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# Increased Insulin Sensitivity and Changes in the Expression Profile of Key Insulin Regulatory Genes and Beta Cell Transcription Factors in Diabetic KKAy-Mice after Feeding with a Soy Bean Protein Rich Diet High in Isoflavone Content

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High content isoflavone soy protein (SBP) (Abalon) has been found in animal studies to possess beneficial effects on a number of the characteristic features of the insulin resistance syndrome. The aim of this study was to investigate whether SBP exerts beneficial effects on metabolism in the diabetic KKAy-mouse. Furthermore, we investigated the long-term in vivo effect of SBP on the expression profile in islets of key insulin regulatory genes. Twenty KKAy-mice, aged 5 weeks, were divided into 2 groups and treated for 9 weeks with either (A) standard chow diet (control) or (B) chow + 50% SBP. Twenty normal C57BL-mice fed with standard chow diet served as nondiabetic controls (C). Blood samples were collected and analyzed before and after intervention. Gene expression was determined in islets by quantitative real-time RT-PCR and Affymetrix microarray. It was demonstrated that long-term treatment with SBP improves glucose homeostasis, increases insulin sensitivity, and lowers plasma triglycerides in diabetic KKAy-mice. SBP reduces fasting plasma glucose, insulin, triglycerides, and total cholesterol. Furthermore, SBP markedly changes the gene expression profile of key insulin regulatory genes *GLUT2*, *GLUT3*, *Ins1*, *Ins2*, *IGF1*, *Beta2/Neurod1*, *cholecystokinin*, and *LDLr*, and proliferative genes in islets isolated from KKAy-mice. After 9 weeks of treatment with SBP, plasma glucose and insulin homeostasis was normalized compared to start levels. The results indicate that SBP improves glucose and insulin sensitivity and up-regulates the expression of key insulin regulatory genes.

**KEYWORDS: KKAy-mice; T2DM; soy bean isolate; islets; insulin sensitizer; islet proliferation; real time RT-PCR; microarray; beta cell transcription factors**

## INTRODUCTION

Type 2 diabetes (T2DM) and the insulin resistance syndrome are currently increasing as a global epidemic. The insulin resistance syndrome is clinically characterized by insulin resistance, visceral obesity, dyslipidemia, and hypertension and is closely associated with coronary artery disease (CAD). Individuals with insulin resistance syndrome are at a 3-fold greater risk of CAD and stroke, and have more than a 4-fold greater risk of cardiovascular mortality (*1, 2*).

Dysfunction of the insulin-producing beta cells of the pancreas during the development of insulin resistance is recognized as a major reason for and precedes escalation of peripheral insulin resistance and progression to overt T2DM. Our understanding of the gene changes in the beta cell during the development of insulin resistance is essential, including changes in genes involved in glucose sensing, transcription factors regulating insulin expression and beta cell function, the insulin signaling pathway, and genes involved in cell proliferation. A strain of obese T2DM mice, the KKAy mice, can serve as an advantageous model for this kind of analysis since these animals progress through several developmental stages into diabetes in a relatively predictable age-dependent fashion, when maintained under standard conditions. Thus, KKAy mice rapidly progress from an insulin-resistant stage with hyperinsulinemia and euglycemia to a hyperglycemic, insulin-deficient stage at the age of approximately 12 weeks.

Diets low in fat and high in carbohydrates from vegetables, grains, and fruits reduce the risk for cardiovascular disease (CVD) (*3*). Soy protein has gained considerable attention for its promising role in improving risk factors for CVD. In October 1999, the US Food and Drug Administration (FDA) approved labeling of foods containing soy protein as protective against CAD and recommended a daily amount of 25 g to lower total and LDL cholesterol (*4*).

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The soy protein hypothesis was strengthened in the late 1970s and early 1980s, as a result of studies by Sirtori et al. (*5*) and Descovich et al. (*6*) who found that diets high in soy protein reduced blood LDL cholesterol by 20% to 30% in severe hypercholesterolemia. A meta analysis of 38 controlled clinical trials indicates that soy protein is effective in lowering plasma cholesterol, LDL cholesterol, and triglyceride (TG) concentrations (*7*). In 29 controlled studies, a trend emerged that soy protein selectively reduced blood cholesterol in direct proportion to the degree of hypercholesterolemia (*7*). A similar beneficial effect has been demonstrated on LDL cholesterol in T2DM subjects (*8*).

Subsequent studies have explored the soy protein hypothesis with greater specificity. In addition, the recognition that soy protein products contain bioactive isoflavones (e.g., genistein, daidzein, and glycitin) with well-known phytoestrogenic activity and saponins (e.g., group A and B soy saponins) added a fascinating new aspect to the soy protein hypothesis (*4, 9, 10*).

Recently, the American Heart Association has assessed 22 randomized trials with isolated soy protein with isoflavones in order to evaluate the effect on LDL and HDL cholesterol, triglycerides, lipoproteins, and blood pressure. They found that isoflavone-containing soy proteins significantly decrease LDL cholesterol, but found no significant changes on other parameters (*4*).

Previously, we have demonstrated in Zucker diabetic fatty rats a beneficial lowering effect of soy bean protein (SBP) on total cholesterol, triglycerides, and free fatty acids (*11, 12*).

The aim of the present study was to investigate the impact of a high isoflavone content soy diet on the intermediary metabolism and the gene expression of key insulin regulatory genes in T2DM KKAy mice. The SBP used in the present study did not undergo heat treatment during the preparation procedure since it has been the subject of debate whether denaturation could destroy the bioactive compounds of the SBP isolate. Our hypothesis was that SBP improves insulin sensitivity and lipid profile as well as changes the gene expression profile of essential insulin regulatory genes and beta cell transcription factors in the isolated islets after long-term in vivo intervention.

## MATERIALS AND METHODS

**Animals.** Twenty male KKAy-mice (Clea Japan, Tokyo, Japan), all 5 weeks-old, weighing 22−25 g were randomized to 2 groups and treated for 9 weeks with A, standard chow diet (control) and B, diet containing 50% standard chow + 50% SPB.

As a non diabetic control group (C), 20 normal C57BL-mice (Taconic, Ry Denmark) were fed with standard chow diet (Altromin 1324, Altromin, GmbH, Lage, Germany) (control to A). The food intake was measured halfway through the study. The composition of the diet dry matter was as follows. Standard chow diet: protein 23.5%, carbohydrate 71.7%, and lipids 4.7%. SBP diet: protein 54.6%, carbohydrate 41.9%, and lipids 3.5%. The SBP (Soy Bean protein isolate (Abalon), Nutri Phama ASA, Vika, Oslo, Norway) was given orally incorporated in the food pellet. The soy bean isolate is nondenaturized, and 100 g of isolated soy protein (Supro; Protein Technologies, St. Louis, MO) contained 340 mg of isoflavones and 40 g of cotyledon fiber (Supro). The Altromin 1324 is heat denaturated and contains no isoflavone. The Danish Council for Animal experiments approved the study.

**Plasma Analysis.** Hormones and lipids were measured from the blood sample at the start and end of the treatment period. Blood samples were taken from the tail of the animals into chilled tubes containing heparin/aprotinin and centrifuged (4000*g*, 60 s, 4 °C), and plasma was frozen for subsequent analysis of insulin, glucose, triglycerides, total cholesterol, and HDL cholesterol. Fasting blood glucose as well as BW was measured once a week.

**Assays.** Plasma blood glucose was determined using the glucose oxidase method (GOD-PAP, Boehringer Mannheim, Mannheim, Germany). Insulin was determined by radioimmunoassay with a guinea pig antiporcine insulin antibody (PNILGP4, Novo Nordisk, Bagsvaerd, Denmark), and mono-[125I]-(Tyr A14)-labeled human insulin (Novo Nordisk) as tracer and rat insulin (Novo Nordisk) as standard. We separated free and bound radioactivity using ethanol (*13*). Interassay and intra-assay variation was below 10%. Triglycerides and cholesterol were determined using colorimetric kits (Boehringer Mannheim).

The fasting glucose−insulin index was calculated as the product of plasma insulin and plasma glucose ([plasma insulin ng/mL] × [plasma glucose mmol/L]/1000) and compared between groups as relative units (*14*).

**Insulin Content.** The Insulin content of isolated islets from the KKAy mice was measured using RIA as described for the Insulin assay. Total islet protein was purified from the organic phase from the RNA Trizol preparation according to the manufacturer's instructions (Gibco BRL, Life Technologies, Roskilde, DK).

**Islet Isolation.** Islets were isolated by the collagenase digestion technique. In brief, the animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally), and a midline laparotomy was performed. The pancreas was filled in a retrograde manner with 3 mL of ice-cold Hanks balanced salt solution ([HBSS] Sigma Chemical, St Louis, MO) supplemented with 0.3 mg/mL collagenase (Boehringer Mannheim GmbH, Germany). The pancreas was subsequently removed and incubated for 19 min at 37 °C. After rinsing in HBSS, the islets were handpicked under a stereomicroscope and immediately transferred to tubes containing 1 mL of Trizol (Gibco, Roskilde, Denmark).

**Isolation of RNA.** For each group, islets from 3−4 mice were pooled in 1 mL of TriZol reagent before RNA purification. Total RNA was extracted according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 and at 280 nm. The quality of the RNA was checked by the Agilent 2100 bioanalyzer (Agilent, Santa Clara, USA).

**Real-Time RT-PCR.** We investigated the expression of *Pdx-1*, *Akt1*, *GLUT2*, *C/EBPalpha*, *CPT1*, *11-Beta-HDS1*, *IR, Visfatin*, *Beta2/NeuroD*, *IRS1*, *Ins1*, and *Ins2* by real-time RT-PCR. cDNA was synthesized using IScript (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Total RNA at 50 ng per 10 $\mu$L of reaction mixture was used for measurement of the target mRNA. The real-time RT-PCR assay was performed using the ABI 7500 FAST machine (ABI; Foster City, CA). Ten microliters of Real-time RT-PCR reaction consists of 5 $\mu$L of 2× TaqMan FAST Universal Master Mix (P/N 43660783, ABI; Foster City, CA), 0.5 $\mu$L of 20× TaqMan Assay/probe (ABI; Foster City, CA), and cDNA equivalent to 50 ng of total RNA in 4.5 $\mu$L of $H_2O$. The thermal FAST cycle program was 20 s at 95 °C, followed by 40 cycles of 3 s at 95 °C, and 30 s at 60 °C. Reactions were set up in triplicate for each sample, and gene expressions were normalized to eukaryotic *18S rRNA* expression (assay Hs99999901_s1; ABI; Foster City, CA). All assays were carried out in 96-well format plates covered with an optical adhesive cover (P/N 4346906 and P/N 4311971 ABI; Foster City, CA). We used the $2^{-\Delta\Delta CT}$ method to calculate the relative gene expression (as described in User Bulletin 2, 1997, from Perkin-Elmer Corp. covering the aspect of relative quantization of gene expression).

The TaqMan Assay used *Pdx-1/Ipf1* (assay Mm00435565_m1), *Akt1* (assay Mm00437443_m1), *GLUT2* (assay Mm00446224_m1), *Igf1* (assay Mm00439561_m1), *IR* (assay Mm00439693_m1), *Beta2/NeuroD* (assay Mm01280117_m1), *IRS1* (assay Mm00439720_s1), *Ins2* (assay Mm00731595_gh), and *Ins1* (assay Mm01259683_g1).

**Affymetrix Microarray Analysis.** *cRNA Preparation and in Vitro Transcription.* In a total of 6 Affymetrix arrays, the MOE430 2.0 probe array cartridge was used, 3 replicas for the KKAy control group and the SBP group. Each of the 6 samples consisted of pooled RNA from 3 to 4 KKAy mice.

Preparation of target for hybridization was prepared from 340 ng of total RNA using MessageAmp II aRNA Amplification Kits (Ambion) according to the manufacturer's instructions.

*Array Hybridization and Scanning.* Fifteen micrograms of cRNA was fragmented at 94 °C for 35 min in a fragmentation buffer containing 40 mmol/L Tris-acetate at pH 8.1, 100 mmol/L KOAc, and 30 mmol/L

MgOAc. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mmol/L Tris at pH 7.6, and 0.005% Triton) was heated to 95 °C for 5 min and subsequently to 45 °C for 5 min before loading onto the Affymetrix MOE430 2.0 probe array cartridge. The probe array was then incubated for 16 h at 45 °C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station 450. The probe array was exposed to 10 washes in 6× SSPE-T at 25 °C followed by 4 washes in 0.5× SSPE-T at 50 °C. The biotinylated cRNA was stained with a streptavidin−phycoerythrin conjugate, final concentration 2 mg/mL in 6× SSPE-T for 30 min at 25 °C followed by 10 washes in 6× SSPE-T at 25 °C. An antibody amplification step followed using normal goat IgG as a blocking reagent, final concentration of 0.1 mg/mL, and biotinylated antistreptavidin antibody (goat), final concentration of 3 mg/mL. This was followed by a staining step with a streptavidin−phycoerythrin conjugate, final concentration of 2 mg/mL in 6× SSPE-T for 30 min at 25 °C and 10 washes in 6× SSPE-T at 25 °C. The probe arrays were scanned at 560 nm using the GeneChip Scanner 3000 System. The raw image files from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software (MAS 5.0) resulting in .cell files containing background corrected probe values.

**Statistical analyses.** . Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance between two groups was evaluated using a two-tailed *t*-test. A $p$ value of less than 0.05 was considered statistically significant. For plasma glucose calculations, analysis of covariance with adjustment for end body weight was performed. For gene expression analysis, one-way ANOVA was applied.

## RESULTS

**Metabolic Effects of SBP.** At the start of the intervention study, no significant difference was found in blood glucose or insulin levels between the three groups (**Figure 1A** and **B**). Importantly, there was no difference between the nondiabetic C57/BL-mouse control group and the diabetic KKAy control group at age 5 weeks, ensuring that the KKAy mice had not developed insulin resistance before intervention started.

After the 9-week treatment period, the KKAy control group developed a significant increase in plasma glucose of 181% (9.4 vs 26.3 mmol/L, $p < 0.001$), whereas the nondiabetic C57/BL control group only experienced an increase of 10% (8.6 vs 9.5 mmol/L, $p = 0.013$). The plasma insulin increased 266-fold (0.9 vs 234.1 ng/mL $p < 0.001$) and 4.5-fold (0.4 vs 1.8 ng/mL $p < 0.001$) for KKAy and C57/BL-mice, respectively. The plasma glucose and insulin was respectively 2.8-fold (9.5 vs 26.3 mmol/L, $p < 0.001$) and 130-fold (1.8 vs 234.1 ng/mL, $p < 0.001$) higher for the KKAy control group compared to the C57/BL control group (**Figure 1A** and **B**). Whereas plasma glucose for the KKAy control group was increased 2.8-fold at the end of the study, the SBP-treated KKAy mice experienced no significant increase (8.9 vs 9.4 mmol/L, $p = 0.58$) (**Figure 1A**). Plasma insulin for the KKAy control group increased 266-fold during the study period, whereas the SBP-treated KKAy mice group did not experience any increase (0.5 vs 35.8 ng/mL $p = 0.21$) (**Figure 1B**). Compared to the KKAy control, SBP reduced plasma glucose by 64% (26.3 vs 9.4 mmol/L, $p < 0.001$) and insulin by 85% (234.1 vs 35.8 ng/mL, $p < 0.001$). Since we found a reduction in body weight for the SBP group, we performed analysis of covariance with adjustment for both start body weight and weight changes. This analysis showed that the reduction in plasma glucose for the SBP cannot be explained by weight loss. The glucose−insulin index (as a measure for insulin resistance) was, as expected, higher for the KKAy control and SBP group compared to the nondiabetic C57/BL group (3.4 vs 8.3 unit, $p < 0,05$ and 3.4 vs 4.5, $p < 0.05$, respectively) (**Figure 1C**). At the end of the study, there was a marked decrease in the glucose−insulin index for the SBP group compared to the KKAy control (6932 vs 65 units, $p < 0.01$), indicating that SBP has decreased the insulin resistance (**Figure 1C**).

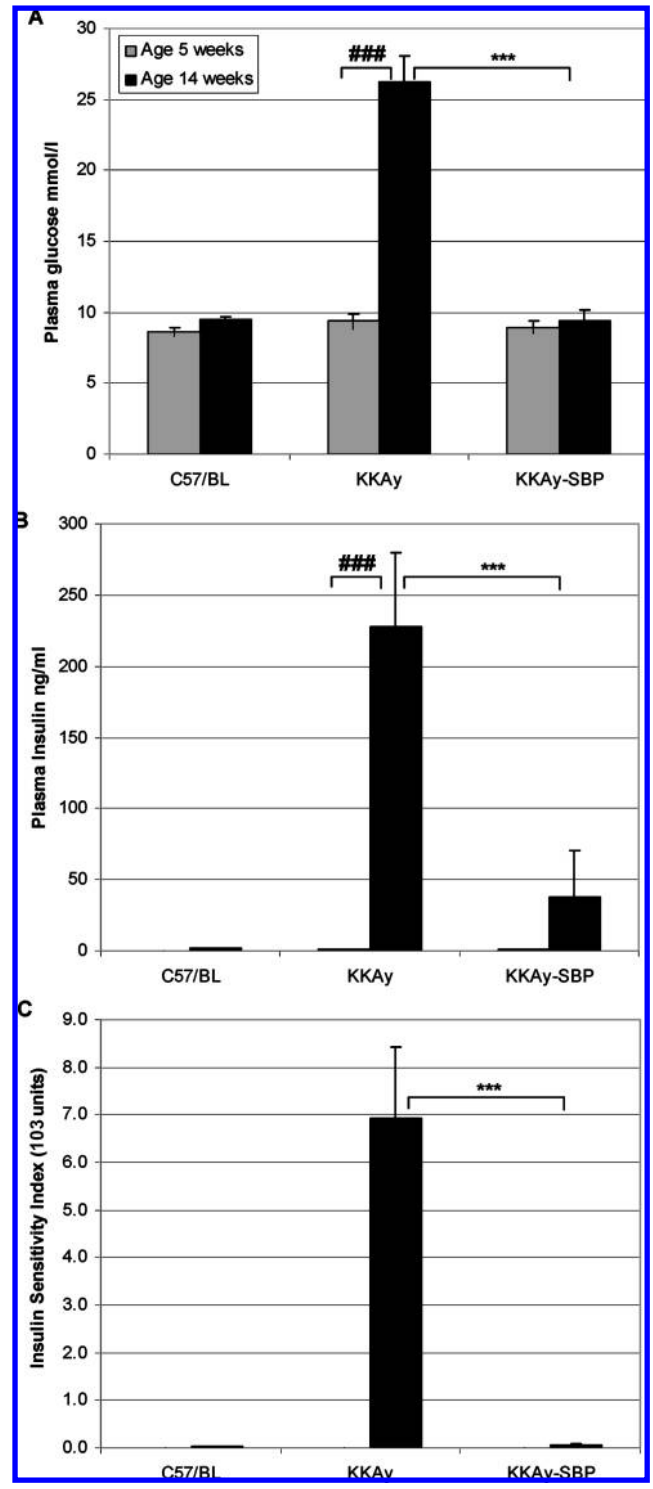


**Figure 1.** Effect of SBP diet on fasting plasma glucose (**A**) and insulin (**B**) and glucose−insulin index (**C**) in KKAy mice before and after the 9-week intervention. The C57/BL and KKAy control groups were fed standard chow diet, and the SBP group was fed standard chow supplemented with 50% SBP by weight. The animals were 5 weeks of age when the treatment began. Data are shown as the mean ± SEM ($n = 10$ in each group). ***$p < 0.001$ (unpaired); ###$p < 0.001$ (paired).

**Changes in Lipid Levels.** At age 14 weeks, the TG level was increased by 192% for the KKAy control group compared to age 5 weeks (1.3 vs 3.9 mmol/L, $p < 0.001$), while the SBP

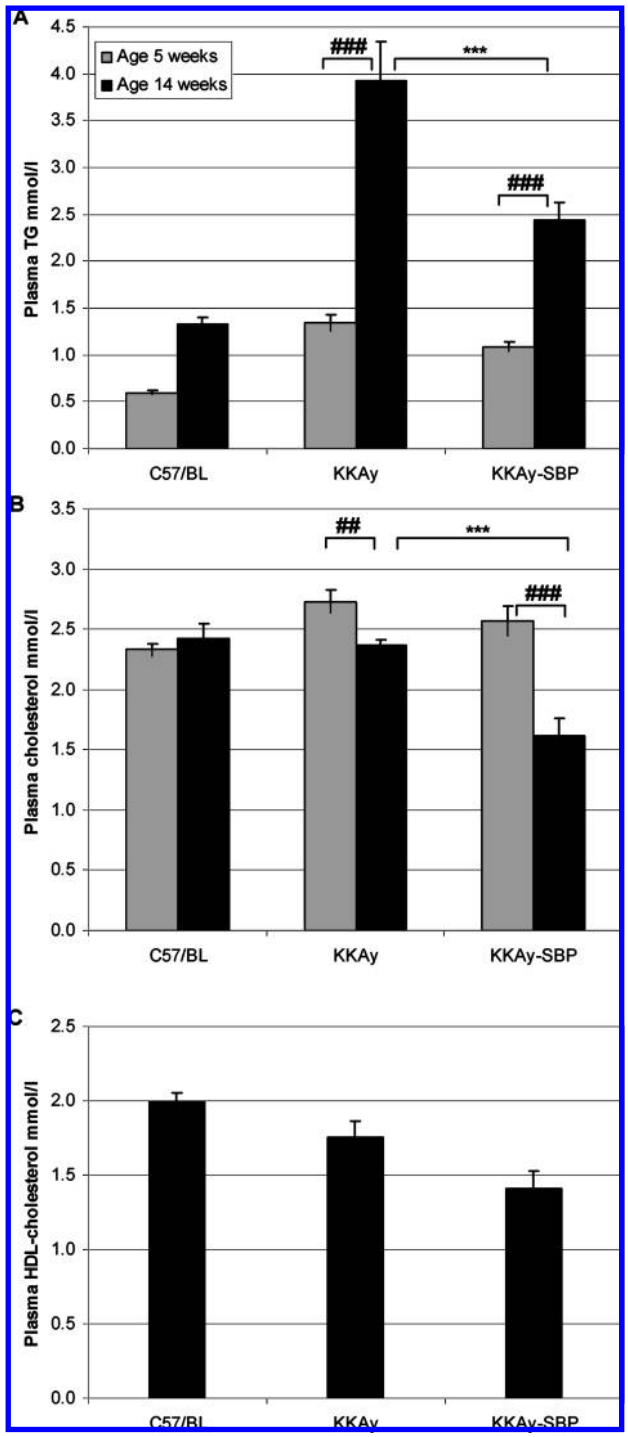


**Figure 2.** Effect of SBP diet on plasma triglycerides (**A**), total cholesterol (**B**), and HDL-cholesterol (**C**) levels in KKAy mice before and after the 9-week intervention. The animals were 5 weeks of age when the treatment began. The C57/BL and KKAy control groups were fed standard chow diet, and the SBP group was fed standard chow supplemented with 50% SBP by weight. Data are shown as the mean ± SEM ($n = 10$ in each group). ***$p < 0.001$ (unpaired); ##$p < 0.01$; ###$p < 0.001$ (paired).

group had increased by 123% (1.1 vs 2.4 mmol/L, $p < 0.001$) (**Figure 2A**). SBP treatment of KKAy mice reduces plasma TG by 38% (3.9 vs 2.4 mmol/L, $p < 0.001$). We measured the total plasma cholesterol level for the KKAy control and SBP group at age 5 and age 14 weeks. At age 5 weeks, there was no difference between groups, and at age 14 weeks, no difference was detected in total cholesterol for the C57/BL and KKAy control (2.42 vs 2.36 mmol/L, $p = 0.66$); however, SBP

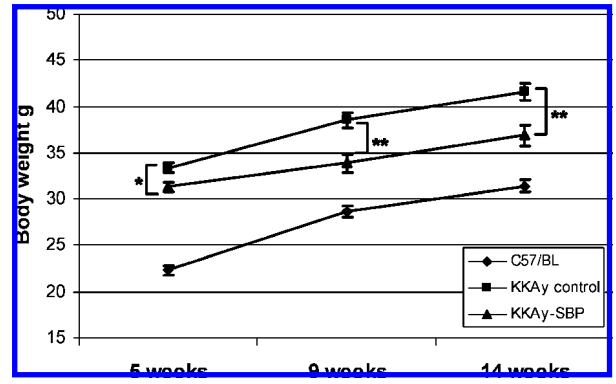


**Figure 3.** Effects of the 9-week treatment with the SBP diet on the body weight of KKAy mice. Data are shown as the mean ± SEMs ($n = 10$ in each group). *$p < 0.05$; **$p < 0.01$.

treatment reduces plasma cholesterol by 31% compared to the KKAy control (2.36 vs 1.62 mmol/L, $p < 0.001$) (**Figure 2B**). Unexpectedly, there was a small decrease of 13% (2.72 vs 2.36 mmol/L, $p < 0.01$) for the KKAy control at 14 weeks relative to 5 weeks.

Interestingly, SBP did not lower the level of HDL cholesterol (1.76 vs 1.41 mmol/L, $p > 0.05$), indicating a tendency to an increase in the ratio of HDL cholesterol to total cholesterol (**Figure 2C**).

**Body Weight.** At the start of the intervention study, a minor difference in body weight of 6% was observed for the body weight between the KKAy control and the SBP groups (33.4 vs 31.4 g, $p = 0.035$) (**Figure 3**). After 9 weeks of treatment, we detected a highly significant difference in body weight between the KKAy control group and the SBP group (41.6 g vs 36.9 g, $p < 0.01$). At the end of the treatment period, the SBP group had an 11% lower average body weight than the KKAy control group, i.e., the SBP treatment apparently induced a net weight reduction of 5% (**Figure 3**). The standard chow used for the KKAy control group contained 4.7% lipid in dry matter, and the dry matter of the SBP group contained 3.5% lipid. The food intake was measured after 5 week treatment, and we found no significant difference between the KKAy control and the SBP group (5.0 vs 4.8 g, $p = 0.63$). As expected, we found a difference between the KKAy control and the smaller C57BL mice (3.8 vs 5.0 g, $p < 0.001$).

**Gene Expression Analysis in Islets.** *Relative Real Time RT-PCR.* We isolated islets from the pancreas of KKAy control and SBP-treated mice. RNA was isolated, and we measured differences in transcript abundance of 9 genes, the protein products of which affect insulin secretion (**Figure 4**). The change in transcript abundance was calculated for the SBP group compared to the nontreated control group (**Figure 4**). Islets from the SBP group versus the KKAy control group experienced an increased expression of glucose transporter 2 *GLUT2* (229%, $p < 0{,}002$), neurogenic differentiation (*Beta2/neuroD*1, 78%, $p < 0{,}025$), insulin1(*Ins1*, 135%, $p < 0{,}025$), insulin2 (*Ins2*, 131%, $p < 0{,}01$), and decreased expression of insulin growth factor 1 (*IGF1,* 56%, $p < 0{,}01$).

The expression of the glucose transporter 2 (GLUT2) was upregulated more than 3-fold, indicating that SBP augments glucose sensitivity. The observed increase in Ins1 and Ins2 expression of more than 2-fold correlates well with the increase in *GLUT2*. In agreement with the increased expression of the insulin genes, the bHLH transcription factor *Beta2/NeuroD*, which is a key activator of insulin gene transcription in pancreatic $\beta$-cells, is upregulated significantly (*15, 16*). The pancreatic duodenal homebox gene-1, *Pdx1*, another important

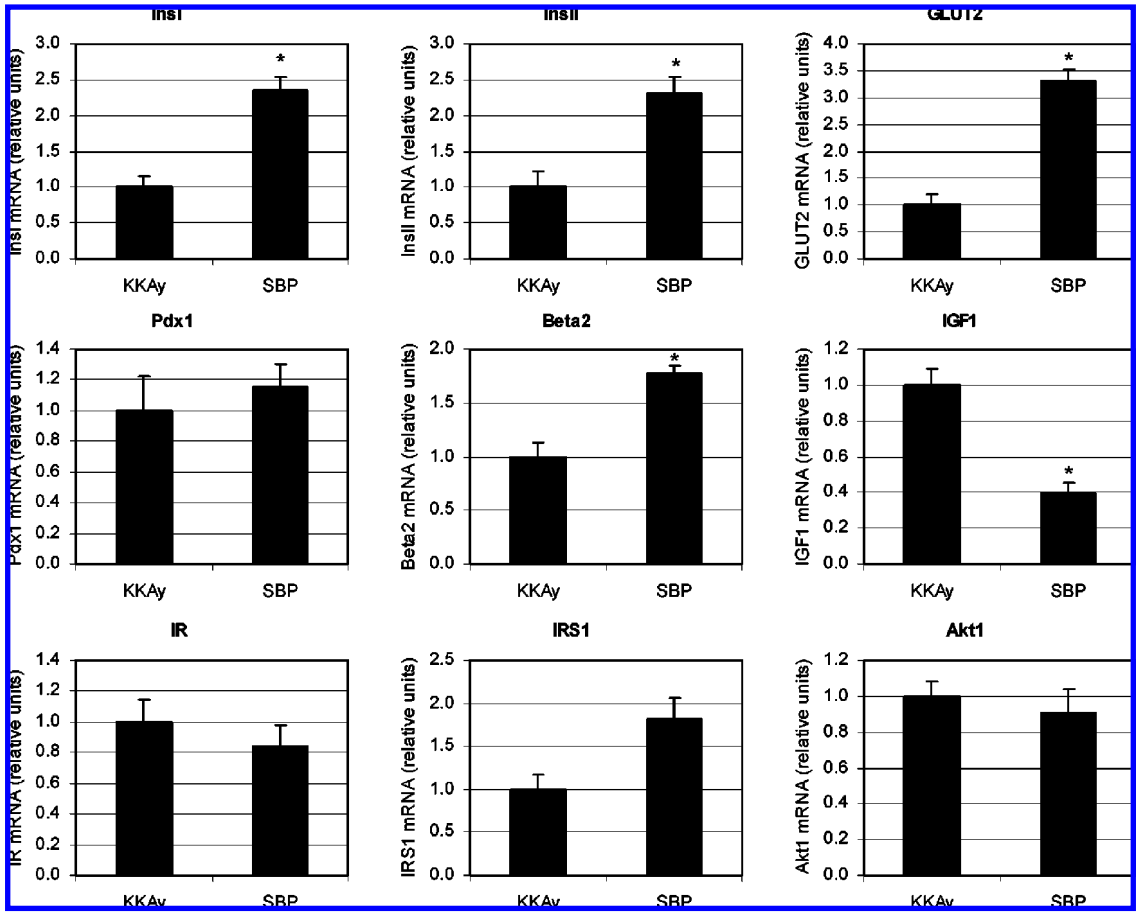


**Figure 4.** RNA from pancreatic islets of KKAy mice treated for 9 weeks with SBP was isolated, and 12 genes related to insulin secretion and regulation were studied for changes in their transcript abundance by real-time RT-PCR using TaqMan assays. Measurements were carried out in triplicate for each sample, and gene expressions were normalized to 18S *rRNA* expression. Changes in mRNA levels were calculated for the SBP group compared to the control group. *$p < 0.05$. ($n = 4$.).

pancreatic transcription factor, showed no changes in expression. The investigated genes are involved in the insulin receptor pathway. The investigated genes in the insulin receptor pathway, the Insulin receptor (IR), Insulin receptor substrate 1 (IRS1) and protein kinase B alpha (Akt1/PKBalpha) were not changes.

**Affymetrix Gene Chip Analysis.** The improved metabolic state in the SBP group and the important changes in islet gene expression prompted us to conduct gene chip analysis on the KKAy control and SBP groups in order to obtain a more detailed picture of the gene changes in the beta cells (**Table 1**). The array analysis confirmed the RT-PCR results found for *GLUT2*, *IGF1*, insulin receptor (IR), *Akt1*, and the insulin receptor substrate 1 (IRS1). Surprisingly, the array analysis does not show any statistically significant increase in *Ins1 or Ins2*; however, the reason for this could be that the insulin mRNA is so abundant in the islet RNA purification that we exceeded the dynamic range of the array. The array data show no significant changes in Beta2 expression. However, the QPCR is more sensitive and therefore more suited for small changes. The transcript abundance of other insulin promoter-binding transcription factors such as Pdx1, NK2 transcription factor related, locus 2 (Nkx2.2), and paired box gene 6 (Pax6) were unchanged or only slightly increased. However, Sterol regulatory element-binding protein (SREBP-1), which activates the insulin promoter, was increased 2-fold. From **Table 1** it can be seen that in addition to GLUT2 expression GLUT3 expression is also increased, suggesting enhanced glucose sensitivity.

IGF1, LDLr, and glucokinase, all involved in insulin secretion, are decreased 3.1-fold and increased 2.2- and 1.8-fold, respectively. Genes of the insulin receptor pathway IRS1−4, Akt1−3, and forkhead box O1 and O3 (FOXO1, FOXO3) are not changes except for forkhead box A2 (Foxa2), which showed a small increase. Finally, several genes involved in cell growth and apoptosis were affected, for example, cyclin A2, cyclin D1-D3, and caspase 9 (**Table 1**).

Using a 2-fold cutoff threshold, 393($p < 0.05$) or 215($p < 0.01$) of the 45100 transcript on the chip were significantly increased in three biological independent islet RNA pools. Likewise 252($p < 0.05$) or 122($p < 0.01$) transcripts were down-regulated.

**KKAy Islet Insulin Content.** In order to clarify whether the increase in Ins1 and Ins2 mRNA becomes translated and processed to insulin protein, the total insulin content from the very limited protein fraction collected from the organic phase during RNA preparation from isolated islets from the KKAY and SBP group was measured using RIA (**Figure 5**). The insulin content in the SBP group was 2.1-fold ($p < 0.05$) higher than the KKAy control, similar to the results of the gene expression analysis.

## DISCUSSION

In the present study, using KKAy mice as a T2DM model, we found that a nondenaturated, soy-based diet with high isoflavone content completely abrogates glycemic abnormalities and insulin resistance, thereby preventing a severe state of

**Table 1.** RNA from Pancreatic Islets of KKAy Mice Treated for 9 Weeks with Standard Chow or SBP Diet Isolated and Used for Microarray Analysis[a]

| mouse 430 2.0 array probe ID | genebank | gene name | gene symbol | fold changes | *P*-value |
|---|---|---|---|---|---|
| 1449067_at | NM_031197 | solute carrier family 2 (facilitated glucose transporter), member 2 | Slc2a2 | +2.9 | 0.0011 |
| 1437052_s_at | BB414515 | solute carrier family 2 (facilitated glucose transporter), member 3 | Slc2a3 | +5.0 | 0.0404 |
| 1426690_a_at | AI326423 | sterol regulatory element binding factor 1 | Srebf1 | +2.0 | 0.0195 |
| 1452174_at | BM123132 | sterol regulatory element binding factor 2 | Srebf2 | +1.1 | 0.6639 |
| 1421112_at | NM_010919 | NK2 transcription factor related, locus 2 (*Drosophila*) | | +1.5 | 0.0196 |
| 1425828_at | AF357883 | NK6 transcription factor related, locus 1 (*Drosophila*) | Nkx6−1 | +1.9 | 0.0342 |
| 1426412_at | BM116592 | neurogenic differentiation 1 | Neurod1 | +1.4 | 0.1872 |
| 1419127_at | NM_023456 | neuropeptide Y | Npy | +4.3 | 0.0022 |
| 1424865_at | BC010821 | peptide YY | Pyy | +2.8 | 0.0123 |
| 1419473_a_at | NM_031161 | cholecystokinin | Cck | −59.2 | 0.0000 |
| 1421195_at | BC020534 | cholecystokinin A receptor | Cckar | −2.9 | 0.0175 |
| 1425303_at | L38990 | glucokinase | Gck | +1.8 | 0.0000 |
| 1426225_at | U63146 | retinol binding protein 4, plasma | Rbp4 | +2.0 | 0.0010 |
| 1448980_at | AB035701 | ghrelin | Ghrl | +1.0 | 0.3123 |
| 1421821_at | AF425607 | low density lipoprotein receptor | Ldlr | +2.2 | 0.0149 |
| 1452014_a_at | AF440694 | insulin-like growth factor 1 | Igf1 | −3.1 | 0.0021 |
| 1422174_at | AK020261 | insulin promoter factor 1, homeodomain transcription factor | Ipf1 | +1.3 | 0.1822 |
| 1419271_at | BC011272 | paired box gene 6 | Pax6 | +1.4 | 0.0071 |
| 1416981_at | AI462296 | forkhead box O1 | Foxo1 | +1.7 | 0.0094 |
| 1422833_at | NM_010446 | forkhead box A2 | Foxa2 | +1.2 | 0.0019 |
| 1450236_at | NM_019740 | forkhead box O3 | Foxo3 | +1.0 | 0.5674 |
| 1416657_at | NM_009652 | thymoma viral proto-oncogene 1 | Akt1 | +1.2 | 0.1441 |
| 1424480_s_at | BC026151 | thymoma viral proto-oncogene 2 | Akt2 | +1.0 | 0.9319 |
| 1435879_at | BB521695 | thymoma viral proto-oncogene 3 | Akt3 | +1.3 | 0.0645 |
| 1423104_at | BB345784 | insulin receptor substrate 1 | Irs1 | +1.3 | 0.0090 |
| 1443969_at | BE199054 | insulin receptor substrate 2 | Irs2 | +1.0 | 0.2021 |
| 1437672_at | AV286336 | insulin receptor substrate 3 | Irs3 | +1.0 | 0.8797 |
| 1441429_at | BB295945 | insulin receptor substrate 4 | Irs4 | +1.0 | 0.9256 |
| 1421380_at | NM_010568 | insulin receptor | Insr | +1.0 | 0.8910 |
| 1422447_at | NM_008386 | insulin I | Ins1 | +1.0 | 0.3989 |
| 1422446_x_at | NM_008387 | insulin II | Ins2 | +1.0 | 0.3848 |
| 1417910_at | X75483 | cyclin A2 | Ccna2 | +2.7 | 0.0044 |
| 1417419_at | NM_007631 | cyclin D1 | Ccnd1 | +1.9 | 0.0022 |
| 1455956_x_at | AV310588 | cyclin D2 | Ccnd2 | +2.7 | 0.0001 |
| 1415907_at | NM_007632 | cyclin D3 | Ccnd3 | +1.0 | 0.3318 |
| 1422439_a_at | NM_009870 | cyclin-dependent kinase 4 | Cdk4 | +1.6 | 0.0047 |
| 1437537_at | BB815299 | caspase 9 | Casp9 | −2.3 | 0.0022 |

[a] In total, 6 Affymetrix arrays, MOE430 2.0 probe arrays, were used, 3 replicas for the KKAy control group and the SBP group, respectively. Each of the 6 samples consisted of pooled RNA from 3 to 4 KKAy mice.

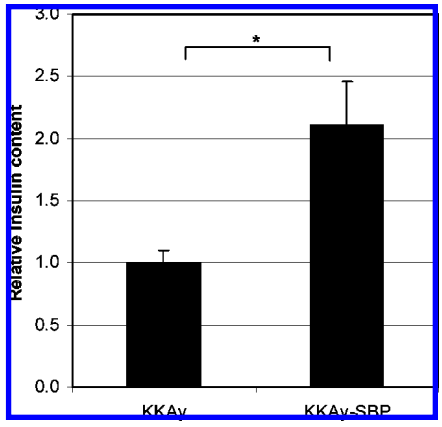


**Figure 5.** Effects on total islet insulin protein content in KKAy mice after the 9-week intervention with SBP. Each bar represents the mean ± SEM from 3 protein purifications, each pooled from 3 to 4 mice ($n = 3$ each bar). *$p < 0.05$, vs control.

diabetes. The antihyperglycemic and antihyperinsulinemic effect of SBP is facilitated by increased insulin and glucose sensitivity in peripheral tissues. Interestingly, a net reduction in body weight of 5% was detected for SBP-treated mice compared to the control group. The difference in lipid content of the chow and SBP diet is negligible (4.7% vs 3.5%) and unlikely to be the explanatory course. To exclude the possibility that the observed lowering of glucose and insulin could be entirely ascribed to a reduction in body weight, we performed an analysis of covariance with adjustment of start body weight and body weight changes. This analysis clearly demonstrated that the body weight reduction naturally contributes positively; however, the *p*-values are still highly significant.

In addition to the lowering in fasting plasma glucose and insulin levels, the lipid profile was also greatly improved for the SBP group, having a 38% lower TG plasma concentration than the KKAy control group. Interestingly, the C57/BL control experienced a 98% increase in TG during the 9 week treatment period, whereas the TG level for the SBP-treated mice increased 123%, meaning that the age related increase is nearly the same, but the start level is shifted due to the genetic differences between the C57/BL and KKAy mice. This normalization of the SBP group is remarkable compared to the 192% TG increase observed for the KKAy control group.

The total plasma cholesterol was unchanged for the C57/BL mice. The SBP group experiences a striking decrease in plasma cholesterol of 31% compared to the KKAy control group. The total cholesterol level was unexpectedly also reduced for the KKAy control at 14 weeks relative to age 5 weeks, however, only 13%. Interestingly, the plasma HDL cholesterol level was unchanged compared to that in the KKAy control. The lowering in total cholesterol indicates that the HDL/total cholesterol may be elevated in the SBP group. HDL plays an important role in the reverse cholesterol transport system, which mediates transportation of cholesterol from the peripheral tissues to the liver,

and HDL is known to be inversely correlated with the risk of CAD (*17*). The finding that SBP apparently causes a substantial rise in the HDL cholesterol ratio is potentially of great interest in targeting HDL cholesterol therapeutically.

Concomitant with the marked changes in metabolic factors, we found correlating changes in the expression of central genes involved in the glucose-stimulated insulin secretory pathway.

The SBP-treated mice have a 3-fold upregulation of GLUT2 expression, suggesting that the glucose sensitivity is increased. *GLUT2* is the glucose transporter with the lowest affinity and the highest capacity for glucose, and this allows beta cells to take up glucose effectively only in times of plenty, when insulin release is needed (*18*). The increased expression of GLUT2 in the SBP may lead to an increased first phase insulin response (*19*). In addition, the GLUT3 glucose transporter is upregulated 5-fold; however, the implication of this is not understood. The observed increase in Ins1 and Ins2 expression of more than 2-fold for the SBP group, as revealed by QPCR analysis, correlates well with the observed increase in GLUT2. Also, Glucokinase (Gck), which catalyzes the conversion of glucose into glucose-6-phosphate and is critical for glucose sensing, is further upregulated 1.8-fold. This supports that the beta cell glucose sensitivity is improved after SBP treatment. Haploinsufficiency of beta cell-specific Gck ($Gck^{+/-}$) leads to mild diabetes associated with impaired insulin secretion in response to glucose (*20*). Gck is now recognized as functioning as a glucose sensor for insulin secretion by pancreatic beta cells (*21*).

No change is found in the expression of IR, IRS1, IRS2, IRS3, IRS4, Akt1, Akt2, and Akt3 that are all components of the insulin signaling pathway. The expression of the islets enriched transcription factor, *Beta2/NeuroD* (reviewed in ref *15*), that has been shown to activate the insulin gene promoter (*22*) and is critical for pancreatic development (*15*) is upregulated in the SBP group (QPCR only) (*22*). The bHLH transcription factor *Beta2/NeuroD* is a key regulator of both insulin gene transcription in pancreatic beta cells in which heterozygous mutations are found associated with the development of T2DM (*15, 16*). Interestingly, we observed a 2-fold increase in SREBP-1. The insulin promoter has three SREBP-1 binding sites (SREs) and is a positive regulator of insulin expression and acts synergistically with Beta2 (*23*). SREBP-1 has been well established as controlling the nutritional regulation of lipogenic genes in the liver; however, the role in beta cells is not fully clarified, although it has been suggested that SREBP-1 action might play a compensatory role in insulin expression in diabetes with beta cell lipotoxicity (*23*). Other insulin promoter binding transcription factors that positively regulate insulin expression were only slightly changed. Pdx1 was unchanged, and Pax6 was increased 1.4-fold.

The SBP group has a 3.1-fold down-regulation of the IGF1 expression. In contrast to liver-specific IGF-I gene deficiency where growth hormone hypersecretion (from the pituitary) can cause insulin resistance and accelerate diabetes, pancreatic-specific IGF-I gene deficiency has been demonstrated by crossing Pdx1-Cre and IGF-I/loxP mice to exert opposite effects, causing enlarged pancreatic islets, hypoglycemia, and significant resistance to islet beta cell damage and diabetes induced by both streptozotocin and high fat diet (*24*). It is therefore possible that locally produced IGF-1 within the pancreas may inhibit islet cell growth and hormone secretion and that a reduction of IGF-1 provides a protective environment to beta cells (*25*).

In the SBP group, we observed a 2.2-fold upregulation of the low density lipoprotein receptor (LDLr). Binding of LDL appears restricted to the beta cells as it is not detected in islets in endocrine nonbeta cells (*26*). Uptake of LDL by islet beta cells and subsequent oxidative reactions can be damaging for the cells. This process can be counteracted by HDL and VLDL, and by antioxidants (*27*). In light of the increase in the HDL/total cholesterol, the observed expression of LDLr may not negatively influence the beta cell mass.

The most profound gene changes were the 59-fold down-regulation of cholecystokinin (CCK). The CCK receptor (CCK-AR) was correspondingly decreased 2.9-fold, suggesting that decreased autocrine CCK stimulation feed back down-regulates the receptor. CCK stimulates the insulin secretion (*28*) as well as the secretion of pancreatic amylase and activates phospholipase A2 in beta cells. Phospholipase A2 facilitates arachidonic acid (AA) production that in turn is a stimulator of insulin secretion (*29*). This could be explained by the normalized plasma glucose/insulin level and enhanced insulin sensitivity, making the recruitment for a high basal insulin secretion unnecessary. In line with this, we found that the pancreatic neuropeptides NPY and PYY were upregulated 4.3- and 2.8-fold, respectively. NPY and PYY are known to inhibit GSIS as studied in rat and mouse pancreas (*30*).

Deficiency, either absolute or relative, of pancreatic beta cell mass underlies the pathogenesis of T2DM. Regulation of postnatal beta cell mass is vital to preserve sufficient insulin secretion in order to appropriately regulate glucose homeostasis. Thus, there is considerable interest in understanding the mechanisms that stimulate pancreatic islet cell growth and differentiation. Several cell cycle regulators, including cyclin D1, cyclin D2, and CDK4, are known to be important for beta cell proliferation (*31*). Cyclin D1 and D2 is shown to be essential for beta cell expansion in adult mice (*32, 33*). Our array data showed that cyclin D1, cyclin D2, and CDK4 were upregulated, (1.8-, 2.6-, and 1.5-fold, respectively), suggesting that the proliferative capacity of the islet is improved for the SBP treated mice. Further corroborating this is the finding that the caspase-9 involved in the apoptotic pathway is down-regulated 2.3-fold. The advanced age of the KKAy control group resulted in relatively high fasting blood glucose levels compatible with a rather severe diabetic state. In contrast, the SBP-treated mice decreased both plasma glucose and insulin levels indicating an improvement in insulin sensitivity. These results may relate to changes in whole body insulin sensitivity, indicating that the SBP acts via inhibition of peripheral insulin resistance in vivo. In the insulin-resistance state, defects in the insulin signal cascade leading to impaired glucose utilization have been proposed as a key role in the pathogenesis of T2DM (*34*). Like glitazones, SBP seems to have a multitude of additional beneficial effects in T2DM, including decreased hyperinsulinemia, improved TG profile, and increased HDL ratio. In contrast to glitazones, which generally are associated with weight gain, this was not induced by SBP that apparently has the opposite effect, i.e., lowering the BW. Some of the positive effects on the glycemic control are possibly in part related to the BW lowering effect. Furthermore, our islet gene expression results shows that SBP has the capability to increase both insulin expression and central genes involved in the proliferation of beta cells. It has been a matter of debate as to which compounds in SBP are active. It is not clear whether the beneficial effect on plasma lipid was due to soy proteins, isoflavones, cotyledon fibers, or saponins. Vedavanam et al. (*35*) suggested that soy isoflavones may be beneficial for diabetic subjects because of their estrogenic activity and ability to prevent glucose-induced lipid peroxidation and to inhibit intestinal glucose uptake by decreasing sodium-dependent glucose transporter, which results in a reduction

in postprandial hyperglycemia. Moreover, the effect on plasma TG and cholesterol could be due to soy saponins. Diet containing soy saponins and no isoflavones has been shown to reduce plasma cholesterol and TG in golden Syrian hamsters (*36*). The beneficial effect of soy may also be due to proteins, as soy proteins are rich in arginine and glycine, which influence insulin and glucagon secretion from the pancreas.

In conclusion, SBP possesses a beneficial impact on a subset of the key factors of the insulin resistance syndrome, i.e., improves blood glucose and insulin sensitivity, and reduces TG, total cholesterol, and reduces body weight. Furthermore, the results indicate that SBP upregulates the gene expression of key insulin regulatory genes and genes involved in beta cell proliferation.

## ABBREVIATIONS USED

Akt1, thymoma viral proto-oncogene 1; CAD, coronary artery disease; CCK, cholecystokinin; GSIS, glucose stimulated insulin secretion; IGF1, insulin growth factor 1; Ins2, insulin 2; InsI, insulin I; Ipf1/Pdx1, insulin promoter factor 1, homeodomain transcription factor; IR, insulin receptor; Neurod1/Beta2, neurogenic differentiation 1; SBP, soy bean protein; T2DM, type 2 diabetes mellitus.

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