (Table 1).

**Effects of IV glucose on insulin and GH (Fig. 1)**

After IV glucose there was no difference in the Δ peak or incremental AUC-glucose between the older GK and normal rats. Plasma insulin peaked within 5 min in normal rats but no response was observed in GK rats. One-way ANOVA for plasma insulin and GH concentrations with time points as the variable factor did not show any change in plasma insulin in GK rats, or GH levels in any animal group after IV glucose.

**Effects of IV GHRH, PD and SS-Ab on GH levels (Table 2)**

16 week rats (Fig. 2)

After GHRH there was an immediate increase in GH concentrations in both groups but the response was significantly greater in normals than in GK rats. PD pretreatment did not alter basal GH concentrations over 30 min in GK or normal rats. In normals, PD pretreatment did not significantly change the GH response to GHRH whereas in GK rats, PD pretreatment resulted in a marked augmentation of the GH response which was significantly greater than in normals. In addition, the GH response to GHRH after PD pretreatment in GK rats was significantly higher than the response in normals during the control study (Δ peak GH. P<0.05; Δ AUC-GH. P<0.01).

Passive immunization with SS-Ab increased basal GH concentrations in both normal and GK rats (P<0.01). There was no difference between GH concentrations at 1 h after passive immunization with SS-Ab in either animal group but SS-Ab induced a significant increase in GH response to GHRH in both normal and GK rats. SS-Ab did not significantly increase the Δ peak GH response to GHRH in normals and the GH response to GHRH after passive immunization with SS-Ab was similar in both GK and normal rats. In addition, the GH response to GHRH after SS-Ab pretreatment in GK rats was significantly higher than the response to GHRH alone in normal rats (Δ peak GH, P<0.05; Δ AUC-GH, P<0.01).

6 weeks rats (Fig. 3).

The GH response to GHRH was not significantly different between GK and normal rats. PD pretreatment did not affect basal GH concentrations and did not increase the GH response to GHRH in normal and GK rats.

Passive immunization with SS-Ab increased basal GH concentrations in both normal and GK rats but there was no significant difference between GH concentrations after passive immunization with SS-Ab in either animal group. SS-Ab did not significantly increase the GH response to GHRH in either normal or GK rats, nor was there any difference between the GH response to GHRH in either group after passive immunization with SS-Ab.

**Pituitary GH and hypothalamic SS contents (Table 1)**

Pituitary GH content was not significantly different between GK and normal rats in either age group. While the pituitary GH content in the normal older rats was significantly higher compared with the normal young rats, there was no difference between the pituitary GH content of young and older GH rats. Hypothalamic SS content was significantly lower in older GK rats compared with normals but there was no difference in the young rats. While hypothalamic SS content in older normal rats was significantly higher compared with young normal rats, there was no difference between the hypothalamic SS content of the young and older GK rats.

In vitro studies of cholinergic control of GH (Figs 4 and 5)

Pir (10^-5 M) did not affect SS release from normal rat hypothalamus but higher doses of PIR stimulated the release of SS in a dose-dependent manner. Therefore in subsequent experiments the maximal releasing dose of PIR (10^-5 M) was used. GK rats showed fasting hyperglycaemia compared with normal rats at sacrifice (GK vs normal 8.4±0.1 vs 4.7±0.1 mM, P<0.001) confirming their diabetic status. In GK hypothalami PIR (10^-5 M) had no effect on SS release in contrast to normal tissue where PIR again stimulated significant SS release. This stimulation in normals was abolished by coinucubation with the acetylcholinesterase inhibitor ESE and ACh. PIR had no significant effects on GHRH or TRH release in either normal or GK hypothalami.

**Discussion**

GK rats were significantly smaller than normal Wistar rats as documented previously [17, 18] and showed fasting hyperglycaemia. In contrast to the work of others [17] 16 week GK rats showed higher fasting plasma glucose levels compared to 6 week GK rats. Although intravenous glucose administration did not reveal any differences in plasma glucose responses in this study, other workers have documented impaired glucose tolerance in GK rats after oral [16, 17] or intraperitoneal glucose [19]. In conjunction with fasting hyperglycaemia, older GK rats also showed fasting hyperinsulaemia although there was no insulin
response to glucose. A loss of the insulin responsiveness to glucose has also been shown in vitro using the isolated perfused pancreas technique (16, 17, 20) and static incubations of isolated pancreatic islets (19, 21) whereas the response to nonglucose stimuli is preserved (17, 20).

The GH response to GHRH was significantly lower in older GK rats compared with normals whereas pituitary GH contents did not differ, as has been demonstrated previously in the more severely diabetic BB W rat (14) but this difference was not apparent in younger rats. Pretreatment with SS-Ab completely reversed the blunted GH response to GHRH in older GK rats and indeed, the GH response after SS-Ab was significantly greater than in normal rats during the control study. These data are similar to previous observations in the BB W rat (14) and indicate a major role for SS in mediating the reduced GH secretion in the GK rat. We went on to study the possible involvement of hypothalamic cholinergic pathways by augmenting cholinergic activity using the acetylcholinesterase inhibitor pyridostigmine (PD). We used this approach because of previous data showing the PD acts via suppression of hypothalamic SS in vitro (22, 23).

Pretreatment of normal rats with PD did not alter the GH response to GHRH which is consistent with previous data suggesting maximal hypothalamic cholinergic activity in normal rats (14, 15, 22). Similarly, PD pretreatment did not affect GH responsiveness in young GK rats with normal GH responses to GHRH. In contrast, PD markedly increased the GH response to GHRH in older GK rats such that the response was even higher than the normal controls. This suggests that hypothalamic cholinergic activity is reduced in the older GK rat with consequent increased release of SS. These data arc quite different to those in BB W rats where PD failed to increase the GH response to GHRH (14). It is interesting to note however that the effects of PD in the GK rat were similar to those in rats fasted for 72 h and also presumed to have high somatostatinergic tone (22).

Anaesthetic agents can affect GH release in the rat and this must be considered when interpreting our results in this study. Thus it is possible that in normal rats, pentobarbital reduced somatostatinergic tone and hence the lack of effect of PD. In contrast, in states of high somatostatinergic tone, the additional effects of pentobarbital and cholinergic agonism may be required to reduce hypothalamic SS release. Even though pentobarbital may affect SS release, our interpretation of the data is valid since animals were treated identically but nevertheless, revealed consistent, significant differences between normal and GK rats.

We have also demonstrated reduced hypothalamic SS content in older GK rats compared with normals, the significance of which is unclear at present. Although this finding may reflect increased hypothalamic SS secretion it contrasts with the unchanged hypothalamic SS peptide and mRNA content in STZ-diabetic and BB W rats (24–28). However, measurement of immunoreactive peptide content in isolation can be misleading since it gives no indication of rates of turnover or release. Hence it is important now to study hypothalamic SS mRNA in the GK rat.

Our in vivo findings are supported by the in vitro data which showed that PDH had no effect on SS release from GK hypothalamati in marked contrast to normal hypothalami. This is consistent with the presence of reduced hypothalamic cholinergic activity in these diabetic animals. The mechanism underlying such reduced cholinergic activity is unclear at present. It may well be due to a functional reduction in cholinergic input to hypothalamic somatostatinergic neurons, since the changes can be easily reversed in vitro by inhibition of acetylcholinesterase with PD. However, our data conflict with those of others (29) since we were unable to demonstrate inhibition of SS release following cholinergic augmentation in vitro with ESE and ACh in either normal or GK rats. This may well be due to limitations of our in vitro system such that high levels of nonspecific neuropeptide release from damaged cells mask the effects of agents which act to reduce SS.
Abnormal GH neuroregulation in the GK rat

Table 2. Basal Plasma GH and Plasma GH Concentrations in Response to GHRH in Normal and GK Rats Pretreated with Normal Rabbit Serum, SS-Ab or PD

<table>
<thead>
<tr>
<th>Control Study</th>
<th>Normal Adults</th>
<th>GK Adults</th>
<th>Normal Young</th>
<th>GK Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal GH (mg L)</td>
<td>31.9 ± 2.0</td>
<td>18.7 ± 3.0</td>
<td>24.2 ± 4.4</td>
<td>15.8 ± 1.7</td>
</tr>
<tr>
<td>Pre-GHRH (mg L)</td>
<td>32.5 ± 1.2</td>
<td>19.4 ± 1.4</td>
<td>30.7 ± 13.0</td>
<td>20.9 ± 3.6</td>
</tr>
<tr>
<td>Δ Peak GH (mg L)</td>
<td>1431 ± 475</td>
<td>266 ± 126*</td>
<td>113 ± 25</td>
<td>257 ± 84</td>
</tr>
<tr>
<td>Δ AUC-GH (mg min L)</td>
<td>143.40 ± 31.34</td>
<td>3390 ± 1681*</td>
<td>1483 ± 524</td>
<td>3380 ± 737</td>
</tr>
</tbody>
</table>

Anti-SS Study

| Basal GH (mg L) | 28.2 ± 1.2 | 21.6 ± 6.0 | 35.6 ± 4.6 | 51.4 ± 8.5 |
| Pre-GHRH (mg L) | 79.6 ± 5.1 | 84.0 ± 12.34 | 140.4 ± 11.8 | 138.7 ± 34.6 |
| Δ Peak GH (mg L) | 3011 ± 1070 | 13805 ± 6241** | 283 ± 111 | 841 ± 420 |
| Δ AUC-GH (mg min L) | 43505 ± 12708** | 125068 ± 64033** | 4422 ± 1895 | 12014 ± 6201 |

PD Study

| Basal GH (mg L) | 27.7 ± 2.2 | 26.1 ± 1.6 | 26.5 ± 2.7 | 20.3 ± 2.1 |
| Pre-GHRH (mg L) | 25.5 ± 6.3 | 22.0 ± 4.4 | 28.6 ± 7.8 | 27.7 ± 3.5 |
| Δ Peak GH (mg L) | 1230 ± 309 | 3669 ± 1096* | 253 ± 77 | 221 ± 40 |
| Δ AUC-GH (mg min L) | 13532 ± 2661 | 36010 ± 9368* | 3939 ± 1042 | 3211 ± 356 |

* P < 0.01; GK vs normal; ** P < 0.01; Anti-SS vs control; P > 0.05; Δ Basal during anti-SS study.

Fig. 2. Plasma GH (mean ± SEM) after GHRH in older normal (A) and GK (B) rats. Upper panels show the area under the GHRH-GH response curve following pre-treatment with normal rabbit serum (control), pyridostigmine (PD) or antisomatostatin (anti-SS). Lower panels show the time-related plasma GH responses to GHRH after the same pretreatments. Normal rabbit serum (■ ■ ■) at -60 min, IV PD (● ● ●) at -30 min or SS-Ab (▲ ▲ ▲) at -60 min. * P < 0.02, ** P < 0.01, *** P < 0.001.

release. It is also important to note that relatively high concentrations of drugs are required to produce biological actions in this sort of in vitro preparation.

Overall, our data support the view that hypothalamic cholinergic inhibition of SS release is reduced in diabetic GK rats which probably contributes to the altered GH secretory status of these animals. The mechanism underlying this age-related, hypothalamic dysfunction is unclear but may well be due to alterations...
in the direct effects of glucose or insulin on hypothalamic neuronal function. Chronic hyperglycaemia or hyperinsulinaemia may lead to the observed reduction in hypothalamic cholinergic activity thus disinhibiting somatostatinergic neurones resulting in increased SS release. Alternatively, or additionally, chronic hyperglycaemia or hyperinsulinaemia may directly desensitise somatostatinergic neurones to the well-established inhibitory actions of glucose (18, 30).

Materials and methods

Male GK rats were obtained from the colony maintained in the Animal Unit of the University of Wales College of Medicine. The colony was started in 1989 with 6 breeding pairs, which were gifts from Professor Y. Coto of Tohoku University School of Medicine, Sendai, Japan. We used normal male Wistar rats obtained from the same Animal Unit (University of Wales College of Medicine) as controls. Both breeds were maintained, 6 to a cage, in a thermostatically controlled room with a 24 h lighting cycle (light period being from 0700 to 1900 h). The animals were fed a standard laboratory diet (Pilsbury's modified rat and mouse breeding diet) and allowed free access to tap water. After an overnight fast, the rats were anaesthetized with intraperitoneal sodium pentobarbitone (50 mg kg\(^{-1}\)) (May and Baker Ltd., Dagenham, Essex, UK). An indwelling cannula was inserted into the internal jugular vein and animals were studied after a 30 min stabilization period.

**Effects of IV glucose on insulin and GH**

Two groups of 6 male rats were studied at 6 weeks (young) and 16 weeks (older). After the 30 min stabilization period, 20% Dextrose (0.5 g kg\(^{-1}\)) was given via the i.v. cannula at t = 0 min. Samples (0.5 ml) were taken at 0, 5, 10, 15 and 30 min for measurement of GH, insulin and glucose. All blood samples were immediately centrifuged and plasma was separated. The red cells were resuspended in heparinized saline and re-injected. The plasma samples were stored at -20 °C until assayed.

**Effects of IV GHRH, PD and SS-Ab on GH levels**

Twelve groups of 6 male rats per group were studied at 6 and 16 weeks. After the 30 min stabilization period, rats were pretreated with either normal rabbit serum (NRS, 0.5 ml) at t = 60 min, SS-Ab (0.5 ml) at t = 60 min or pyridostigmine bromide (PD, Mestinon, Roche, Switzerland, 200 µg kg\(^{-1}\)) at t = 30 min. Human GHRH 1-44 (5 µg kg\(^{-1}\), Sanofi, Manchester, UK) was injected at 0 min. Blood samples (0.5 ml) were drawn at 30 min after cannulation (basal sample at t = 60 min for the control study and SS-Ab study and -30 min for PD study), immediately before GHRH injection (0 min) and at 5, 10, 15 and 30 min after GHRH injection. Following the last sample, the animals were killed by cervical dislocation and decapitated. The anterior pituitaries and hypothalami were dissected out for measurement of GH and SS content respectively.
SS-Ab was prepared by immunizing rabbits with synthetic SS (Peninsula Laboratories, St Helens, Merseyside, UK) conjugated to Keyhole Limpet Hemocyanin (Sigma, UK) by the glutaraldehyde method. The conjugated SS (20 μg) was added to sterile PBS 1600 ml and emulsified with 1.4 ml Freund's Complete Adjuvant (Sigma). The rabbits received multiple (20-30) intradermal injections (12 ml) of the mixture. A booster injection (10 μg of conjugated SS in Freund's Incomplete Adjuvant) was given after 8 weeks. The animals received a further booster of 10 μg conjugated peptide after a further 2 weeks and were bled 3 weeks later. The serum was separated and stored at -20°C. [125I]-Tyr-SS-labelled tracer showed approximately 40% specific binding when 12,500 cpm in 100 μl was incubated with 100 μl of 1:5000 antibody diluted with an NSB of 14%. The biological activity of the SS-Ab was confirmed by demonstrating an acute rise in circulating GH levels following t.I. administration.

In vitro studies of cholinergic control of GH

For the in vitro studies static incubation techniques were used as previously described (31). Briefly, 16 week normal and GK rats were decapitated with minimum stress after an overnight fast and the brains removed rapidly. Trunk blood was obtained for fasting plasma glucose measurement. The hypothalami, defined by the posterior margin of the optic chiasma and the anterior margin of the mammary bodies to a depth of approximately 2 mm, was dissected out and cut longitudinally through the median eminence. The two halves were placed together in each well of a multiwell tissue culture plate containing 300 μl of Krebs-Ringer bicarbonate (KRB) solution (5 mM glucose, 25 mM NaHCO3, 4.75 mM KCl, 1.18 mM MgSO4, 0.85 mM KH2PO4, 2.52 mM CaCl2, 118.5 mM NaCl, pH 7.4) with 0.1% (w/v) bovine serum albumin and bacitracin (300 μg/ml) (to reduce non-specific peptide adsorption and prevent enzymatic degradation respectively). The hypothalami were incubated for 20 min periods to allow stabilization of basal peptide release, during which fresh medium was replaced and then discarded. The experimental treatment was applied during a final 20 min incubation period. Medium was then removed, centrifuged and aliquoted for somatostatin, TRH and GHRH assay.

Initially a dose-response study of SS release was performed using hypothalamic tissue from normal rats and various concentrations of the cholinergic muscarinic agonist, pilocarpine (PIR) in the incubating medium. The concentrations of PIR and the number of rats (n) used were $10^{-7}$ M ($n=7$), $10^{-5}$ M ($n=11$) and $10^{-3}$ M ($n=12$). In subsequent studies GHRH and TRH were also measured as these peptides have GH releasing actions. Furthermore, since small changes in the ambient glucose concentration can markedly alter hypothalamic neurosecretory release (31), it was essential to maintain in vitro glucose concentrations throughout the in vitro studies. Hence, KRB containing 5 mM D-glucose was used for normal hypothalami and 8 mM D-glucose for diabetic hypothalami since these concentrations matched the in vitro plasma glucose levels at the time of animal sacrifice. To control for osmolarity, 3 mM L-glucose was added to the incubating medium for normal hypothalami. In each experiment, equal numbers of diabetic (n = 9) and normal (n = 9) hypothalami were used. Hence, a total of 9 incubations were carried out for each treatment group which comprised the following: untreated control; PIR $10^{-3}$ M; PIR $10^{-5}$ M + acetylcholine (ACH) $10^{-8}$ M + eserine (E; anticholinesterase inhibitor) $10^{-5}$ M; ACH $10^{-3}$ M + ESE $10^{-3}$ M. 

**Assays**

Rat GH was measured by standard RIA using hypophysectomized rat serum as the standard curve and Protein A for separation. The materials were obtained from the NIADDK (Baltimore, MD, USA). Assay sensitivity was 0.02 pg/tube. Intraassay and interassay variations averaged 8 and 12% respectively. SS was measured by standard RIA using a fully characterized antibody 693 (32). Appropriate dilutions of labelled [125I]-TriI SS and antibody were used to obtain 10,000 cpm and 20% specific binding. Twenty-four h preincubation and 24 h incubation times were used at 4 C and non-specific binding was 2 to 4%. Assay sensitivity was less than 1 pg/tube and SS-14 and SS-28 compete on an equimolar basis for binding of label to this particular antibody (33, 34).

TRH was also measured by a well characterized RIA (33) which was set up as for SS. Twenty-four h preincubation and 24 h incubation times were used at 4 C and non-specific binding was less than 5%. Assay sensitivity was less than 0.5 pg/tube. Rat GHRH was measured using a two-site immunoenzymometric assay (35). In this assay, a C-terminal antibody was attached to a chemiluminescent probe and the N-terminal was attached to the solid phase. Assay sensitivity was 5 pg/ml and the coefficient of variation was less than 10% at dose levels of 0.5 to 100 pg. In each assay procedure a standard curve was set up in tubes containing the appropriate amount of KRB. Plasma glucose was measured using a YSI Model 2300 STAT Plus Glucose Analyser (Yellow Springs Instruments Ltd., Yellow Springs, Ohio, USA). Rat plasma insulin was measured using a commercial radio-immunooassay kit from Novo Nordisk, Copenhagen, Denmark. The materials supplied included rat insulin, [125I]-[152]A[14]-human insulin and anti-porcine insulin guinea pig serum. M 8309 (dilution 1:300). The sensitivity limit of the assay was 0.2 pg/l.

**Data presentation and analysis**

In vitro data are presented as mean ± SEM and the GH secretors responses are expressed as Δ peak changes (pg/I) (calculated by subtracting the peak value from the value at t = 0) and incremental area under the response curve (Δ AUC). The AUC was calculated by the trapezoidal rule. Because of the positively skewed distribution of GH concentrations, these summary measures were log transformed before statistical analysis but only untransformed data have been presented for clarity. Comparisons of the log transformed summary measures utilized the two sample t-test (36). Analysis of variance (ANOVA) was used where appropriate, as indicated.

In vitro data are presented as mean ± SEM of the amount of neurosecretory release during the test incubation (ng/mg T) for each group as well as the test incubation alone. GH release was to allow comparison of more than one experiment and reduce experimental variability. Because of their non-parametric distribution, the data were analysed using the Kruskal-Wallis test. P < 0.05 was considered as statistically significant.

Accepted 28 February 1995

**References**


Abnormal GH neuroregulation in the GK rat


