Pancreatic Insulin Secretion in Rats Fed a Soy Protein High Fat Diet Depends on the Interaction between the Amino Acid Pattern and Isoflavones*

Lilia Noriega-López‡§*, Armando R. Tovar†, Marcela Gonzalez-Granillo***, Rogelio Hernández-Pando†, Bruno Escalante†, Patricio Santillán-Doherty**, and Nimbe Torres††

From the ‡1 Depto. de Fisiología de la Nutrición, **2 Depto. de Cirugía Experimental, and †3 Depto. Patología Experimental Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”, D.F. 14000, the ‡4 Depto. de Biomedicina Molecular, CINVESTAV, D.F. 07360, and the §5 Universidad Iberoamericana, México, D.F. 01219, México

Obesity is frequently associated with the consumption of high carbohydrate/fat diets leading to hyperinsulinemia. We have demonstrated that soy protein (SP) reduces hyperinsulinemia, but it is unclear by which mechanism. Thus, the purpose of the present work was to establish whether SP stimulates insulin secretion to a lower extent and/or reduces insulin resistance, and to understand its molecular mechanism of action in pancreatic islets of rats with diet-induced obesity. Long-term consumption of SP in a high fat (HF) diet significantly decreased serum glucose, free fatty acids, leptin, and the insulin/glucagon ratio compared with animals fed a casein HF diet. Euglycemic-hyperinsulinemic clamps showed that the SP diet prevented insulin resistance despite consumption of a HF diet. Incubation of pancreatic islets with isoflavones reduced insulin secretion and expression of PPARγ. Addition of amino acids resembling the plasma concentration of rats fed casein stimulated insulin secretion; a response that was reduced by the presence of isoflavones, whereas the amino acid pattern resembling the plasma concentration of rats fed SP barely stimulated insulin release. Infusion of isoflavones during the hyperglycemic clamps did not stimulate insulin secretion. Therefore, isoflavones as well as the amino acid pattern seen after SP consumption stimulated insulin secretion to a lower extent, decreasing PPARγ, GLUT-2, and SREBP-1 expression, and ameliorating hyperinsulinemia observed during obesity.

Obesity is a major health problem around the world because of chronic overnutrition and increase in the sedentary life style (1, 2). The development of obesity is accompanied by several metabolic changes including insulin resistance, hyperinsulinemia, dyslipidemia, hyperleptinemia, and hepatic steatosis among others, known as the metabolic syndrome (3, 4). Thus, the search for therapies to prevent the development of metabolic syndrome has increased over the last few years, including pharmacological and dietary therapies (5–11).

Previous studies have shown that long term consumption of soy protein diet reduces hyperinsulinemia, which in turn decreases the expression of the sterol regulatory element–binding protein (SREBP)2-1c in liver, reducing hepatic steatosis (12). Furthermore, recent evidence showed that soy protein is able to reduce hepatic lipotoxicity even in the presence of hyperinsulinemia and hyperleptinemia by a reduction in the expression of lipogenic genes and an increase in oxidative pathways (13). This evidence suggests that the type of dietary protein may play an important role in preventing the development of the metabolic syndrome.

It is not clear whether the effect of consumption of soy protein on serum insulin concentration is associated with changes in the mechanism of insulin secretion or peripheral insulin sensitivity. Insulin secretion by pancreatic β-cells is mainly regulated by plasma glucose concentration (14); in addition, other nutrients such as free fatty acids (FFA) and amino acids are involved in this process (15). Glucose uptake by the pancreatic β-cell is carried out through the non-insulin-dependent glucose transporter GLUT-2 (16). Once inside the cell, glucose is oxidized in the glycolysis and Krebs cycle generating ATP and raising the ATP/ADP ratio in the cytoplasm. The high ATP/ADP ratio provokes the closure of the ATP-sensitive K⁺ channels that leads to membrane depolarization and opening of voltage-sensitive calcium channels. Ca²⁺ influx through these channels raises the intracellular Ca²⁺ concentration and triggers exocytosis of insulin-containing granules (14, 17, 18). Fatty acids (FA) also increase insulin secretion by increasing FA β-oxidation leading to a high ATP/ADP ratio or by activating some protein kinases (19–21). On the other hand, some amino acids such as arginine, alanine, glutamine, and glycine modify

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† To whom correspondence should be addressed; Dept. Fisiología de la Nutrición, Instituto Nacional de Ciencias Médicas y Nutrición, Vasco de Quiroga No 15, Tlalpan, México, D. F., 14000, México. Tel.: 525-6553038; Fax: 525-6551076; E-mail: nimbet@quetzal.imss.mx.

2 The abbreviations used are: SREBP-1, sterol regulatory element binding protein-1; FFA, free fatty acids; GLUT-2, glucose transporter-2; PPAR, peroxisome proliferator activator receptor; RPMI, Roswell Park Memorial Institute media; HOMA, homeostasis model assessment; C, casein; S, soy protein; CSF, casein high in saturated fat diet; SSF, soy protein high in saturated fat diet; UCP-2, uncoupling protein-2; TG, triglycerides; HBSS, Hanks’ balanced salt solution.
the membrane potential, opening the voltage-sensitive calcium channels, and increasing insulin secretion (22–24). Interestingly, the isoflavones, genistein and daidzein, the main phytosterogens present in soy protein, also modify insulin secretion. In vitro studies incubating insulin-secreting cell lines or pancreatic islets with genistein, the most abundant phytosterogen present in soy protein, have shown controversial results; some studies show that genistein increases insulin secretion by its tyrosine kinase inhibitor activity, and others demonstrate the opposite effect (25–27). However, the phytosterogen effect on insulin secretion has not been demonstrated in vivo.

During obesity, there is hypersecretion of insulin associated with an enhanced pancreatic β cell glucose metabolism, FFA uptake, and hyperplasia (28–32). Thus, it is important to study whether long-term consumption of a soy protein diet containing isoflavones can modulate the process of insulin secretion in the β cell during obesity.

In the present work, the hyperglycemic clamp showed that long-term soy protein consumption stimulates to a lesser extent insulin secretion by the pancreas, even in the presence of saturated fat, when compared with animals fed the casein diet. This effect was associated with a decrease in pancreatic islet area, insulin, and PPAR mRNA abundance, which in turn prevents the induction of GLUT-2 and, therefore, insulin secretion. These effects were also observed in isolated pancreatic islets incubated with the amino acid concentration resembling those found after soy protein consumption as well as with isoflavones. Our data also revealed that infusion of isoflavones rapidly reduced insulin secretion in vivo. Furthermore, animals fed soy protein showed an improvement in insulin sensitivity measured by euglycemic clamp when compared with those fed the casein diet, indicating that these animals require less insulin to maintain glucose in the normal range. Our results suggest that insulin secretion is regulated by the type and concentration of amino acids sensed by the pancreas as well as the presence of isoflavones. The combined effect of amino acids and isoflavones present in the soy protein explain the prevention of hyperinsulinemia, even in animals fed a high fat diet.

**EXPERIMENTAL PROCEDURES**

**Animal Care and Maintenance**—Sprague-Dawley rats were purchased from Harlan-Teklad (México, DF) with a weight between 200 and 220 g. The day after arrival, rats were placed in individual cages, maintained on a constant 12 h light/dark cycle at 22 °C, and randomly assigned to four experimental diets. Rats had free access to their experimental diets and water for 130 days. Diets were administered in dry form, and the composition of each diet is described in Table 1. Isolated soy protein used in these studies contained 1.38 and 0.71 mg/g protein of genistein and daidzein, respectively. Animals were weighed every other day, and food consumption was determined every day. The animal protocol was approved by the Animal Committee of the National Institute of Medical Sciences and Nutrition, Mexico City.

**Serum Measurements**—Rats were deprived of food overnight before killing by decapitation after being immobilized with CO₂. Blood was collected in tubes with gel and clot activator (Beckton Dickinson, Franklin Lakes, NJ) and centrifuged at 1,000 × g for 15 min to obtain serum. Fasting serum glucose was measured using an YSI2700 select Biochemistry Analyzer (YSI Incorporated, Yellow Spring, OH). Cholesterol and triglycerides were quantified by the SERA-PAK Plus kit (Bayer, Buenos Aires, Argentina). Free fatty acids were determined using a FFA Microtest kit (Roche Applied Science). Insulin, glucagon, and leptin were measured using rat-specific radioimmunoassay kit (LINCO Diagnostics, St Charles, MO).

**Euglycemic Clamp**—Animals were cannulated as described above. Insulin infusion was performed at a rate of 10 milliunits/kg/min for 2 h. Blood glucose was clamped at 100 mg/dl by

### Table 1

| Ingredient (%) | Casein | Soy | Casein fat | Soy fat |
|---------------|--------|-----|------------|--------|
| Casein        | 30     | 0   | 30         | 0      |
| Soy protein   | 0      | 30  | 0          | 30     |
| Cornstarch    | 19.5   | 19.5| 19.5       | 19.5   |
| Dextrose      | 5      | 5   | 5          | 5      |
| Corn oil      | 5      | 5   | 5          | 5      |
| Mineral mixture| 1     | 1   | 1          | 1      |
| Vitamin mixture| 0.0165| 0.0165| 0.0165| 0.0165 |
| Lard          | 0      | 0   | 20         | 20     |

**Hyperglycemic Clamp in the Presence of Phytoestrogens**—The procedure was similar to that described above using Sprague-Dawley rats fed Chow diet with a weight of 400 g, and were never exposed to a soy protein diet or isoflavones. Five minutes prior to the glucose bolus, genistein, daidzein, or equol, a metabolite of genistein, were infused to give a final plasmatic concentration of 0.4, 0.15, or 2.55 μmol/liter, respectively. These phytosterogens concentrations correspond to those found in the plasma of rats fed a soy protein diet containing phytosterogens (37). Serum glucose and insulin were determined every 5 min. At the end of the clamp, the pancreas was removed and frozen for gene expression analysis.

**Euglycemic Clamp**—Animals were cannulated as described above. Insulin infusion was performed at a rate of 10 milliunits/kg/min for 2 h. Blood glucose was clamped at 100 mg/dl by
determining the blood glucose concentration at 5-min intervals. The glucose infusion rate during the second hour was taken as a response parameter, indicating insulin sensitivity (38).

Islet Isolation—Islets were isolated from rats in each group as described before (39). Briefly, the pancreas was infused through the bile duct with Hanks’ balanced salt solution (Invitrogen), and the tissue was isolated, minced, and digested with 3 mg of collagenase P (Roche Applied Sciences) for 14 min at 37 °C with constant agitation. Islets were washed with Hanks’ balanced salt solution, centrifuged at 100 × g for 3 min at 4 °C, and then decanted and resuspended; this procedure was repeated three times. Islets were separated on a Ficoll step density gradient and handpicked under a stereomicroscope to exclude any contaminating tissue.

Isolation of Total RNA and Real Time PCR—Total RNA was extracted from islets by the TRizol isolation method according to the manufacturer’s protocol (Invitrogen). For quantitative real-time PCR, the first strand cDNA was synthesized from 300 ng of total RNA using the oligo (dT) primer and MMLV reverse transcriptase (Invitrogen). Samples were subjected to quantitative amplification using the TaqMan probe and primer sets for rat GLUT-2 (Rn00563565_m1), SREBP-1 (ATOVARPE1-PE1), PPARα (Rn00440945_m1), and PPARγ (Rn00566193_m1), and PPARα (Rn00566193_m1).

PCR amplification was carried out in triplicate for each sample and performed in a total volume of 10 μl containing 30 ng of cDNA, 900 nM of each primer, 250 nM of the respective probe, and 6 μl of Taq Man Universal PCR Master Mix. The conditions of amplification and detection were described previously (13). Gene expression was normalized with the expression of the housekeeping gene actin. Probes and primers were obtained from PE Applied Biosystems (Pre-Developed TaqMan Assay Reagents Control kits).

Histological Analysis—The pancreas was rapidly removed. A fraction was fixed in tissue-tak and immediately frozen for red oil staining. Another fraction was fixed in 10% formalin and stored at 4 °C for morphological studies. Briefly, the pancreas was fixed by immersion in ethanol and embedded in paraffin. Sections were cut at a thickness of 4 μm at different levels of depth. Every section was mounted on glass slides and stained with hematoxylin and eosin. Morphological analysis was performed to determine pancreatic islet area and number using a Leica Qwin image-analyzer system on a Leica DMLS microscope.

Pancreatic Islet Cultured with Phytoestrogen or Amino Acids—Islets were obtained from male Sprague-Dawley rats (200–250 g) by collagenase digestion as described above. Islets were maintained overnight in suspension culture in 12-well plates at 37 °C in an atmosphere of 5% CO₂ and 95% air. The culture conditions were as follows:

- Casein
- Soy
- Casein fat
- Soy fat

| Parameter          | Casein | Soy | Casein fat | Soy fat |
|--------------------|--------|-----|------------|---------|
| Body weight (g)    | 430.03 | 414.10 | 463.11     | 443.74  |
| Food intake (kcal/d)| 64.15  | 63.22 | 64.61      | 64.95   |
| Glucose (mg/dL)    | 91.05  | 90.15 | 123.00     | 112.33  |
| Triglyceride (mg/dL)| 59.67  | 40.33 | 73.5        | 64.33   |
| Cholesterol (mg/dL)| 88.83  | 80.33 | 96.83      | 86.5    |
| Free fatty acids (mM)| 1.13 | 0.99 | 1.72       | 1.26    |
| Insulin (ng/ml)    | 0.54   | 0.99 | 0.60       | 0.59    |
| Glucagon (pg/ml)   | 75.24  | 79.05 | 59.45      | 86.84   |
| Insulin/glucagon ratio | 7.18 | 4.11 | 10.25      | 6.82    |
| Leptin (ng/ml)     | 3.08   | 2.12 | 8.07       | 4.14    |

Results are means ± S.E. (n = 4). Superscript matched letters indicate significant differences among each group (p < 0.05).

FIGURE 1. Plasma glucose and insulin concentrations during hyperglycemic clamps of rats fed a high fat diet with different types of protein. Plasma glucose (A) and insulin (B) concentrations were determined every 10 min before and every 5 min after a glucose bolus of 375 mg/kg in rats fed 30% casein or soy protein with or without 20% saturated fat. The glucose infusion rate after the glucose bolus was not statistically different among the four groups (p < 0.05). Values are means ± S.E. (n = 4). Different letters or * indicate significant differences among groups (p < 0.05).
medium consisted of RPMI 1640 (Invitrogen) supplemented with 11 mM glucose, 2 mM glutamine, 10% of heat-inactivated fetal bovine serum, and 5% of Antibiotic-Antimycotic (Invitrogen). Then, islets were washed and incubated for 2 h at 37 °C in Hanks' balanced salt solution (HBSS) enriched with 11 mM glucose, and 0.2% NaHCO₃, with 5 μM genistein, daidzein, or equol. Furthermore, another sample of islets were incubated for 2 h at 37 °C under the same conditions as above in HBSS containing the amino acid concentration present in soy protein or casein and with the plasma amino acid concentration resembling those found after casein or soy protein consumption (40) with and without isoflavones. After incubation, RNA was extracted as described above. Also, medium was collected and frozen for insulin determination as described above.

Statistical Analysis—All data are presented as mean ± S.E. and were analyzed with Statview software (Abacus Concepts) using one-way analysis of variance combined with the Fischer protected least-significant difference test. p < 0.05 was considered to be statistically significant.

RESULTS

Soy Protein Modified Biochemical and Hormonal Parameters—As can be seen in Table 2, animals fed high fat diet had higher body weight, serum glucose, triglycerides (TG), cholesterol, FFA, leptin, and insulin:glucagon (I/G) ratio, than those fed control diets. However, consumption of soy protein in the high fat diet reduced significantly the body weight, glucose, FFA, I/G ratio, and leptin in comparison with animals fed the casein high fat diet, suggesting that not only the presence of saturated fat in the diet but the type of protein modifies biochemical and hormonal parameters during the development of obesity.

Soy Protein and Insulin Secretion—To determine if soy protein modified insulin secretion in a
high fat diet, hyperglycemic clamps were performed in rats fed 30% casein (C) or 30% soy protein (S), 30% casein + 20% saturated fat (CSF), and 30% soy protein + 20% saturated fat (SSF). Plasma glucose concentration was similar in the basal period for the groups C, S, and SSF (88.6 ± 3.3 mg/dl), whereas the group of CSF presented significantly higher plasma glucose concentration than the rest of the groups (154.0 ± 4.8 mg/dl), indicating an increased insulin resistance due to the presence of animal protein and saturated fat in the diet. In the hyperglycemic period, after the glucose bolus, there was an increase in plasma glucose concentrations up to 277.0 ± 14.2 mg/dl for the groups C, S, and SSF, whereas the CSF group reached 335.0 ± 5.8 mg/dl indicating that the group fed CSF is less sensitive to glucose utilization (Fig. 1A). On the other hand, fasting basal plasma insulin concentration (Fig. 1B) was similar in the 4 groups (0.155 ± 0.01 ng/ml). After the glucose bolus infusion, the 4 groups showed the same first peak of insulin secretion at minute 5. This initial increase is due to secretion of preformed insulin, which is soon significantly depleted. The secondary rise in insulin reflects the considerable amount of newly synthesized insulin (41). As can be seen in Fig. 1B, insulin release significantly increased in the CSF group (0.77 ± 0.13 ng/ml) over the C group (0.43 ± 0.1), and this in turn was higher than the SSF group (0.28 ± 0.03). Finally, the SSF group was higher than the S group (0.08 ± 0.01). These results indicate that rats fed soy protein secreted less insulin to maintain glucose at normal values.

Increase in Insulin Secretion Involved an Increase in Pancreatic Islet Area, Number, and Lipid Depot, as well as Insulin and SREBP-1 mRNA Expression—Increased insulin secretion is in part related to pancreatic islet hyperplasia (31, 42, 43). Total pancreatic islet area in CSF rats was ~45–50% bigger than the rest of the groups (Fig. 2B). In addition, there was no difference in the number of islets less than 20,000 µm² in the four groups; however, the number of pancreatic islets in the CSF group with an area >20,000 µm² doubled over the number of the rest of the groups (Fig. 2A). These data suggest that after chronic consumption of a high fat diet, islets increased in area and number to secrete more insulin to try to maintain glucose homeostasis. This was not possible due to the development of insulin resistance during obesity. Insulin mRNA abundance was significantly lower in the S and SSF groups than the C and CSF groups (Fig. 2C). These results indicated that soy protein prevented the induction of insulin gene expression even in the presence of saturated fat in comparison with rats fed the casein diet. Furthermore, normalization of insulin mRNA expression by the area of pancreatic islets significantly correlated with the amount of insulin secreted after 30 min of a glucose bolus (Fig. 2D).

On the other hand, pancreatic islet area is in part related to its intracellular lipid content (44). The transcription factor SREBP-1 mediates the expression of lipogenic genes. As shown in Fig. 3A, there was a 62% reduction in SREBP-1 expression in rats fed the S diet with respect to those fed the C diet. Interestingly, the presence of fat increased SREBP-1 expression in the SSF group without reaching the levels seen in the CSF group. However, changes in SREBP-1 expression did not correlate with the lipid depots observed in the islets. As can be seen in Fig. 3B, the only group that showed lipid depots was the CSF group.

These results suggest that the main responsibility of lipid accumulation is the influx of circulating FFA (Table 2) rather than lipogenesis mediated by SREBP-1.

Soy Protein Reduced GLUT-2 Transporter by Reducing PPARγ Induction in a High Fat Diet—The reduced stimulation of insulin secretion seen in the S and SSF groups could be in part...
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FIGURE 5. Insulin secretion and SREBP1c, PPARγ and PPARα mRNA expression in pancreatic islets incubated with amino acids and isoflavones. Pancreatic islets were incubated in Hanks’ balanced salt solution with amino acids resembling the casein and soy protein amino acid pattern. Concentration of amino acid in the medium resembling casein amino acid pattern (µM): Ala, 314; Arg, 195; Asp, 473; Cys, 21; Glu, 1393; Gly, 213; His, 167; Ile, 396; Leu, 625; Lys, 492; Met, 174; Phe, 272; Pro, 825; Ser, 476; Thr, 327; Trp, 49; Tyr, 259; Val, 520. Concentration of amino acid in the medium resembling soy protein amino acid pattern (µM): Ala, 460; Arg, 430; Asp, 894; Cys, 108; Glu, 1461; Gly, 559; His, 167; Ile, 373; Leu, 617; Lys, 431; Met, 87; Phe, 327; Pro, 477; Ser, 495; Thr, 310; Trp, 73; Tyr, 220; Val, 384. Pancreatic islets also were incubated with 5 µM genistein, daidzein, or equol for 2 h. After incubation, secreted insulin was determined by RIA kit (A). Total RNA was obtained from each culture and detection of SREBP-1 (B), PPARγ (C), and PPARα (D) mRNA abundance was performed by real-time PCR. mRNA abundance is relative to the expression of each gene in pancreatic islets incubated only with HBSS. Results are means ± S.E. (n = 4). Different letters indicate significant differences among groups (p < 0.05).

due to changes in the glucose transporter (GLUT-2). GLUT-2 mediates the entrance of glucose to the β cell to initiate insulin secretion. The GLUT-2 gene has a response element for PPARγ, and an increase in PPARγ expression raises the level of glucose-stimulated insulin secretion (45, 46). As shown in Fig. 3D, there was a 53.5% increase in PPARγ expression in rats fed the CSF diet with respect to the SSF group (Fig. 3D). Thus, animals fed the S diet showed lower GLUT-2 mRNA abundance than the C group (Fig. 3C) suggesting less glucose entrance to the β cell. The presence of saturated fat in the diet increased significantly the expression of this transporter (Fig. 3C), although the increase in the SSF group was lower than animals fed the CSF diet. These results indicate that the S and SSF groups stimulate to a lower extent insulin secretion by a decrease in GLUT-2 mRNA expression through PPARγ.

PPARα Is Not Modified by Soy Protein in Pancreatic Islets—It is known that PPARα regulates insulin secretion (47). Expression of PPARα was not modified in rats fed the CSF diet (Fig. 4A), and it was significantly reduced in animals fed the C, S, or SSF diet (relative expression -0.6). As a consequence, there was a reduction in some of the PPARα target genes such as the uncoupling protein (UCP)-2 (Fig. 4B) and CPT-1 (Fig. 4C). These results indicate that PPARα is not involved in the reduction of insulin secretion, although the oxidative capacity of pancreatic islets is reduced. This could be explained in part by the age of the animals, because the expression of PPARα, CPT-1, and UCP-2 was lower compared to rats at the beginning of the experiment. The presence of saturated fat in the diet reduced even more UCP-2 gene expression (Fig. 4B).

The Casein or Soy Protein Amino Acid Pattern and Phytoestrogen Content Modified SREBP-1, PPARα, and PPARγ Expression—It is not known if the amino acid pattern of the soy protein, or the phytoestrogens tightly associated to this protein, are responsible for a lower stimulation of insulin secretion. Therefore, pancreatic islets were incubated with concentrations of amino acids resembling those found in casein or soy protein or with different phytoestrogens. Incubation with the amino acid pattern of casein stimulated insulin secretion by about 100% above the control, whereas incubation of islets with the amino acid pattern of soy protein stimulated by only 30% (Fig. 5A). Incubation with genistein, daidzein, or equol resulted in a decrease in insulin secretion by 12–30% (Fig. 5A).

To determine if insulin secretion in islets incubated with amino acids or phytoestrogens was associated with the expression of lipogenic or oxidative genes, SREBP-1, PPARγ, and PPARα mRNA expression were measured after 120 min of incubation. There was an increase in SREBP-1 expression in islets incubated with the amino acid mixture of soy protein (Fig. 5B). Incubation of pancreatic islets with genistein or daidzein...
resulted in a 50% reduction in SREBP-1 expression (Fig. 5B). These results indicated that phytoestrogens were effective in reducing insulin secretion of pancreatic islets in vitro and down-regulate SREBP-1 expression.

In view of the fact that a reduced stimulation of insulin release in rats with long term soy protein consumption was associated with lower PPARγ mRNA expression, we evaluated its expression in islets incubated with the amino acid pattern resembling those of casein or soy protein or phytoestrogens. Islets incubated with the amino acid pattern of soy protein significantly decreased PPARγ mRNA expression with respect to the casein amino acid pattern. As can be seen, daidzein and equol also reduced the expression of PPARγ (Fig. 5C). These results indicate that the amino acid pattern as well as phytoestrogens are responsible for the effects seen in in vivo studies.

To make clear if PPARα was involved in insulin secretion in short-term in vitro studies, we measured its expression in pancreatic islets. PPARα was reduced by the soy amino acid pattern or the presence of genistein in comparison with the casein amino acid pattern (Fig. 5D). These results suggest that the proportion of amino acids as well as the phytoestrogen content play an important role in insulin secretion and in the expression of transcription factors involved in lipid metabolism.

**Serum Amino Acid Concentration after Consumption of Soy Protein and Phytoestrogens Stimulate Insulin Secretion to a Lower Extent**—Pancreatic islets do not detect "protein" per se, but rather the postprandial increase in circulating amino acids. Thus, we assessed the effect of the amino acid pattern found in plasma after the consumption of soy protein, and the combined effect of this amino acid pattern simultaneously with phytoestrogens on insulin secretion. The results indicate that the plasma amino acid pattern observed after soy protein consumption had significantly lower stimulation of insulin secretion than the plasma amino acid pattern after casein consumption. Interestingly, the presence of the three phytoestrogens decrease significantly the insulin secretion observed with the casein amino acid pattern. In the case of the plasma amino acid pattern after soy protein consumption, the presence of phytoestrogens did not have an additional effect on insulin secretion (Fig. 6A). To demonstrate if in in vitro studies PPARγ and GLUT-2 were involved in the reduction of insulin secretion, we measured their mRNA abundance in pancreatic islets. As shown in Fig. 6, B and C, there was a decrease in PPARγ and GLUT-2 in pancreatic islets incubated with the plasma amino acid pattern after the consumption of soy protein. Furthermore, the presence of phytoestrogens significantly decreased PPARγ and GLUT-2 even with the plasma amino acid pattern of the casein protein.

**Phytoestrogens Decreased Insulin Secretion in Vivo**—To assess whether phytoestrogens modify insulin secretion in vivo, hyperglycemic clamps were carried out in the presence of genistein, daidzein, or equol at concentrations observed in plasma of rats after the consumption of a soy protein diet (37). The results showed that the presence of daidzein and equol after the glucose bolus did not modify the first peak of insulin secretion; however, after 10 min, daidzein and equol maintained insulin near to basal levels, whereas in the control group there was a continuous increase in insulin secretion (Fig. 7B).
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**FIGURE 7.** Hyperglycemic clamps and PPARγ and GLUT-2 mRNA abundance of pancreas of rats infused with genistein, daidzein, and equol. Plasma glucose (A) and insulin (B) concentrations were determined every 10 min before and every 5 min after a glucose bolus of 375 mg/kg in rats infused with genistein, daidzein or equol. Detection of PPARγ (C) and GLUT-2 (D) mRNA abundance was performed by real-time PCR. Total RNA was obtained from pancreas of rats at the end of hyperglycemic clamp. mRNA abundance is relative to the expression of each gene in the pancreas of rats without infusion of phytoestrogens. Results are means ± S.E. (n = 4). Different letters indicate significant differences among groups (p < 0.05).

Genistein almost suppressed the elevation of insulin after glucose infusion. At the end of the clamp, the pancreas was removed, and RNA was isolated to determine expression of insulin, PPARγ, and GLUT-2 mRNA. As observed in Fig. 7, C and D, genistein was the phytoestrogen more effective to down-regulate the expression of PPARγ and GLUT-2 mRNA, and it was associated with a significant reduction in insulin secretion. Infusion of equol barely increased PPARγ expression and decreased GLUT-2 at the same extent of genistein. Daidzein did not change PPARγ and GLUT-2 mRNA expression. These results demonstrated that isoflavones were capable of decreasing insulin secretion in vivo.

**Soy Protein Prevented Insulin Resistance after Chronic Consumption of High Saturated Fat Diet**—To determine if the reduction in insulin secretion was associated with changes in insulin sensitivity, a euglycemic-hyperinsulinemic clamp was performed. As shown in Fig. 8B, the glucose infusion rate in the CSF group was significantly lower than the SSF group, and this was significantly lower than the C and S groups (Fig. 8B). These results indicate that SSF group had more insulin sensitivity than the CSF group despite the high content of saturated fat in their diets. No difference was observed between the C and S groups.

**DISCUSSION**

There are compensatory mechanisms that buffer the metabolic consequences of short-term overnutrition; however, these mechanisms are incapable of compensating chronic changes in energy imbalance, leading in the development of obesity. The fact that individuals who are clinically normal show insulin resistance, increased central fat distribution, and high plasma TG, has prompted attention by the scientific community to understand the molecular basis of metabolic consequences during the development of obesity.

Consumption of high fat and high carbohydrate diets are associated with the development of obesity (2, 48, 49). In the course of obesity, hyperinsulinemia appears and stimulates lipogenesis mediated by the transcription factor SREBP-1 in the liver and adipose tissue (48). However, not only dietary fat or carbohydrates have an influence in the development of insulinemia and regulation of genes involved in lipid metabolism, but also dietary protein may play an important role. Previous studies have shown that long-term consumption of soy protein decreases hyperinsulinemia and hepatic steatosis by reducing the expression of SREBP-1 and enzymes involved in lipogenesis (12). In addition, the type of protein modifies the I/G ratio, which in turn regulates the expression of lipogenic genes. The high I/G ratio is associated with an increased risk of developing cardiovascular diseases because of its hyperlipidemic and atherogenic effect (50). Generally, consumption of plant proteins give low I/G ratios associated with low serum lipids, whereas animal protein produces the opposite effect (51). As observed in Table 2, there was a lower I/G ratio in animals who were long-term-fed the SSF diet than in animals fed the CSF diet. This was associated with a decrease in serum glucose, FFA, and leptin, indicating that the type of protein may improve the metabolic consequences of the consumption of a high saturated fat diet.

It is not fully understood if long-term consumption of a specific type of protein in a high fat diet may regulate insulin secretion or modify insulin sensitivity. The results in the present study indicate that long-term soy protein consumption stimulates to a lower extent insulin secretion (Fig. 1B). To understand the possible mechanism(s) by which soy protein stimulates insulin secretion to a lower extent, we studied pancreatic islet area and lipid content, because it has been demonstrated that hyperinsulinemia is related to hyperplasia of pancreatic islets attributed to the accumulation of lipids (44). We observed a significant increase in pancreatic islet area and lipid content in rats fed the CSF diet, whereas animals fed the SSF diet did not show a difference in comparison with the control groups (Figs. 2B and 3B). It has been demonstrated that endogenous pancre-
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GLUT-2 and PPARγ. GLUT-2 is the main transporter of glucose in pancreas, and it is regulated by PPARγ because the GLUT-2 gene promoter has a PPAR response element (45, 55). Our data showed that animals fed the CSF diet had significantly higher PPARγ and GLUT-2 expression than animals fed SSF (Fig. 3, C and D). These results suggest more glucose uptake by pancreatic islets resulting in more insulin secretion.

It has been demonstrated that another possible modulator of insulin secretion is PPARα (47). PPARα in turn regulates the transcription of CPT-1 and UCP-2 (56). However, our results showed that there was no induction of PPARα or its target genes, CPT-1 and UCP-2 (Fig. 4, A–C). Thus, the difference in lipid content in pancreatic islets between CSF and SSF groups seems to depend on differences in FA accumulation as TG rather than FA oxidation.

On the other hand, it has been demonstrated that high fat diets decrease insulin sensitivity (1). As seen in Fig. 8B, the type of dietary protein may modulate insulin sensitivity despite the high saturated fat content in the diet. The results with the euglycemic-hyperinsulinemic clamp clearly showed that animals fed SSF required more glucose infusion to maintain euglycemia in comparison with those fed the CSF diet indicating that rats fed SSF are significantly more sensitive to insulin action (Fig. 8B). This explains in part why animals fed SSF secrete less insulin when measured by the hyperglycemic clamp (Fig. 1B).

On the other hand, it is not clear which component of soy protein is responsible for reducing insulin secretion. First, despite the observation that soy protein and casein are high quality proteins, their amino acid patterns are different, and they stimulated to different extents insulin secretion. As seen in Figs. 5A and 6A, the amino acid pattern resembling soy protein or plasma amino acid concentration after consumption of a soy protein diet stimulates to a significantly lower extent insulin secretion. This reduction of insulin secretion will prevent stimulation of adipogenesis and lipogenesis, which are central elements in the development of metabolic syndrome. Second, other possible responsibilities of insulin secretion could be the amino acid pattern resembling soy protein or plasma amino acid concentration after consumption of a soy protein diet stimulates to a significantly lower extent insulin secretion. This reduction of insulin secretion will prevent stimulation of adipogenesis and lipogenesis, which are central elements in the development of metabolic syndrome. Second, other possible responsibilities of insulin secretion could be the amino acid pattern resembling soy protein or plasma amino acid concentration after consumption of a soy protein diet stimulates to a significantly lower extent insulin secretion. This reduction of insulin secretion will prevent stimulation of adipogenesis and lipogenesis, which are central elements in the development of metabolic syndrome.

We performed an in vivo study using a hyperglycemic clamp infusing these phytoestrogens to maintain physiological concentrations in the plasma observed in rats fed a soy protein diet. The results showed that these compounds, mainly genistein, reduced rapidly the release of insulin, implying that there must be a short-term mechanism regulating this process by altering possibly the membrane polarity. Furthermore, we also observed that infused isoflavones decreased the expression of PPARγ, which may mediate long-term reduction of insulin secretion.
Soy Protein and Insulin Secretion

In summary, our findings showed that chronic consumption of a saturated fat diet increased insulin secretion associated with an increase in pancreatic islet area and lipid content, and insulin mRNA by a mechanism that involves an increase in PPARγ and GLUT-2 mRNA abundance. These effects were ameliorated by the presence of soy protein in a high saturated fat diet. The mechanism by which soy protein reduces insulin secretion is mediated by the amino acid pattern as well as the isoflavones tightly bound to this protein. Also, soy protein consumption increases insulin sensitivity despite the consumption of a high fat diet. These beneficial effects will prevent hyperinsulinemia and reduce the formation of fatty liver and lipotoxicity.

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