Plasma Glucagon and Free Fatty Acid Responses to a Glucose Load in the Obese Spontaneous Hypertensive Rat (SHROB) Model of Metabolic Syndrome X

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Metabolic Syndrome X is a cluster of abnormalities including insulin resistance, hyperlipidemia, hypertension, and obesity. We sought to determine if excess plasma glucagon and free fatty acids (FFA) might contribute to the insulin resistance in the obese spontaneous hypertensive rat (SHROB), a unique animal model of leptin resistance and metabolic Syndrome X. SHROB were extremely hyperinsulinemic and mildly glucose intolerant compared with lean SHR. SHROB had elevated fasting plasma glucagon and FFA, and showed paradoxical responses to an oral glucose challenge, with increased glucagon at 30 and 60 min postchallenge (200% ± 45% and 91% ± 13%, respectively; n = 9). In lean SHR, glucagon was nearly unchanged by glucose loading (<30% increase, $P > 0.05$; n = 5). Plasma FFA were not affected by a glucose load in SHROB, whereas SHR showed a decrease of 40% ± 6% (n = 5–9). The I/G molar ratio changed in opposite directions in the two genotypes, with a decrease in SHROB at 30 and 60 min, in contrast to the appropriate increase at 30 and 60 min postchallenge in the lean SHR ($P < 0.01$; n = 5–9). Administration of 500 ng/kg exogenous glucagon to SHR raised glucagon 56% ± 5% to a level that was similar to fasting SHROB. This level of circulating glucagon was sufficient to elevate glucose and insulin during the 7 hr of observation (n = 9). Based on these results, we suggest that fasting hyperglucagonemia and impaired suppression of glucagon secretion and FFA in response to an oral glucose load may contribute to insulin resistance and glucose intolerance in the SHROB model of metabolic Syndrome X.

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Metabolic Syndrome X is characterized by insulin resistance, hypertension, hyperlipidemia, and obesity (1). Also referred to as insulin resistance syndrome, metabolic Syndrome X often precedes type 2 diabetes and atherosclerosis. Several animal models show one or more features of this syndrome (2), but few reproduce all the abnormalities.

The obese spontaneously hypertensive rat or Koletsky strain (SHROB/Kol) is a unique animal model with phenotypic features that strongly resemble metabolic Syndrome X (3). The SHROB has monogenetic obesity superimposed on a hypertensive genetic background. The obesity mutation is a recessive trait, designated fa^k, which is a non-sense mutation of leptin receptor gene resulting in a premature stop codon in the leptin receptor extracellular domain. Lean siblings of the SHROB, carrying one or no fa^k alleles, are spontaneously hypertensive rats (SHR) with elevated blood pressure but only mild insulin resistance relative to normotensive rats (4). The SHROB carries two fa^k alleles, is leptin resistant, and has circulating leptin levels 30-fold higher than its lean siblings. This mutation renders the SHROB incapable of central and peripheral responses to leptin (5).

The role of glucagon in the SHROB model of metabolic Syndrome X is unknown. Glucagon increases hepatic glucose production by stimulating hepatic glycogenolysis and gluconeogenesis and by promoting ketogenesis. These effects are antagonistic to those of insulin. Data on levels of plasma glucagon in animal models of insulin resistance are conflicting. In the fasted state, higher (6–8) and lower (9–11) glucagon levels relative to normal controls have been reported. In a Zucker rat model of insulin resistance, basal fasting levels of plasma glucagon have been reported to be lower than lean Zucker (7, 10). Zucker fatty rats also have blunted glucagon responses to hypoglycemia, but hypersecretion in response to arginine infusion (10). In the fed state, higher plasma glucagon concentrations have been reported in *ob/ob* mice and Zucker *fa/fa* rats relative to lean controls (6, 7).

Glucose loading would be expected to decrease circulating glucagon levels because glucagon is secreted in response to hypoglycemia. Surprisingly, glucagon responses to an oral glucose challenge are inconsistent between animal models of insulin resistance. Unchanging or lower plasma glucagon concentrations have been reported in obese and lean Zucker rats (12). Increases in glucagon secretion in response to a glucose challenge were noted in *ob/ob* mice (13). Elevation or lack of suppression of plasma glucagon have been reported after an oral glucose challenge among human diabetic subjects (14, 15). In contrast, normal human subjects show a fall in glucagon levels in response to an oral glucose challenge.

The implication of elevated plasma free fatty acids (FFA) in the insulin resistance of obesity was first proposed by Randle (16) more than 35 years ago. Randle postulated that increased availability of FFA decreased glucose oxidation in muscle through substrate competition. More recently, elevated FFA have been reported to induce hepatic insulin resistance (17), inhibit insulin secretion in isolated pancreatic islet cells (18), and promote gluconeogenesis (19). We hypothesized that elevated plasma glucagon is one of the factors contributing to insulin resistance in a leptin receptor knockout model of metabolic Syndrome X. In addition, we hypothesized that impaired reductions in plasma glucagon and FFA in response to a glucose load contribute to the glucose intolerance in the SHROB model of metabolic Syndrome X.

Materials and Methods

Animals. Experiments were performed on homozygous male and female SHROB (fa^k/fa^k; weight: 530 ± 13 g). Age and sex-matched lean SHR littermates (Fa^k/fa^k or Fa^k/Fa^k) were used in these experiments as well (weight: 281 ± 9 g). Animals were housed individually and were provided food (Tek lab formula 8664) and water *ad libitum*. Animals were on a 12:12-hr light:dark cycle (lights on from 0700–1900 hr) and were maintained at a constant temperature of 21°C. These procedures were carried out with the approval of the Case Western Reserve University Animal Care and Use Committee.

$n = 8$ for insulin and glucose were from our most recent age and sex matched samples from littermates in which these parameters were measured. $n = 20$ for glucagon comes from all recent fasting blood samples containing sufficient volumes, as the glucagon assay requires a large plasma sample > 100 μl. $n = 50$–51 for FFA comes from our initial experiments in which we collected minimal blood volumes to examine genotype differences.

Oral Glucose Challenge. The oral glucose challenge was conducted on 18-hr fasted rats. Rats were administered by gavage a 50% glucose solution at a dose of 6 g/kg body weight. Blood (0.2 ml) was obtained from the tail vein of unrestrained, conscious animals at 0, 30, 60, 120, 240, and 360 min and glucose was measured in whole blood by colorimetric glucose oxidase assay (One-Touch; Lifescan, Milpitas, CA). The remaining blood samples were allowed to clot on ice, and were centrifuged for 20 min at 5000*g* at 4°C and serum frozen at −70°C until assayed for insulin, glucagon, and FFAs.

Glucagon Challenge. SHR were injected i.p. with 500 ng/kg glucagon, and tail blood samples were obtained as described above for the glucose challenge, except that a 420-min sample was also obtained.

Plasma Analysis. Insulin and glucagon radioimmunoassay kits were used with rat insulin and glucagon standards and antibodies directed against rat insulin and glucagon, respectively (Linco, St. Charles, MO). The glucagon antibody used in this assay has no detectable cross-reactivity with glucagon like-peptides. FFAs were determined by an enzymatic kit (Wako Chemicals, Neuss, Germany). Assays were conducted in duplicate; intraassay variation was less than 5% and interassay coefficient of variation was less than 10%.

Statistical Methods. Results are presented as means ± SEM. Comparisons between groups were made using two-way analysis of variance (ANOVA) using Prism (Graph Pad Software, San Diego, CA) with *post hoc* analyses by Neuman-Keuls test. Pearson correlation coefficients were used to determine the relationship between body weight, plasma glucagon, and FFA concentrations.

Results

Baseline Characteristics. Table I represents the fasting metabolic profile of SHROB and lean SHR. The SHROB are normglycemic, showing no significant elevation in fasting blood glucose relative to lean SHR. SHROB had significantly higher plasma insulin, glucagon, insulin/glucagon (I/G) molar ratio, and FFA concentrations compared with lean littermates. Insulin showed the greatest dif-

Table I. Baseline Characteristics in SHROB and Lean SHR

	SHROB	SHR	SHROB:SHR
Insulin (ng/dl)	22.03 ± 5.06[a] (8)	0.55 ± 0.15 (8)	40.5
Glucose (mg/dl)	59 ± 2 (8)	56 ± 2 (8)	1.05
Glucagon (pg/dl)	136.9 ± 13.0[a] (20)	79.7 ± 4.2 (20)	1.71
Insulin/glucagon molar ratio	406 ± 127[a] (8)	16 ± 4.9 (8)	25.4
FFA (mM)	1.81 ± 0.09[a] (50)	1.45 ± 0.05 (51)	1.25

Note. Data are means ± SEM. (n).
[a] Significantly different from SHR ($P < 0.01$).

ference between SHROB and SHR, followed by I/G ratio, glucagon, and FFA ($P < 0.01$).

In order to evaluate the relationship of body weight to metabolic characteristics, correlation coefficients were determined. In contrast to the expected positive correlation, body weight and glucagon were unrelated in both SHROB and lean SHR rats ($r = -0.42$; $P = 0.08$ and $r = -0.14$; $P = 0.56$, respectively). In addition, there was a nonsignificant relationship between body weight and FFA in SHROB rats ($r = -0.25$; $P = 0.08$), but a slight positive correlation was found in lean SHR rats ($r = 0.28$, $P = 0.05$).

Oral Glucose Challenge. Figure 1 shows that despite fasting normoglycemia, SHROB have elevated blood glucose levels following a glucose load at all time points tested ($P < 0.01$, two-way ANOVA with repeated measures). Insulin levels are shown on a logarithmic scale to allow inclusion of SHROB and SHR on the same graph. In response to a glucose load, insulin reached peak values at

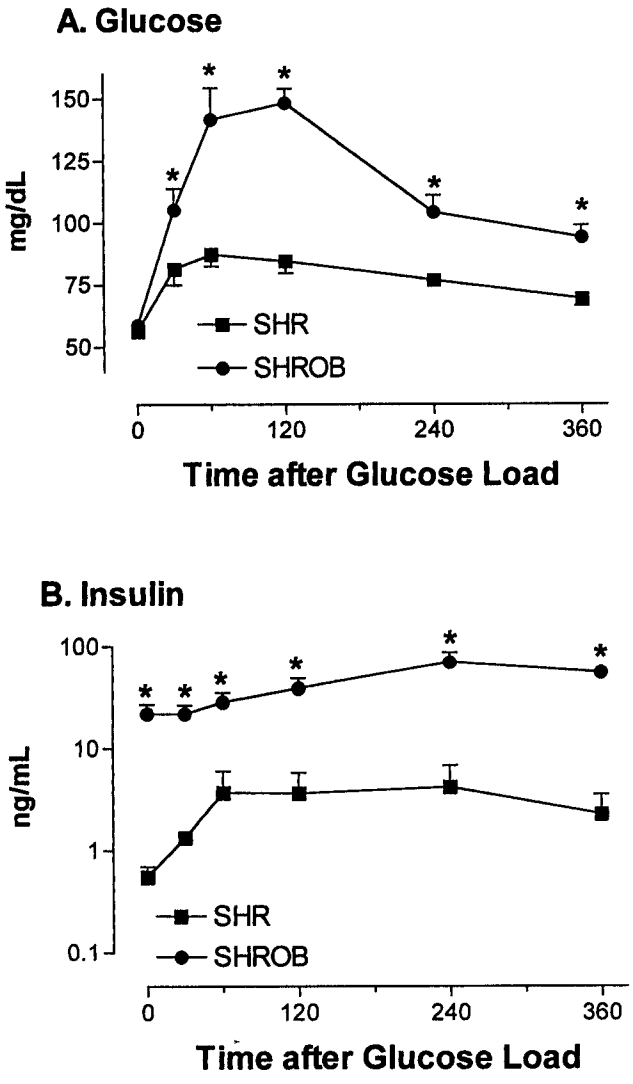

Figure 1. Glucose (A) and insulin (B) response to an oral glucose challenge in SHROB and lean SHR. Animals were fasted for 18 hr and were administered a 6-g/kg glucose load by oral gavage at time 0. Values are means ± SEM for eight animals, $^*P < 0.05$ verses lean SHR at same time point.

240 min in both genotypes, with insulin rising 8-fold in SHR, but was significantly attenuated only 3-fold elevation in SHROB. These data confirm that SHROB are extremely insulin resistant and mildly glucose intolerant compared with lean SHR.

In response to the glucose challenge, plasma glucagon levels showed a significant interaction between time and genotype ($P < 0.001$). As shown in Figure 2, plasma glucagon concentrations were significantly increased in SHROB at 30 and 60 min postchallenge (200% ± 45% and 91% ± 13%, respectively), whereas glucagon levels did not change significantly over time following glucose challenge in lean SHR ($P > 0.05$ by two-way repeated measures ANOVA). SHROB showed higher glucagon levels than lean SHR at all time points ($P < 0.0001$).

Glucagon secretion can be elevated in response to stress. Therefore, we sought to determine whether glucagon is increased by the nonspecific stress associated with the gavage procedure. Plasma glucagon levels did not change in response to an oral saline (0.9% NaCl) gavage in SHROB ($n = 6$; $P > 0.05$; data not shown).

A significant interaction between time and genotype was also present for the I/G molar ratio ($P < 0.02$). The I/G molar ratio was changed in opposite directions in the two genotypes, with a decrease in SHROB at 30 and 60 min, whereas lean SHR responded with an appropriate increase at 30 and 60 min postchallenge ($P < 0.01$).

The suppression of plasma FFA in response to a glucose challenge was absent in SHROB compared with lean SHR. At 30 min, plasma FFA levels were unchanged in SHROB compared with a drop of 30% ± 9% in lean SHR. At 60 min, plasma FFA levels were again unchanged from baseline in SHROB, yet had fallen 40% ± 6% in lean SHR ($P < 0.0001$; Fig. 2). To examine whether a blunted relative change in plasma insulin levels could account for the lack of FFA suppression during the oral glucose challenge in the SHROB, we determined the correlation between the percentage of change of insulin and m*M*/l change in FFA levels relative to baseline fasting values in all subjects. No correlation between the changes in insulin and those in FFA was detected ($r^2 = 0.001$; $n = 27$; $P = 0.87$).

Glucagon Challenge. Thus far, the data indicate that fasting glucagon is elevated by 72% in SHROB relative to SHR. This raises the question whether an elevation in plasma glucagon of this magnitude has any impact on circulating levels of glucose and insulin. To test this hypothesis, we injected fasted lean SHR with a low dose of glucagon and followed the evolution of glucose and insulin levels. We reasoned that if the higher glucagon levels in SHROB contributed significantly to their insulin resistance, then raising glucagon levels in lean SHR should reproduce a portion of the insulin resistance seen in SHROB. As shown in Figure 3, both glucose and insulin rose sharply in the first 30 min, and remained elevated throughout the 7-hr postinjection period. Plasma FFA levels were, however, unchanged after the glucagon injection. The peak increase of

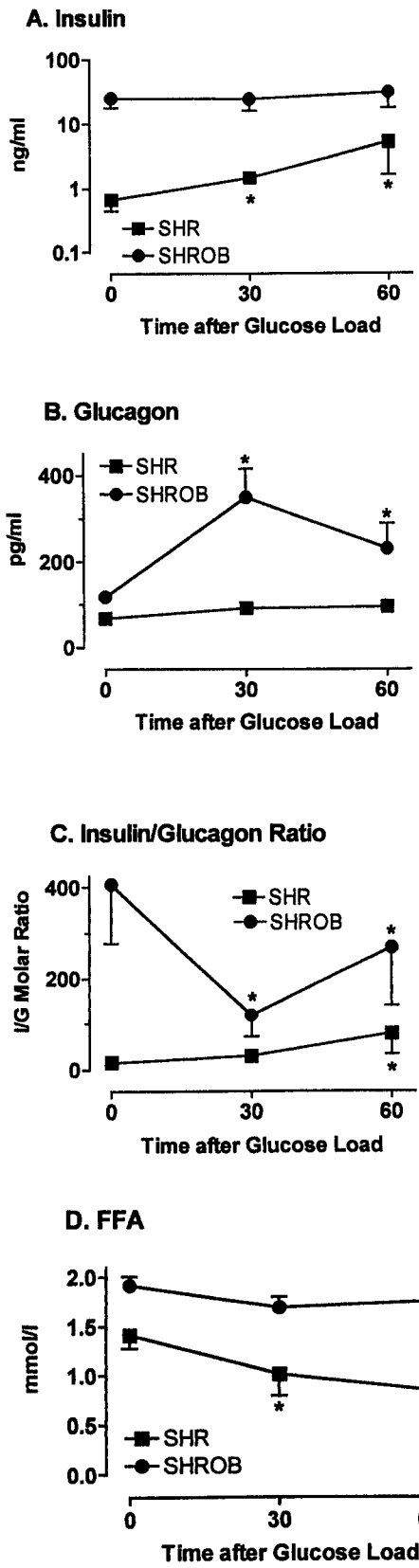

Figure 2. The metabolic effects of an oral glucose challenge in SHROB and lean SHR. Plasma insulin (A), plasma glucagon (B), insulin/glucagon molar ratio (C), and FFA (D). Animals were fasted for 18 hr and were administered a 6-g/kg glucose load by oral gavage at time 0. Values are means ± SEM for five to nine animals. An asterisk indicates a significant effect of the glucose challenge, $P < 0.05$ verses baseline (time 0) by Neuman-Keuls test.

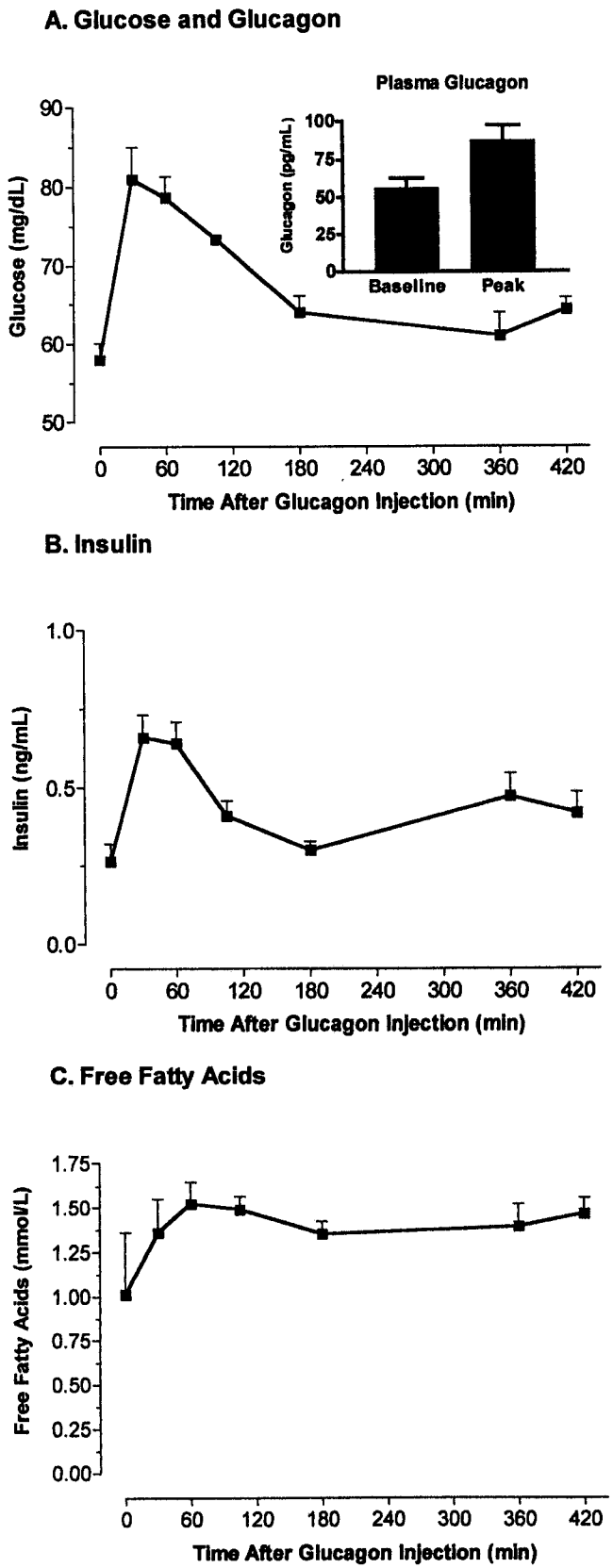

Figure 3. The metabolic effects of a low-dose glucagon injection in lean SHR. Plasma glucose (A), insulin (B), and FFA (C) levels following i.p. injection of 500 ng/kg glucagon in lean SHR. Peak levels of glucagon are shown in the inset of A. Animals were fasted for 18 hr, and blood samples were taken at the indicated times for determination of glucose, insulin, and glucagon. Values are means ± SEM for nine animals.

plasma glucagon following injection of exogenous glucagon was only 56% ± 5% above preinjection baseline (see inset), and was considerably below the baseline fasting levels of SHROB. Thus, the elevated level of plasma glucagon in SHROB, if present in their lean SHR littermates, would be sufficient to induce sustained elevations of plasma glucose and insulin in the fasted state. Glucagon is not likely to contribute to elevated FFA in SHROB.

Discussion

The data confirm that SHROB, despite fasting normoglycemia, are hyperinsulinemic and glucose intolerant compared with lean SHR. We show for the first time that SHROB have significantly higher fasting plasma glucagon concentrations and I/G molar ratios compared with lean SHR littermates. Based on the response of lean SHR to exogenous glucagon, the excess circulating glucagon is sufficient to alter glucose and insulin levels in a counterregulatory fashion. SHROB also exhibit significantly higher fasting plasma FFA concentrations then lean SHR. Furthermore, SHROB respond inappropriately to an oral glucose challenge in regards to changes in plasma glucagon and FFA concentrations.

During an oral glucose challenge, SHROB are glucose intolerant and have a delayed and exaggerated insulin response. In SHROB, plasma insulin concentrations typically peak between 180 and 240 min during an oral glucose tolerance test (OGTT) and are still elevated 360 min postchallenge. SHROB exhibited a 40-fold higher fasting insulin concentration than lean SHR despite normoglycemia, implying severe insulin resistance. Possible cellular mechanisms for insulin resistance include lower levels in SHROB relative to SHR of insulin receptor β-chain, insulin receptor substrate 1 (IRS-1), phosphorylated insulin receptor and IRS-1, and glucose transporter isoform-4 (20, 21). Thus, in face of reduced insulin signaling and lower expression of glucose transport proteins, it is reasonable to suggest that hypersecretion of insulin occurs as a compensatory mechanism.

The primary role of glucagon is to increase hepatic glucose production by increasing hepatic glycogenolysis and gluconeogensis. In addition, *in vitro* glucagon increases plasma FFA concentration by increasing lipolysis via a cAMP-dependent pathway (22). *In vivo*, lipolytic actions of glucagon have also been observed (23), but not in all studies (24). SHROB show a 72% elevation of fasting plasma glucagon concentrations compared with lean SHR. These experiments did not determine whether this elevation was due to hypersecretion or reduced catabolism. However, the rapid increase in plasma glucagon levels in response to a glucose challenge is more likely to reflect hypersecretion than reduced catabolism. In the *ob/ob* mouse model of insulin resistance, glucagon is also elevated (6). Furthermore, neutralization of glucagon by administration of glucagon antibody reduced glucose and insulin levels in those mice (25). Boron *et al.* (26) have suggested that among type 2 diabetics, 60% of basal hepatic glucose production is sustained by fasting plasma glucagon concentrations and that fasting hyperglucagonemia is largely responsible for the apparent insulin resistance and hyperinsulinemia. In addition, glucagon potentates glucose-induced insulin secretion in β cells during fasting (27, 28). Diabetes mellitus may be a dual disorder of pancreatic islet cell function with relative insulin deficiency and glucagon excess. Thus, hyperglucagonemia may be a potential contributing mechanism to the hyperinsulinemia in the SHROB.

In support of this contention, a small dose of glucagon given to lean SHR, sufficient to increase plasma glucagon by a maximum of about 50%, increased blood glucose and triggered a compensatory rise in plasma insulin. Plasma FFA levels were not significantly different after the glucagon injection compared with baseline. The lack of a lipolytic action of glucagon may be caused by the opposing actions of insulin, which was elevated in response to the glucagon injection. Thus, the increase in plasma glucagon detected in SHROB relative to SHR is probably of sufficient magnitude to be physiologically meaningful.

Interestingly, leptin has been reported to antagonize the hepatic effects of glucagon (29, 30). Zhao *et al.* (30) reported that leptin activates components of the insulin signaling pathways in hepatocytes, leading to decreased levels of cAMP. Central administration of leptin into rats and mice was reported to increase skeletal muscle glucose uptake (31, 32), but not when administered peripherally into *ob/ob* mice (33). Leptin administration to leptin-deficient *ob/ob* mice was reported to lower hepatic glucose production in the fasted state (34). Because the SHROB have no functional central or peripheral leptin receptors, the negative feedback system between leptin and glucagon would be lost and glucose production and metabolism would go unchecked in an insulin resistant state.

SHROB responded to an oral glucose challenge with a tripling of glucagon at 30 min postchallenge, whereas only a modest rise of less than one-third was seen among the lean SHR. In addition, SHROB had an impaired plasma FFA response at 30 and 60 min postchallenge compared with lean SHR. The absent of a decrease in plasma FFA levels is probably due to the lack of an early increase in insulin secretion (delayed first phase insulin secretion) and insulin resistance of lipolysis. During an OGTT, nonobese, glucose-tolerant humans respond by reducing circulating plasma glucagon and FFA concentrations (35). Type 2 diabetic, obese, and glucose-intolerant humans have abnormal glucagon and FFA responses to an OGTT. These human subjects respond with an elevation in plasma glucagon and FFA concentrations or with an impaired suppression postchallenge (14, 15, 36–38). Thus, the SHROB model shows similarities to human type 2 diabetes. The SHROB model could be used to study pharmacological agents that modify the various metabolic problems found in Syndrome X.

The molar ratio of circulating I/G has been implicated as a determinant of fatty acid metabolism (39, 40). The fasting I/G molar ratio was greatly elevated in SHROB (406 ± 127) compared with lean SHR (16 ± 4.9). In response to an oral glucose challenge, the I/G molar ratio changed in opposite directions. One would normally expect an increase in the I/G molar ratio, represented by increased insulin and decreased glucagon secretion during an oral glucose challenge. The SHROB in our study showed a decreased I/G molar ratio at 30 and 60 min postchallenge, whereas lean SHR responded with an 85% and 387% increase at 30 and 60 min postchallenge, respectively. Similar to the present results in SHROB rats, a higher I/G molar ratio has been reported in Zucker *fa/fa* compared with lean controls (7). In humans, an inappropriately low I/G ratio has been reported to increase hepatic glucose production (41). Thus, an inappropriate decrease in the I/G molar ratio may be an underlining feature contributing to impaired glucose tolerance in SHROB.

SHROB displayed 25% higher fasting plasma FFA concentrations than lean SHR, consistent with previous reports from our laboratory (21). The higher fasting plasma FFA concentration observed among the SHROB may be a consequence of decreased antilipolytic actions of insulin due to severe insulin resistance. Elevated plasma FFA concentrations may be involved in substrate competition with glucose and thus exacerbate insulin resistance (16). On the other hand, the relatively small elevation in fasting plasma FFA in SHROB alone may not be sufficient to drive insulin resistance.

Taken together, both elevated fasting plasma glucagon and FFA concentrations in the presence of reduced insulin-signaling proteins may be minor but significant contributors to insulin resistance in SHROB. The abnormal responses to an oral glucose challenge in the SHROB could impair glucose tolerance by several mechanisms. First, increased glucagon secretion concentrations could contribute to increase hepatic glucose production. Second, impaired suppression of plasma FFA concentrations could abate glucose utilization through the glucose-fatty acid cycle, thereby worsening glucose tolerance. Based on our current results showing that SHROB have higher fasting plasma glucagon and FFA concentrations compared with lean SHR, and that they have abnormal responses to an oral glucose challenge with an elevation in glucagon and an impaired suppression of plasma FFA, we suggest that these abnormalities together may exacerbate insulin resistance in this model of metabolic Syndrome X, with the role of glucagon being more significant than that of FFA.

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