

Mechanism of Compensatory Hyperinsulinemia in Normoglycemic Insulin-resistant Spontaneously Hypertensive Rats

Augmented Enzymatic Activity of Glucokinase in β -Cells

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Abstract

The cause of compensatory hyperinsulinemia in normoglycemic insulin-resistant states is unknown. Using spontaneously hypertensive rats (SHR), we tested the hypothesis that a lowered β -cell set-point for glucose causes a hypersecretion of insulin at a normal glucose level. Islets isolated from normoglycemic hyperinsulinemic SHR were compared to age-matched (12 wk old) Wistar-Kyoto (WK) rats. The ED_{50} for glucose-induced insulin secretion was 6.6 ± 1.0 mM glucose in SHR versus 9.6 ± 0.5 mM glucose in WK ($P < 0.02$). Glucokinase enzymatic activity was increased 40% in SHR islets ($P < 0.02$) without any change in the glucokinase protein level by Western blot. The level of the β -cell glucose transporter (GLUT-2) was increased 75% in SHR islets ($P < 0.036$).

In summary, the β -cell sensitivity for glucose was increased in these normoglycemic insulin resistant rats by an enhanced catalytic activity of glucokinase. We have identified a regulatory system for glucokinase in the β -cell which entails variable catalytic activity of the enzyme, is modulated in response to variations in whole-body insulin sensitivity, and is not dependent on sustained changes in the plasma glucose level. (*J. Clin. Invest.* 1994, 94:399–404.) Key words: insulin secretion • hexokinase • glucose metabolism • islets of Langerhans • GLUT-2 • glucose transporters

Introduction

The islet β -cell precisely maintains the blood glucose level through a feedback loop between the level of blood glucose and insulin secretion (1). As such, changes in insulin secretion are presumed to occur secondary to fluctuations in glycemia. However, states of insulin resistance such as obesity (2, 3) are characterized by normal blood glucose levels and an increase in insulin secretion (4). The mechanism of the β -cell hyperfunction in euglycemic insulin resistant states is unexplained.

Studies in insulin-resistant rat models have suggested a mechanism. The LAN-cp rats, Zucker fatty rats, and pregnant rats, all have lowered set-points for glucose-induced insulin

secretion (5–8) which could explain exaggerated insulin output at a normal glucose level. However, none of the previous studies characterized the plasma glucose levels carefully, and hyperglycemia is known to shift the glucose concentration–insulin secretion curve to the left (9–15). We examined rats that were hyperinsulinemic and normoglycemic after a 48-h iv glucose infusion to identify the mechanism for their hypersecretion of insulin (16). The hyperinsulinemia was associated with a lowered set-point for glucose-induced insulin secretion, and an increased islet glucokinase activity, which reinforces the role of this important enzyme in regulating the β -cell glucose sensitivity (17). Surprisingly, the glucokinase protein level was not increased. Thus, glucokinase in the β -cell appears to be regulated not only by the previously known effect of the plasma glucose level to control its cellular content (17, 18), but also through altering the activity of the enzyme. However, the relevance of our previous studies to euglycemic insulin resistant states was unclear because in the animal model we used (48-h glucose infusions), the rats were hyperglycemic during the first 6 h of the infusion (16).

We now have investigated the basis for compensatory hyperinsulinemia in spontaneously hypertensive rats (SHR).¹ Of key importance, these insulin resistant rats (19, 20) are normoglycemic, even when challenged with oral or intravenous glucose (21, 22). We hypothesized that SHR would be hyperinsulinemic because of a shift in the glucose concentration–insulin secretion relationship to lower glucose levels, and that the mechanism would be an increase in the catalytic activity of the key β -cell “glucose sensor” enzyme, glucokinase.

Methods

SHR model and islet isolation. SHR and Wistar Kyoto (WK) rats (Taconic Farms Inc., Germantown, NY) were studied at 12 wk of age. All rats had free access to standard rat chow and tap water except during the overnight fasts which preceded the oral glucose tolerance test (OGTT) and the meal challenge. On the morning of islet isolation, tail vein blood was obtained from unanesthetized, nonfasting rats for plasma glucose and insulin measurements. Islets were isolated using an adaptation of the method of Gotoh et al. (23): pancreatic duct infiltration with collagenase, Histopaque gradient separation (Sigma Chemical Co., St. Louis, MO), and hand picking. All experiments used fresh islets that had undergone a 90-min preincubation at 37°C in KRB, 10 mM HEPES, 2.8 mM glucose, and 0.5% BSA except for the Western blot experiments where islets were frozen on dry ice following isolation and stored at -70°C until studied.

OGTT and meal challenge. Both tests were preceded by an overnight fast. For the OGTT, age-matched SHR and WK rats were administered

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1. Abbreviations used in this paper: SHR, spontaneously hypertensive rats; WK, Wistar-Kyoto.

Table 1. General Characteristics of 12-wk-old SHR and WK Rats

Group	Weight	Plasma glucose	Plasma insulin
	g	mM	ng/ml
Wistar-Kyoto (8)	359±5	8.0±0.3	1.40±0.20
SHR (8)	280±4	8.3±0.2	2.81±0.30
P	0.0001	NS	0.0015

The data are expressed as $m \pm SEM$. All values were obtained in unanesthetized, nonfasting rats.

300 mg of glucose (0.5 g/ml) by gavage tube. Blood for plasma glucose was obtained by tail snipping at 0, 30, 60, and 120 min. 3 d later, a meal challenge was performed. The SHR and WK rats were given free access to chow (time 0), and plasma glucose values were measured at 0, 30, 60, and 120 min.

Insulin secretion. Triplicate batches of 10 islets were placed in glass vials containing 1 ml KRB, 0.5% BSA, 2.8–27.7 mM glucose, and incubated 60 min in a 37°C shaking water bath. The medium was separated from the islets by gentle centrifugation (500–750 rpm 5 min at 10°C) and stored at –20°C pending insulin measurements by RIA (24).

Glucose utilization. Islet glucose usage was measured by the method of Ashcroft et al. (25). Triplicate groups of 20 islets were incubated in 100 μ l KRB, glucose (2.8–27.7 mM), 2 μ Ci D-[5-³H]glucose (NEN, Boston, MA). The incubation was carried out in a 1-ml cup contained in a rubber-stoppered 20-ml scintillation vial that had 500 μ l of distilled water surrounding the cup. After 90 min at 37°C, glucose metabolism was stopped by injecting 100 μ l 1 M HCl through the stopper into the cup. After overnight incubation at 37°C to allow the [³H]H₂O to equilibrate with the distilled water, the distilled water underwent liquid scintillation counting. The recovery of [³H]H₂O, calculated for each experiment with known amounts of [³H]H₂O (NEN), averaged 45–55%. Glucose utilization (pmol glucose/90 min/islet) was calculated as:

$$((\text{cpm per tube} - \text{blank cpm}) \div \text{total cpm added per tube})$$

$$\times \text{pmol cold glucose added per tube} \div \% \text{ recovery } [^3\text{H}] \text{H}_2\text{O} \div 20 \text{ islets.}$$

Glucose phosphorylation. Glucose phosphorylation was measured in islet extracts as the conversion of NAD⁺ to NADH by exogenous glucose-6-phosphate dehydrogenase (26). 300 islets from a single rat were homogenized on ice in 300 μ l buffer (1 mM EDTA, 20 mM

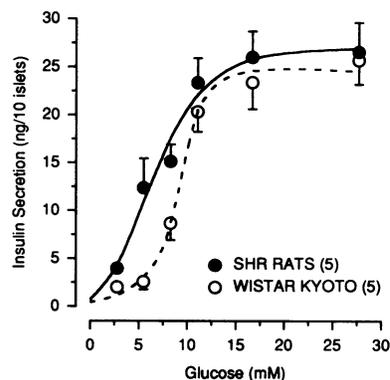


Figure 2. Glucose-induced insulin secretion in isolated islets from SHR (●—●) and WK (○—○) rats. 10 islets were incubated for 60 min at the glucose concentrations shown. The half maximal insulin response (ED₅₀) occurred at 6.6±1.0 mM glucose in SHR versus 9.6±0.5 mM glucose in WK (P < 0.02).

K₂HPO₄, 110 mM KCl, 5 mM dithiothreitol) by 25 strokes of a machine-driven Teflon pestle in a Kontes glass homogenizer (0.004–0.006 inch). Aliquots (10 μ l × 3) were stored at –70°C for DNA content (27). After a 10-min centrifugation at 12,000 g to remove mitochondrial-bound hexokinase (28), 7- μ l aliquots were added to 100 μ l of a reaction buffer that consisted of 50 mM HEPES/HCl, pH 7.6, 5 mM ATP, 100 mM KCl, 7.4 mM MgCl₂, 15 mM β -mercaptoethanol, 0.5 mM NAD⁺, 0.05% BSA, glucose (0.03–100 mM), and 0.7 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim, Indianapolis, IN). After 90 min at 30°C, the reaction was stopped with 1 ml 500 mM NaHCO₃ pH 9.4. Triplicate samples were performed at each glucose concentration in parallel with reagent blanks (no homogenate). The tissue blanks were the islet homogenate in reaction buffer that contained 0 mM glucose. The standard curve used glucose-6-phosphate standards (0.3–3 nmol) in reaction buffer that contained 100 mM glucose. Glucokinase and hexokinase V_{max} and K_m were calculated by linear regression from an Eadie-Scatchard plot (v/[s]) after extrapolating the data to 37°C assuming a Q₁₀ of 2 (26, 29), followed by 10 cycles of the method of Spears et al. (30) to separately identify each enzyme's activity.

Glucokinase and GLUT-2 Western blots. 300–500 islets from a single rat were lysed in 5% SDS, 80 mM Tris/HCl, pH 6.8, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 μ g DNAase, and 0.2 mM N-ethylmaleimide. Protein content was measured by the BCA assay (Pierce, Rockford, IL) with BSA as standard. 20- μ g aliquots were resolved by electrophoresis on a 0.75-mm 10% polyacrylamide gel containing SDS, and electroblotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) (31). Filters were blocked overnight at 4°C

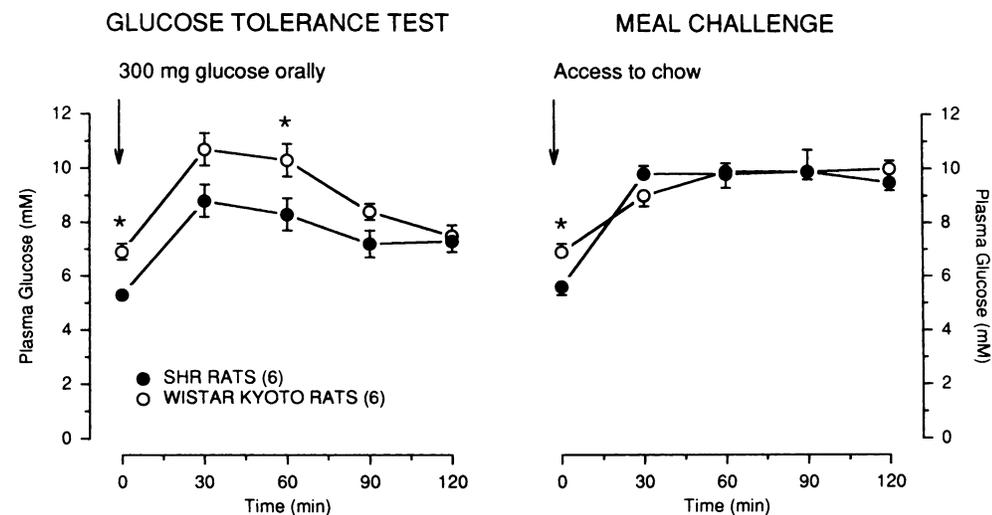


Figure 1. Plasma glucose responses during an oral glucose tolerance test (left) and a meal challenge (right) in 12-wk-old SHR (●—●) and WK (○—○) rats. *P < 0.05.

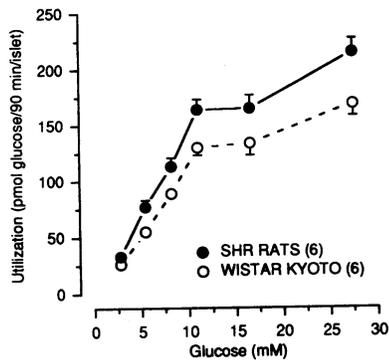


Figure 3. Glucose utilization in isolated islets from SHR (●—●) and WK (○—○) rats. 20 islets were incubated for 90 min at the glucose concentrations shown in combination with 2 μ Ci D-[5- 3 H]glucose. Glucose metabolism was measured as the production of [3 H] H_2O .

in 5% nonfat dry milk, 0.01% Tween 20, 20 mM Tris/HCl, pH 7.4, then incubated at room temperature with the specific antisera. Glucokinase: (1) sheep antiserum raised against an *E. coli*-derived B1 isoform of rat glucokinase (gift from Dr. Mark Magnuson, Vanderbilt University, Nashville, TN) at 1:500 dilution for 3 h, (2) rabbit anti-sheep IgG (Sigma Chemical Co.) at 1:1,500 dilution for 1 h. GLUT-2: rabbit antiserum raised against a polypeptide that corresponded to amino acids 513–522 of rat GLUT-2 (gift from Dr. Bernard Thorens, Lausanne, Switzerland) at 1:100 dilution for 2 h. Bound antibody was detected with 125 I-conjugated protein A (ICN, Costa Mesa, CA). Band intensity was quantified by densitometry using IMAGE 1.4 software (NIH, Bethesda, MD). Extracts were used for glucokinase and GLUT-2 only once so that the “n” cited in the text is the number of rats studied.

Analytical methods. Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman Instruments, Inc., Fullerton, CA). The insulin RIA used charcoal separation (24) and rat insulin standards (Lilly, Indianapolis, IN).

Data presentation and statistical methods. All data are expressed as mean \pm SEM. The listed “n” in all experiments is the number of rats studied. The densitometry results from the Western blots are expressed in relative terms by comparing the SHR extract on each gel to the WK extract (assigned a value of 100%). Statistical significance was determined by the unpaired Student’s *t* test. The one-way *t*-test was used for the Western blot results.

Results

General characteristics of SHR rats (Table I). The body weights of 12-wk-old SHR rats were lower than age-matched WK rats. Insulin resistance was confirmed by the twofold higher basal plasma insulin level ($P < 0.0015$) in conjunction with a normal plasma glucose. To further characterize the plasma glu-

ucose level of SHR, an OGTT and a meal challenge were performed. An OGTT usually entails administering an amount of glucose that is adjusted for the rat’s weight. With this approach (1 g/kg), SHR rats had lower fasting and post challenge blood glucose values (data not shown). However, the lower body weight complicated the interpretation of these results because of the smaller dose of glucose. For that reason, the test was repeated by giving the same amount of glucose (300 mg) to all rats. Fasting blood glucose levels were again lower in SHR (5.3 ± 0.2 mM versus 6.9 ± 0.3 mM WK, $P < 0.002$), and the post glucose values were either reduced or the same as WK (Fig. 1). Similar results were obtained during the meal challenge.

Insulin secretion. Insulin secretion was measured at varying glucose concentrations in islets isolated from SHR and WK rats (Fig. 2). The curve in the WK islets was sigmoidal with a half maximal insulin response (ED_{50}) of 9.6 ± 0.5 mM glucose versus 6.6 ± 1.0 mM glucose in SHR ($P < 0.02$) without any change in the maximal response. At 8.3 mM glucose (reproducing the basal plasma glucose level of both groups), insulin secretion from SHR was twice that of WK (15.2 ± 1.8 ng/10 islets versus 8.7 ± 1.8 ng/10 islets WK, $P < 0.031$).

Glucose utilization. The glucose concentration–glucose utilization curve was also shifted to the left in SHR (Fig. 3). However, the pattern differed from that for insulin secretion. Glucose utilization was 20–40% higher in the SHR islets at all glucose concentrations including 27.7 mM (215 ± 12 pmol glucose/90 min/islet versus 169 ± 11 pmol glucose/90 min/islet WK, $P < 0.019$). As such, the ED_{50} for the SHR curve was identical to that of WK (8.0 ± 0.6 mM glucose SHR versus 7.8 ± 0.1 mM in WK).

Glucose phosphorylation. The V_{max} and K_m values for glucokinase and hexokinase are listed in Table II. The glucose phosphorylation data (expressed as mol glucose-6-phosphate/60 min/kg DNA) are shown in Fig. 4. The DNA contents of the SHR and WK islets were the same, as were the V_{max} and K_m values for hexokinase. In contrast, the glucokinase V_{max} was increased 40% in SHR ($P < 0.02$) without any change in the K_m .

Islet glucokinase and GLUT-2 protein levels. The etiology of the increased glucokinase V_{max} was investigated by quantifying the protein level of glucokinase by Western blot (Fig. 5). A total of seven SHR and WK rats were studied. Single bands at 52 kD characteristic of glucokinase were found in both groups. Identical levels of glucokinase were found in the SHR and WK

Table II. Kinetic Parameters for Glucokinase and Hexokinase in SHR and WK Islets

Animal	Hexokinase		Glucokinase		Islet DNA content ng
	V_{max}	K_m	V_{max}	K_m	
	mol glucose/kg DNA/60 min	mM glucose	mol glucose/kg DNA/60 min	mM glucose	
Wistar-Kyoto (5)	5.1 ± 1.2	0.04 ± 0.01	8.0 ± 0.4	10.2 ± 1.6	17.7 ± 2.5
SHR (5)	6.0 ± 0.8	0.03 ± 0.01	10.9 ± 1.4	11.8 ± 1.4	17.4 ± 2.6
<i>P</i>	NS	NS	0.02	NS	NS

The data are expressed as $m \pm$ SEM. Glucose phosphorylation was determined in islet extracts as described in the text. Hexokinase activity was determined from a glucose range of 0.03–0.5 mM, glucokinase activity from a glucose range of 6–100 mM. V_{max} and K_m for hexokinase and glucokinase were calculated by linear regression from Eadie-Scatchard plots ($v/[s]$ versus v) and corrected 10 times using the method of Spears et al. (30) after extrapolating the data to 37°C.

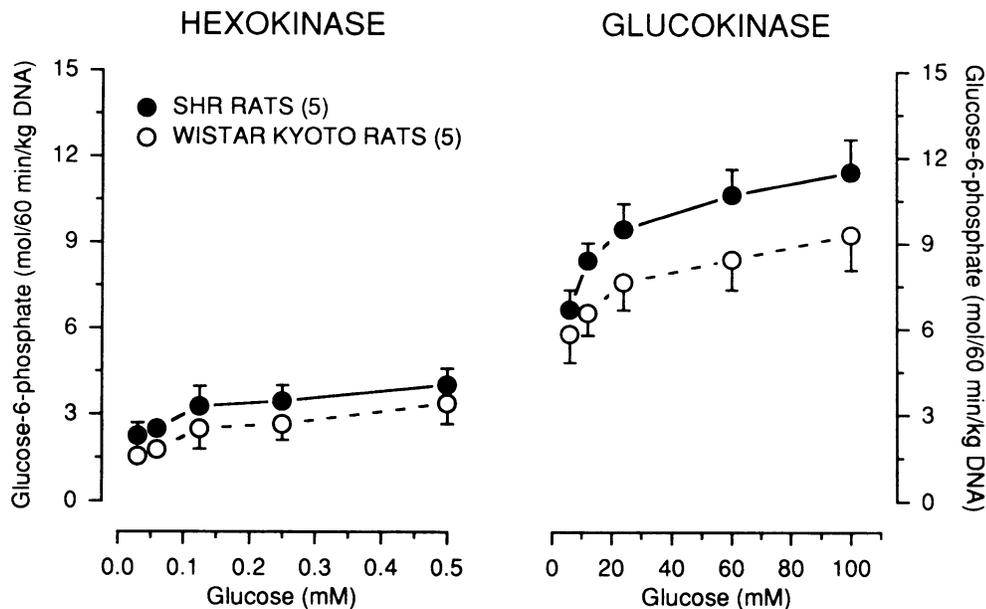


Figure 4. Glucose phosphorylation by islet extracts from SHR (●) and WK (○) rats measured after 90-min incubations at the glucose concentrations shown. The reaction was measured at 30°C as the appearance of NADH from NAD⁺ by exogenous glucose-6-phosphate dehydrogenase. (Left) Incubations carried out at 0.03–0.5 mM glucose representing hexokinase activity. (Right) Incubations carried out at 6–100 mM glucose representing glucokinase activity.

islets (SHR 95±3% of WK). It has recently been speculated that the level of the β -cell glucose transporter, GLUT-2, affects glucokinase activity (32). Therefore, GLUT-2 was also quantified in SHR and WK islets (Fig. 6). A total of 10 WK and SHR rats were studied. The GLUT-2 band was located at 55 kD in both groups. Its level was raised in SHR islets to 176±31% of WK ($P < 0.036$).

Discussion

Glucose phosphorylation by glucokinase plays a central role in determining the β -cell sensitivity to glucose (17, 18). Our finding of an augmented catalytic activity of this enzyme in SHR islets expands the understanding of how this β -cell “glucose sensor” is regulated. It is well-known that the β -cell content

of glucokinase is modulated by the plasma glucose level (16–18). We have identified an additional level of control through alterations in the catalytic activity. Supporting this idea, we made a similar observation in normoglycemic glucose-infused rats (16). Also, Iynedjian et al. (33) observed no change in the level of glucokinase in islets from 72-h fasted rats despite a reported 30% reduction in glucokinase activity (34). Importantly, in both of our studies, this effect was noted in normoglycemic rats. Thus, sustained changes in plasma glucose are not required to mediate the effect on glucose sensing. These data provide a mechanism for compensatory adaptations of insulin secretion under normoglycemic conditions.

Potential cellular mechanisms by which the catalytic activity of glucokinase could be altered have received recent attention. Malaisse et al. (35) postulated that islets contain the inhibitory

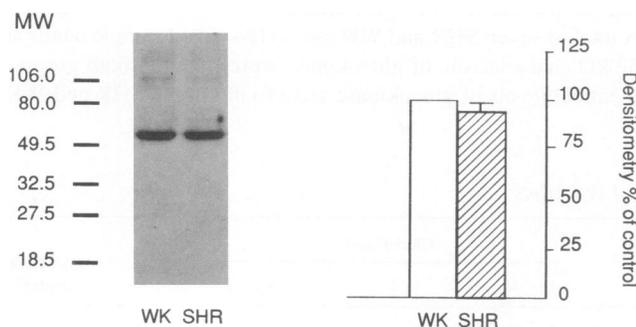


Figure 5. Western blot for glucokinase in isolated islets from SHR and WK rats. Protein aliquots (20 μ g) were resolved by electrophoresis on a polyacrylamide gel and transferred onto nitrocellulose. Filters underwent sequential incubation with a polyclonal sheep antiserum raised against an *E. coli*-derived B1 isoform of rat glucokinase, then rabbit anti-sheep IgG. Bound antibody was detected with ¹²⁵I-conjugated protein A. (Left) Representative gel showing single glucokinase bands at 52 kD. (Right) Combined densitometry data from a total of seven SHR and seven WK rats expressed in relative terms (WK extract was designated as 100% on each gel).

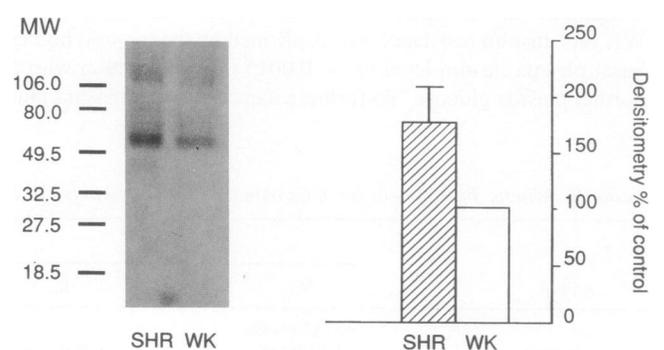


Figure 6. Western blot for GLUT-2 in isolated islets from SHR and WK rats. Protein aliquots (20 μ g) were resolved by electrophoresis on a polyacrylamide gel and transferred onto nitrocellulose. Filters underwent incubation with a rabbit antiserum raised against a polypeptide that corresponded to amino acids 513–522 of rat GLUT-2. Bound antibody was detected with ¹²⁵I-conjugated protein A. (Left) Representative gel showing single GLUT-2 bands at 55 kD. (Right) Combined densitometry data from a total of 10 SHR and 10 WK rats expressed in relative terms (WK extract was designated as 100% on each gel).

protein for glucokinase enzymatic activity that has been identified in liver. However, this explanation seems unlikely to account for the results in the SHR islets, since this protein is a competitive inhibitor of glucokinase which alters the K_m rather than V_{max} of this enzyme (36). An alternate suggestion is that the β -cell glucose transporter, GLUT-2, regulates glucokinase activity (32). Ferber et al. (37) observed in RIN cells (rat insulinoma cell line) transfected with GLUT-2 that glucokinase activity was increased. Furthermore, coincubating human erythrocyte glucose transporters (GLUT-1) with yeast glucokinase increased the glucokinase V_{max} without changing the K_m (38), i.e., the pattern found in the SHR islets. Our intriguing finding of the increased GLUT-2 level in SHR islets may provide additional support for this idea although coincidental occurrences clearly cannot be ruled out.

The concept that glucokinase activity determines the β -cell sensitivity to glucose is based on the understanding that glucose-stimulated insulin secretion is dependent on β -cell glucose metabolism, and that glucokinase is the rate-limiting step (17). Why then were the patterns for insulin secretion and glucokinase activity different in the SHR islets: a lowered ED_{50} in the secretion curve without a change in V_{max} versus an increased V_{max} for glucokinase without a change in K_m ? We have postulated that the maximal limit for glucose-stimulated insulin secretion is set by a factor other than β -cell glucose metabolism (16). In that case, an increase in glucokinase activity would enhance insulin output at submaximal levels of glucose without changing the maximal insulin response. The glucose utilization data support this suggestion, since in SHR the curve for glucose utilization matched that of phosphorylation. These data suggest that insulin secretion is uncoupled from β -cell glucose metabolism at high glucose concentrations which explains the lack of an increase in maximal glucose-induced insulin secretion in SHR islets.

An important caveat is to determine if the functional changes in SHR are real, or if there are dissimilar masses of β -cells in the SHR and WK islets. This analysis is particularly important, since insulin resistance causes a compensatory increase in pancreatic β -cell mass (39). For unclear reasons, this effect apparently does not occur in SHR. A morphometric study in pancreases from 4-mo-old SHR and WK rats found identically sized islets with the same proportions of insulin, glucagon, and somatostatin containing cells (40). Also, two findings in this study are consistent with a similar β -cell mass in SHR and WK islets: identical islet DNA content and identical maximal insulin response to glucose.

In summary, the insulin-resistant SHR rats were hyperinsulinemic because of an increase in the β -cell sensitivity for glucose due to an augmented catalytic activity of glucokinase, which in turn may be regulated by the β -cell glucose transporter. These findings provide a mechanism for β -cell hyperfunction in the presence of normoglycemia.

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