Genistein Induces Pancreatic β-Cell Proliferation through Activation of Multiple Signaling Pathways and Prevents Insulin-Deficient Diabetes in Mice

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Abstract:

Genistein, a flavonoid in legumes and some herbal medicines, has various biological actions. However, studies on whether genistein has an effect on pancreatic β-cell function are very limited. In the present study, we investigated the effect of genistein on β-cell proliferation and cellular signaling related to this effect and further determined its antidiabetic potential in insulin-deficient diabetic mice. Genistein induced both INS1 and human islet β-cell proliferation after 24 h of incubation, with 5 μm genistein inducing a maximal 27% increase. The effect of genistein on β-cell proliferation was neither dependent on estrogen receptors nor shared by 17β-estradiol or a host of structurally related flavonoid compounds. Pharmacological or molecular intervention of protein kinase A (PKA) or ERK1/2 completely abolished genistein-stimulated β-cell proliferation, suggesting that both molecules are essential for genistein action. Consistent with its effect on cell proliferation, genistein induced cAMP/PKA signaling and subsequent phosphorylation of ERK1/2 in both INS1 cells and human islets. Furthermore, genistein induced protein expression of cyclin D1, a major cell-cycle regulator essential for β-cell growth. Dietary intake of genistein significantly improved hyperglycemia, glucose tolerance, and blood insulin levels in streptozotocin-induced diabetic mice, concomitant with improved islet β-cell proliferation, survival, and mass. These results demonstrate that genistein may be a natural antidiabetic agent by directly modulating pancreatic β-cell function via activation of the cAMP/PKA-dependent ERK1/2 signaling pathway.

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Article:

Genistein, an isoflavone in legumes and some Chinese herbal medicines, has well-known weak estrogenic effect and is a pharmacological inhibitor of tyrosine kinase. It has also been extensively explored for its potential hypolipidemic and antioxidative effects. Recent studies performed in animals (1) and humans (2) have shown that ingestion of isoflavones containing soy protein moderated hyperglycemia. However, it is not clear whether genistein primarily contributes to this beneficial effect. Emerging studies reported that administration of isoflavones or genistein lowered plasma glucose in diabetic animals (3, 4, 5) and postmenopausal women (6), suggesting that genistein may be a plant-derived antidiabetic agent. However, the mechanism of genistein action in diabetes is unknown. Whereas data from one study showed that genistein intake exerted a hypolipidemic effect in obese diabetic rats (3), other studies demonstrated that genistein lowered plasma glucose without affecting lipid profile or insulin sensitivity in obese diabetic animals (7) and humans (6). There is a line of evidence showing that oxidative stress and reactive oxygen species (ROS) play a potential role in the initiation of diabetes (8, 9, 10, 11). Genistein has been reported to exhibit antioxidant activity in aqueous phase systems (12, 13). However, the antioxidant effect of genistein is achieved only at concentrations ranging from 25 to 100 μm, suggesting that genistein is not a physiologically effective antioxidant because the achievable levels of total plasma genistein in both humans (14, 15) and rodents (16, 17) through dietary supplementation is no more than 10 μm. Indeed, intake of isoflavones has no antioxidative effect in healthy postmenopausal women (18). Consistently it has been shown that genistein is a relatively poor ROS scavenger (19, 20).

Loss of β-cell mass and insulin secretory function, leading to the deterioration of glycemic control over time, is central to the development of both type 1 and type 2 diabetes (T2D) (21,22). Recent studies provided evidence that β-cells have the potential to regenerate by proliferation of preexisting β-cells in both physiological and pathological conditions (23, 24). As such, a strategy that induces β-cell proliferation, thus preserving functional β-cell mass, could be one of the essential strategies to prevent the onset of diabetes (21, 23, 25, 26, 27,28). Several earlier studies reported that genistein directly acts on β-cells, leading to insulin secretion (29, 30), whereas other studies found an inhibitory effect (31, 32). We recently discovered that genistein is a cAMP signaling agonist by activation of adenylate cyclase in pancreatic β-cells (33). It has been recently shown that several growth factors induce β-cell proliferation and exert their antidiabetic effects via activation of cAMP signaling (34, 35). Given this background, we investigated in the present study the effect of genistein on β-cell proliferation and cellular signaling related to this effect.

Materials and Methods

Cell and human islet culture

INS1 cells were cultured as we previously described (33). Human pancreatic ductal cells (PANC1s), NIH3T3 preadipocytes (American Type Culture Collection, Manassas, VA), human aortic endothelial cells (HAECs), and rat vascular smooth muscle cells (RVSMCs; Lonza, Gaithersburg, MD) were grown using standard methods. Human islets were obtained through the National Institutes of Health-supported islet cell resource centers and the Islet Distribution Program at the Juvenile Diabetes Research Foundation. The islet purity was 80–90% and viability was 80–97%. Before the experiment, INS1 cells were synchronized in serum-free, 3 mm glucose RPMI 1640 (Sigma, St. Louis, MO) for 24 h, and the islets were maintained in CMRL-1066 medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum (HyClone, Logan, UT).

Cell proliferation assay

INS1 cells or human islets were incubated with various concentrations of genistein (Sigma) in RPMI 1640 at 37 C. The culture medium contained 1 mm glucose for INS1 cells and 2.8 mmglucose for human islets. PANC1s, HAECs, and NIH3T3, and RVSMCs were incubated with genistein in RPMI 1640, M199, and DMEM, respectively. Twenty-four hours later, the cultures were continued for an additional 4 h in the presence of 5-bromo-2-deoxyuridine (BrdU; 10 μm). In some experiments, INS1 cells or the islets were preincubated with PD 098,059 (PD), H89, ICI 182,780 (ICI), or vehicle (dimethyl sulfoxide) for 30 min before addition of 1 μm genistein for 24 h. Cell proliferation was assessed by BrdU incorporation measurements with an ELISA kit (Roche Applied Science, Indianapolis, IN).

In vitro free radical scavenging activity assay

Free radical scavenging activity of genistein was determined by oxygen radical absorbance assay as described (36).

Immunoblot analysis

Equal amounts of protein from cell extracts were resolved on 10% SDS-PAGE gels, blotted onto nitrocellulose membranes, and probed with antiphospho-ERK1/2 or anticyclin D1 (Cell Signaling, Danvers, MA) as we previously described (37). Membranes were then stripped and reprobed with anti-ERK1/2 or anti-β-actin to monitor for equal sample loading.

Intracellular cAMP and protein kinase A (PKA) activity assays

Intracellular cAMP concentration and PKA activity in the lysates of human islets were measured as previously described (33).

Antibody transfection

Polyclonal antibodies against PKA catalyticα plus catalyticβ or preimmune IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into INS1 cells using a Chariot reagent

according to the manufacturer's protocol (Active Motif, Carlsbad, CA). After 5 h in complete RPMI 1640 and 12 h in serum-free medium containing 3.0 mm glucose after transfection, the cells were treated with 1 μm genistein or vehicle for 24 h followed by cell proliferation assay.

Adenoviral PKA inhibitor gene construct and infection

Replication-deficient adenovirus containing the complete sequence of endogenous PKA inhibitor cDNA (AdPKI) was constructed as previously described (38). For determining infection efficiency, human islets were exposed to purified adenovirus at 100–400 multiplicity of infection (MOI)/cells in RPMI 1640 medium for 1 h at 37 C and then cultured in RPMI 1640 containing 5% fetal bovine serum for 24 h at 37 C. AdPKI null virus served as controls. For MOI calculation, it was assumed that each islet equivalent consists of 1000 cells. After infection, the islets were treated with 10 μm forskolin and 0.2 mm 3-isobutyl-1-methylxanthine for 15 min. The enzymatic activity of PKA in the lysates of islets was determined. For proliferation assay, islets were infected with AdPKI or AdPKI null virus at 200 MOI/cell and then treated with 1 μm genistein or vehicle for 24 h.

Animals and treatment with genistein

Four-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed in a room maintained on a 12-h light, 12-h dark cycle under constant temperature (22–25 C) with*ad libitum* access to food and water. The protocol of this study was approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University. Mice were initially fed a modified AIN-93G rodent diet with corn oil substituted for soybean oil (Dyet, Inc., Bethlehem, PA) for 2 wk and then were randomly divided into three groups with 12 mice per group and fed a diet containing either 0 g (groups 1 and 2) or 0.25 g (group 3) genistein per kilogram. This genistein dosage was used (approximately a human intake of 25–200 mg/d) because it is within the range that humans can realistically consume through taking supplements (39). We confirmed by performing HPLC analysis that the basal diet is free of genistein. After 2 wk, diabetic mice were induced with ip injection of streptozotocin (STZ) dissolved in 0.1 mm cold sodium citrate buffer (pH 4.5) at 40 mg/kg daily for 5 consecutive days. Control mice received ip citrate buffer. After this procedure, mice were continually treated with the control or genistein diet. Body weight and feed intake were recorded weekly throughout the study. To confirm the results, we repeated this animal experiment using the same study protocols.

Plasma glucose, insulin, and lipid measurements

At the beginning of the experiment, the fasting blood glucose levels in tail vein blood sample were measured using a glucometer (Roche) to ensure that the mice were euglycemic. After STZ injection, the levels of blood glucose were measured weekly to assess the onset of hyperglycemia (nonfasting blood glucose > 250 mg/dl) (40). Plasma insulin concentration was measured by ELISA (Mecodia, Winston-Salem, NC) in mice fasted for 4 h. Fasting plasma total cholesterol

and triacylglycerides were measured in triplicate by enzymatic methods using a Pointer 180 analyzer (Pointe Scientific, Canton, MI) as described (41).

Plasma genistein measurements

Blood samples were drawn 2 h after food intake from the retrobulbar plexus through heparinized capillary tubes. Plasma was collected by centrifugation at $16,000 \times g$ for 15 min. An aliquot of 250 μl serum per sample was used for extraction of genistein using a previously described method (42). Genistein in the extracted samples was determined by using the HPLC system (Waters2695; Waters Co., Milford, MA) with a Luna phenyl-hexyl column (5 μm C_{18} 100 Å; Phenomenex Inc., Torrance, CA) (43).

Glucose and insulin tolerance tests

For glucose tolerance tests, mice were fasted 4 h and injected ip with a single bolus of glucose (2 g/kg body weight). Glucose levels were measured at time points of 0, 15, 30, 60, and 120 min after glucose administration. For insulin tolerances tests, mice were injected ip with insulin (0.75 U/kg body weight), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after insulin administration.

Hepatic antioxidant and other enzyme assays

The harvested livers were homogenized in ice-cold 50 mm phosphate buffer containing 2 mmEDTA on ice and then centrifuged at $10,000 \times g$ for 15 min at 4 C. The resulting supernatants were collected and kept on ice for measuring the level of glutathione (GSH) and activity of GSH reductase (GR), GSH peroxidase (GPx), GSH S-transferase (GST), catalase, superoxide dismutase, and nicotinamide adenine dinucleotide phosphate oxidase-quinone oxidoreductase 1 (NQO1) as previously described (44).

Immunohistochemistry and islet morphometry

Twelve mice per group were injected (ip) with BrdU (100 mg/kg body weight). Ten hours after injection, the pancreata were dissected, fixed in 4% (vol/vol) formaldehyde buffer (pH 7.2), and embedded in paraffin. A series of tissue sections (5 μm thickness) were prepared; mounted on glass slides; and immunofluorescently stained for determining β-cell mass, proliferation, and apoptosis. The β-cell area was measured using images acquired from serial insulin-stained pancreatic sections (500 μm interval). The β-cell mass was calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue and multiplying by the pancreas weight (45). Proliferating β-cells were identified by examining the incorporation of BrdU in β-cells, which was determined by sequential immunolabeling pancreatic sections with sheep anti-BrdU and guinea pig antiinsulin antibodies (Abcam, Cambridge, MA) (46). Apoptotic β-cells were detected by labeling the sections with a terminal deoxynucleotidyl transferase-mediated

deoxyuridine 5-triphosphate nick-end labeling (TUNEL) kit (Roche Applied Science) as described (47), followed by staining with a guinea pig antiinsulin antibody to visualize β-cells.

Statistical analysis

Data were analyzed with one-way ANOVA or Student's paired *t* test when designated using SAS program (SAS Institute, Cary, NC). Treatment differences were subjected to Tukey's test. A $P < 0.05$ was considered significant.

Results

Genistein stimulates β-cell proliferation

We first examined whether genistein has an effect on clonal β-cell proliferation. As shown in Fig. 1A, genistein dose-dependently stimulated INS1 cell proliferation. The effect of genistein on β-cell proliferation was significant at 0.1 μm concentration, with a maximal increase at 5 μmgenistein (27% over control, $P < 0.01$). We further evaluated the nuclear event that mediates genistein effect on cell proliferation and found that exposure of INS1 cells to genistein for 3 h elicited a 61.6% increase ($P < 0.05$) in the expression of cyclin D1 protein (Fig. 1B), a major cell-cycle regulator essential for β-cell growth (48), suggesting that genistein may stimulate cell cycle progress involving cyclin D1expression. We considered the possibility that the stimulation of genistein on β-cell proliferation might simply represent the repair of oxidation-induced damage to the DNA. Our studies excluded this possibility based on these observations. First, we directly counted the cells using a microscope cell counting chamber after genistein treatment. We observed that exposure of INS1 cells to genistein (1 and 5 μm) increased cell number by 28.9–35.6%. Second, we observed that exposure of genistein to β-cells for 24 h had no effect on cell viability (data not shown), suggesting that the increased cell proliferation by genistein is not due to a change in cell apoptosis. Third, whereas pharmacological dose of genistein (10–20 μm) showed significant free radical scavenging activity, it had no effect at 1 μm (data not shown). Fourth, as described below, a variety of flavonoids that are reportedly potent antioxidants failed to stimulate β-cell proliferation (Fig. 2).
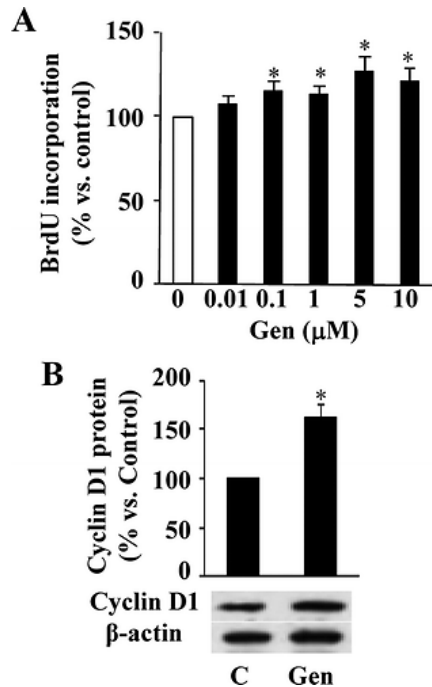
Fig. 1. Genistein (Gen) stimulates proliferation of β-cells. A, INS1 cells were incubated with various concentrations of Gen or vehicle in RPMI 1640 medium for 24 h, followed by addition of BrdU (10 μm) for 4 h. Cell proliferation were determined by measuring DNA synthesis using BrdU ELISA kit. Data were expressed as mean ± se of observations from four to five separate experiments, each performed in triplicate sample. B, INS1 cells were incubated with Gen (1 μm) or vehicle [control (C)] for 3 h. The level of cyclin D1 in the treated cell extracts was measured by Western blot and normalized to β-actin content from the same sample. The bar graphs (mean ± se) represent four independent experiments. *, $P < 0.05$ vs. vehicle-alone-treated control.
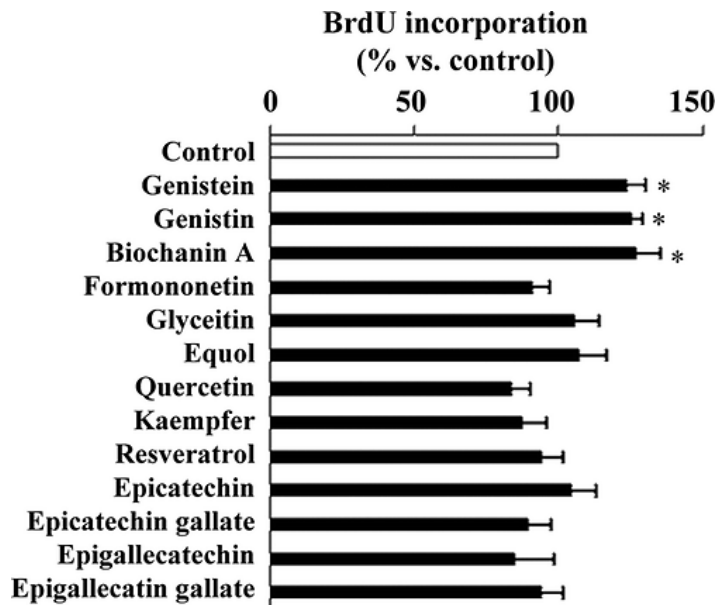
Fig. 2. The stimulatory effect of genistein on β-cell growth may be structure specific. INS1 cells were cultured with various flavonoids (1 μm) in RPMI 1640 containing 1 mm glucose for 24 h. Cell proliferation was determined using an ELISA kit. Data are expressed as mean ± se derived from three independent experiments performed in triplicate each. *, $P < 0.05$ *vs.* vehicle-alone-treated cells.

Genistein has no effect on the proliferation of PANC1s, NIH3T3, HAECs, and RVSMCs

Increased cell proliferation may not always be desirable for other tissues. We therefore tested the effects of genistein on proliferation of PANC1s, NIH3T3, HAECs, and RVSMCs. We found that genistein at the same doses used for β-cell proliferation study had no effect on proliferation of these cells (data not shown), suggesting that the stimulatory effect of genistein on cell proliferation may be restricted to β-cells.

The stimulatory effect of genistein on β-cell proliferation may be structure specific

To determine the specificity of this genistein effect, we tested a host of structurally related flavonoid compounds in parallel to genistein. This analysis revealed the following data (Fig. 2): 1) isoflavones [genistin (a glycoside form of genistein) and biochanin A (a precursor of genistein), which are largely converted to genistein in the intestine (49), increased INS1 cell proliferation by 25 and 27%, respectively, a magnitude comparable with that of genistein (24%), whereas formononetin, glycerin, and equol had no effect; 2) flavones (quercetin and kaempferol) slightly decreased cell proliferation by 15.8 and 12.7%, respectively; 3) flavanols (epicatechin, epicatechin gallate, epigallacatechin, epigallacatechin gallate) had no effect; 4) resveratrol, a natural phytoalexin, was also inactive. These results demonstrated the unique effect of genistein on β-cell growth that is possibly not shared by other structurally related flavonoid compounds.

Estrogen receptor (ER)-independent effect of genistein on β-cell proliferation

Because genistein has well-known weak estrogenic effects in some tissues by binding to ERs (50), we examined whether genistein-induced cell proliferation was mediated through ERs. As shown in Fig. 3A, genistein increased INS1 cell proliferation by about 30%. The ER antagonist ICI had no effect on genistein-induced cell proliferation. The activity of ICI used in this study was validated through blocking 17β-estradiol-induced Akt phosphorylation in endothelial cells as we recently demonstrated (51). In addition, exposure of INS1 cells to 17β-estradiol for 24 h failed to stimulate cell proliferation (Fig. 3B). These results suggest that the effect of genistein on β-cell proliferation is independent of estrogen signaling mechanisms.
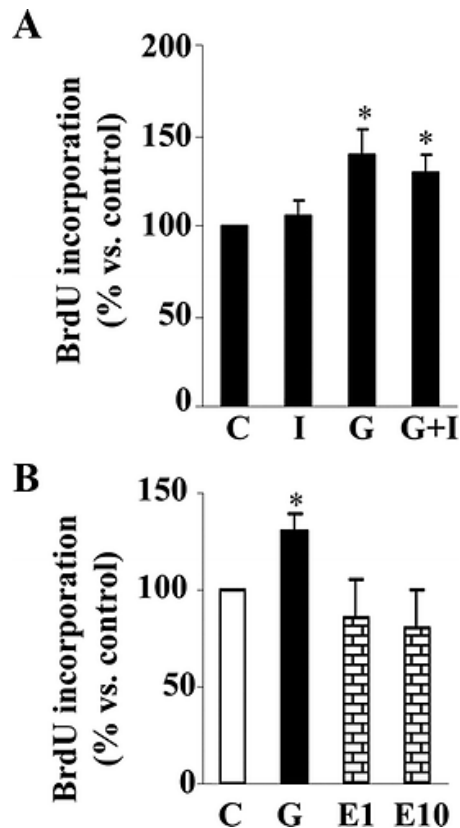
Fig. 3. Genistein (G)-induced β-cell proliferation is ER independent. A, INS1 cells were preincubated with ICI (I; 2 μm) or vehicle for 30 min, followed by addition of G (1 μm) or vehicle (C). B, INS1 cells were incubated with G (1 μm), 17β-estradiol (E1; 1 nm; E10; 10 nm) or vehicle (C) in RPMI 1640 containing 1 mm glucose. Twenty-four hours later, cell proliferation was measured with ELISA. Data obtained from five separate experiments in triplicate determinations each are expressed as mean ± se. *, $P < 0.05$ *vs.* vehicle-alone-treated cells.

Stimulation of β-cell proliferation by genistein is mediated through PKA-dependent activation of ERK1/2 signaling

Recent studies suggested that the cAMP/PKA- and ERK1/2-mediated pathways are two important signaling cascades mediating various stimuli-induced β-cell proliferation (52). We recently reported that genistein activates the cAMP/PKA signaling in clonal β-cells and mouse islets (33). Here we showed that incubation of INS1 cells in genistein for 15 min stimulated the phosphorylation of ERK1/2, with 5 μm genistein inducing a maximal increase (Fig. 4A), a pattern that is consistent with its effect on cell proliferation. However, the expression of ERK1/2 protein was not changed in these studies (Fig. 4B).

Next, we determined whether the activation of cAMP and ERK1/2 signaling is involved in genistein-induced β-cell proliferation. We showed that incubation of INS1 cells with the PKA inhibitor H89 or the MAPK kinase (MEK)-1/2 inhibitor PD completely abolished the genistein-

stimulated proliferation of INS1 cells (Fig. 4C). Consistent with this result, inhibition of PKA or MEK1/2 blocked genistein-induced phosphorylation of ERK1/2 (Fig. 4D), suggesting that PKA acts upstream of MEK1/2 to mediate ERK1/2 phosphorylation and subsequent cell proliferation. To further confirm the role of PKA, we delivered PKA Cα- plus Cβ-antibodies into INS1 cells (33). Transfection of the cells with PKA antibodies significantly attenuated genistein-induced β-cell proliferation (Fig. 4E), whereas preimmune IgG had no effect. These results indicate that activation of PKA is sufficient for genistein-induced β-cell proliferation.
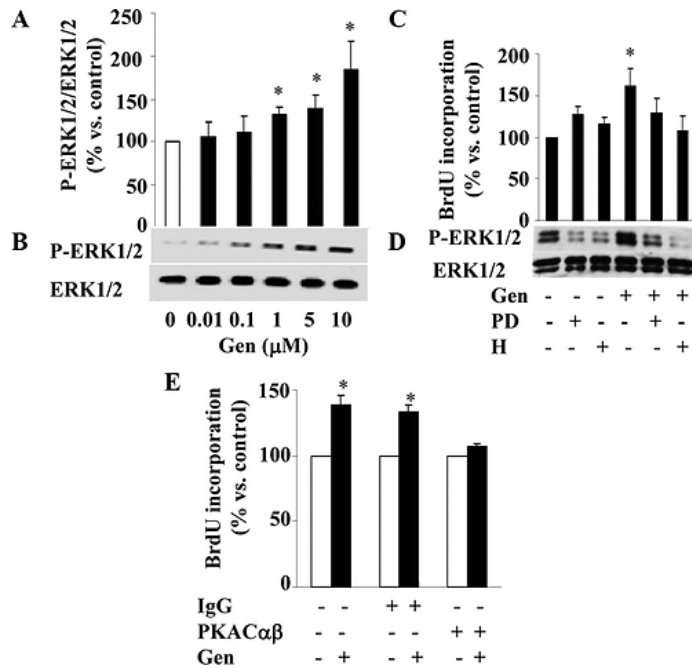

Fig. 4. Genistein (Gen)-stimulated β-cell proliferation is mediated through the PKA and ERK1/2 signaling mechanisms. A, INS1 cells were incubated in RPMI 1640 containing 1 mm glucose with various concentrations of Gen for 15 min at 37 C. Western analysis was performed per protocol described in *Materials and Methods* to detect phosphorylated ERK1/2 (P-ERK1/2), which was normalized to total ERK 1/2 (B) from the same sample. C and D, INS1 cells were preincubated with PD (2 μm), H89 (H; 10 μm) or vehicle (Control) for 30 min. Cells were then treated with Gen (1 μm) or vehicle (Control) in the continued presence or absence of inhibitors at 37 C. Incubation was either terminated after 15 min to determine ERK1/2 phosphorylation by Western blotting or continued for 24 h to determine cell proliferation. E, INS1 cells were transfected with antibodies against PKA Cα plus Cβ or preimmune IgG per protocol as described in *Materials and Methods*. Transfected cells were treated with Gen (1 μm) or vehicle for 24 h followed by performing cell proliferation assay. Data are expressed as mean ± se of three to six experiments in triplicate each. *, $P < 0.05$ *vs.* vehicle alone or preimmune IgG-alone-treated cells.

Genistein activates cAMP and ERK1/2 signaling and subsequently stimulates human islet cell proliferation

We tested whether genistein has a similar effect on cell proliferation in human pancreatic islets. As shown in Fig. 5A, genistein significantly induced cell proliferation in human islets, with 5 μm genistein inducing 44.5% increase in cell proliferation over control. Because not all cells in the islets are β-cells, we performed an immunofluorescence study to determine whether cell proliferation in islets induced by genistein is β-cell specific. Islets treated with or without genistein were double immunostained with BrdU and insulin antibodies. The result showed that proliferative cells in human islets induced by genistein are β-cells (Fig. 5B).

To determine whether the same signaling pathways that mediate genistein effect on INS1 cell proliferation also operate in human islets, we first tested whether genistein elevates intracellular cAMP levels and activates PKA in human islets. The result showed that genistein also significantly elevated cAMP (Fig. 5C) and induced PKA activity (Fig. 5, D and E), consistent with the dose-response pattern as that obtained in islet proliferation study.

To determine the role of PKA and ERK1/2 in the regulation of genistein effect in human islets, we incubated the islets with genistein in the presence or absence of PD or H89 for 24 h. In line with observations in INS1 cells, inhibition of MEK1/2 or PKA completely abolished the genistein-stimulated cell proliferation (Fig. 5F) and ERK1/2 phosphorylation in human islets (Fig. 5G), further confirming a central role of PKA and a cross talk between cAMP/PKA and MEK/ERK signaling pathways in mediating genistein action. To further confirm the role of PKA in mediating this genistein effect, human islets were infected with AdPKI. Treatment of islets with AdPKI greatly attenuated PKA activity (Fig. 5H) and genistein-induced cell proliferation (Fig. 5I), whereas control virus was inactive. Taken together, these results indicate that genistein induces islet β-cell proliferation via activation of the cAMP/PKA/MEK/ERK signaling cascade.
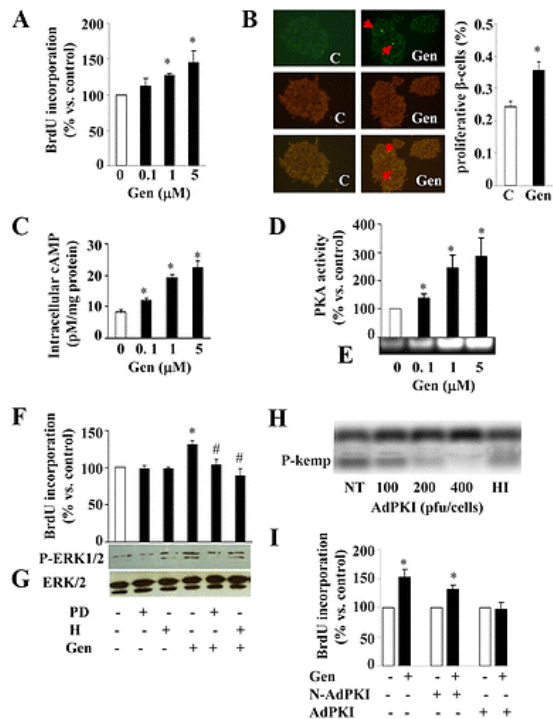
Fig. 5. Genistein (Gen) stimulates intracellular cAMP accumulation, activates PKA and ERK1/2, and subsequently stimulates cell proliferation in human islets. Human islets were incubated with various concentrations of Gen; panel A or 1 μm Gen panel B in RPMI 1640 containing 2.8 mmglucose for 24 h, followed by labeling with BrdU (10 μm) for 4 h. Islet cell proliferation was either measured by ELISA (panel A) or visualized by double immunostaining for BrdU (*green*) and insulin (*red*) (panel B). The *light green* BrdU+ nuclei as identified by the *arrows* from merged images (*brown*) show that Gen increases β-cell proliferation. C, Vehicle (control). Panels C and D, Human islets were stimulated with various concentrations of Gen or vehicle in RPMI 1640 in the presence of 2.8 mm glucose at 37 C for 20 min. Intracellular cAMP concentration (panel C) and PKA activity (panel D) in cell extracts were measured, with a representative photograph of the agar gel used for PKA activity assay shown (panel E). Panels F and G, Human islets were preincubated with PD (10 μm), H89 (H; 10 μm), or vehicle (control) for 30 min. The islets were then stimulated with Gen 1 μm) or vehicle (control) in the continued presence or absence of inhibitors. Incubation was either terminated after 20 min to determine ERK1/2 phosphorylation by Western blotting (panel G) or continued for 24 h to determine cell proliferation by ELISA (panel F). Panel H, Human islets were infected with 0, 100, 200, 400 MOI AdPKI/cell, or control virus (N-AdPKI), followed by incubation with 10 μm forskolin and 0.2 mm 3-isobutyl-1-methylxanthine for 15 min. PKA activity in the cell lysates was then determined by measuring phosphorylation of kemptide (P-kemp). Panel I, Human islets were infected with 200 MOI AdPKI/cell and then treated with Gen (1 μm) or vehicle for 24 h, followed by cell proliferation assay. Data are expressed as mean ± se obtained from three to four independent experiments in triplicate each. *, $P < 0.05$ *vs.* vehicle-alone-treated cells; #, $P < 0.05$ *vs.* genistein-alone-treated cells.

Dietary genistein intake ameliorates hyperglycemia in STZ-induced diabetic mice

To investigate whether our *in vitro* and *ex vivo* findings are biologically relevant, we performed an animal study assessing whether genistein has potential to prevent diabetes. As expected, dietary supplementation of genistein significantly elevated plasma genistein levels. Under our experimental conditions, plasma genistein levels in STZ-induced diabetic mice fed basal or 0.25 g/kg genistein diet were 0 and 6.84 ± 0.59 μm, respectively. Our data showed that genistein significantly ameliorated STZ-induced hyperglycemia in diabetic mice (Fig. 6A). By the 28th day after STZ injection, 88% of STZ-alone-treated mice became hyperglycemic, whereas only 13% were diabetic in the genistein-treated group. Consistently, dietary genistein ingestion prevented body weight loss (Fig. 6B), whereas food intake was not affected in diabetic mice (data not shown). Mice fed genistein showed significantly higher blood insulin levels (Fig. 6C) and improved glucose tolerance (Fig. 6D), whereas insulin tolerance was not altered by genistein treatment (data not shown), suggesting that genistein has no effect on insulin sensitivity. To confirm the results from this study, we conducted a second animal trial and similar results were obtained.
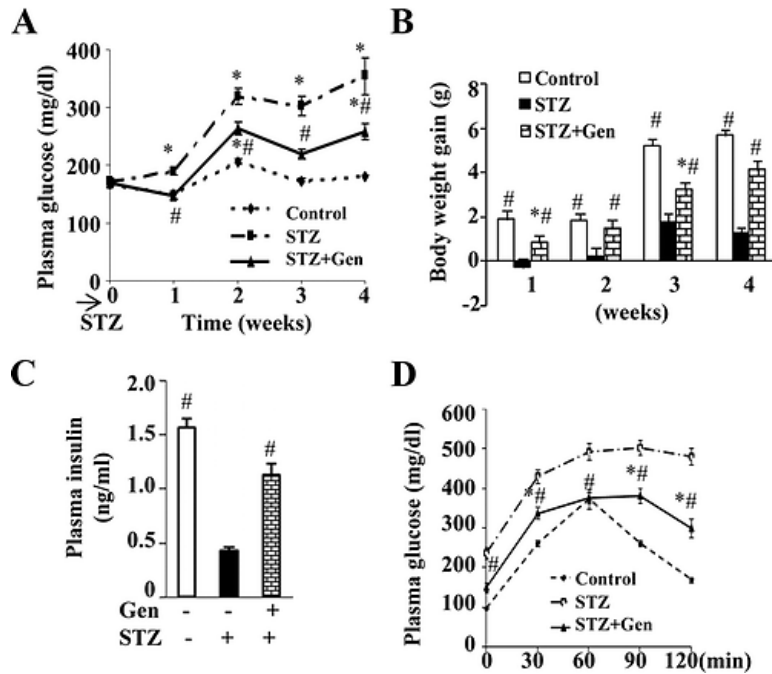
Fig. 6. Dietary intake of genistein (Gen) prevents STZ-induced diabetes in mice. Male C57B/6J mice (4 wk old) were fed a control diet (C; STZ) or Gen-supplemented diet (0.25 g/kg diet) for 2 wk before administration of STZ (40 mg/kg body weight for 5 d) and continued on the same diet for 4 wk. The mice in the control group were injected with citrate buffer. Panel A, Nonfasting blood glucose levels were measured before and 1, 2, 3, and 4 wk after STZ administration. Panel B, Body weight gain was measured weekly during the whole period of animal study. Panel C, Plasma insulin levels in fasted mice were measured by ELISA. Panel D, Glucose tolerance was determined. Data are expressed as mean ± se (n = 12 mice). *, $P < 0.05$ *vs.* healthy control; #, $P < 0.05$ *vs.* STZ-alone-treated mice.

The antidiabetic effect of genistein is not due to modulating plasma lipid profiles or scavenging ROS

We first measured plasma lipid levels in mice treated or untreated with genistein. The results showed that genistein had no effect on plasma cholesterol (Fig. 7A) and triglyceride concentrations (Fig. 7B). We have not observed significant differences in body weight and food intake between control and healthy mice fed a diet containing 0.25 g/kg genistein (data not shown). This result suggests that a moderate level of dietary genistein used in this study had no effect on appetite, energy expenditure, or lipogenesis, although it can reduce adipose deposition at higher doses (0.5–1.5 g/kg diet) in mice (17). Next, we evaluated hepatic antioxidant defense system in these animals. Our data demonstrated that genistein intake had no significant effects on the activities of NQO1, GST, GPx, GR, and catalase that play important role in scavenging ROS (Fig. 7C), although the activities of some of these enzymes were significantly increased in diabetic mice compared with normal mice.
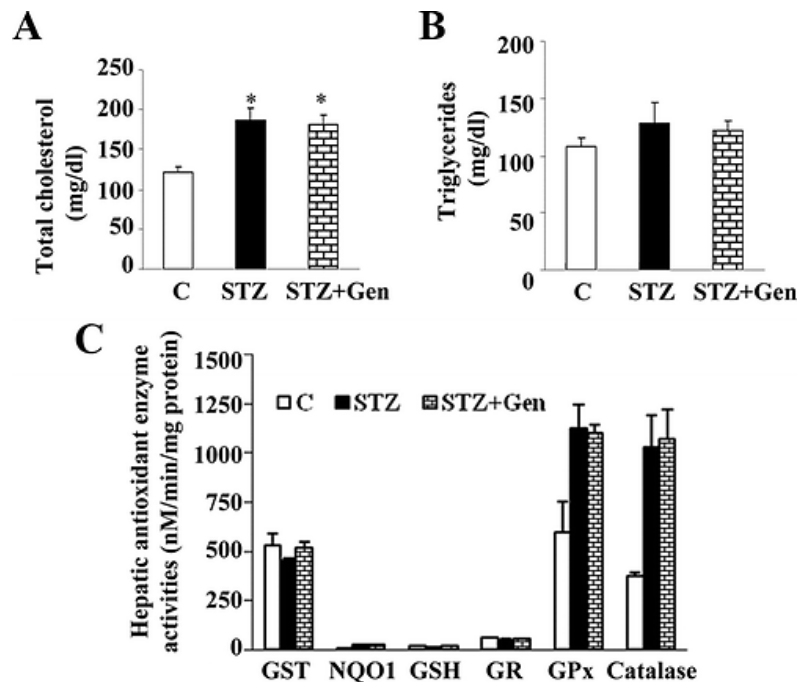
Fig. 7. Genistein (Gen) has no effect on plasma lipid profiles and hepatic antioxidant enzyme activities in STZ-induced diabetic mice. Diabetic mice were induced with STZ and fed a basal or Gen diet as described in Fig. 6. Plasma total cholesterol (panel A), triglycerides (panel B), and hepatic activities of GST, NQO1, GSH, GR, GPx, and catalase (panel C) were measured as described in *Materials and Methods*. Data are expressed as mean ± se (n = 6–8 mice). *, $P < 0.05$ *vs.* healthy control (C).

Dietary intake of genistein improves pancreatic β-cell proliferation, survival, and mass in diabetic mice

We evaluated pancreatic islet cell mass through the immunohistochemical technique. We found that STZ administration severely decreased β-cell mass and disrupted the islet architecture (Fig. 8, A and B), which is essential for normal islet function. However, dietary provision of genistein significantly improved islet β-cell mass. Next, we measured the proliferation and apoptosis of β-cells, which may represent the mechanisms by which genistein improves β-cell mass. STZ treatment increased residual β-cell proliferation, an observation consistent with previous findings that destruction of β-cells by STZ leads to subsequent regeneration of β-cell mass primarily from proliferation of preexisting and surviving β-cells (53, 54, 55). Dietary provision of genistein significantly enhanced islet β-cell proliferation (Fig. 8, C and D). Furthermore, we performed *in situ* detection of DNA fragmentation by TUNEL assay to assess whether genistein has an effect on apoptosis of mouse islets. In addition, the islets were costained for insulin to identify β-cells. Genistein significantly reduced STZ-induced β-cell apoptosis (Fig. 8, E and F). This result suggests that genistein may improve pancreatic β-cell mass in diabetic mice through its mitogenic and antiapoptotic effects on islet β-cells.
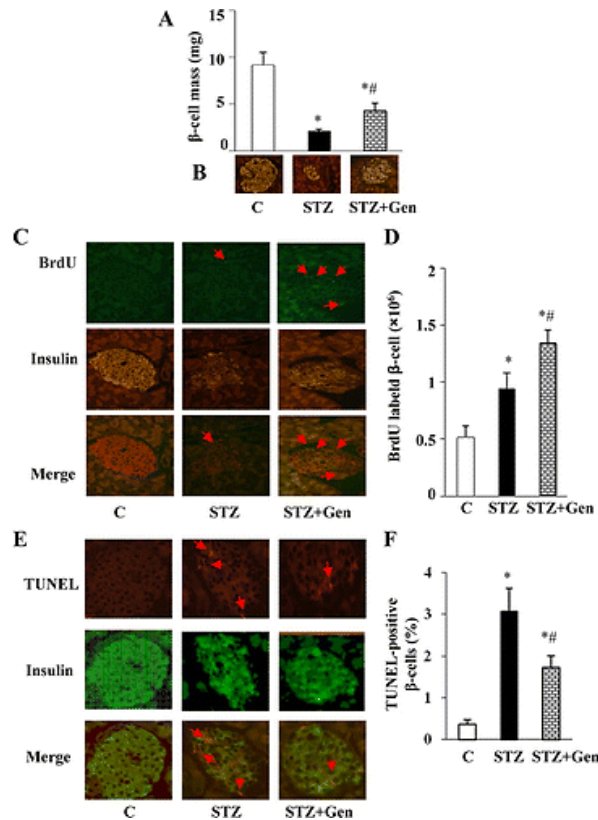
Fig. 8. Dietary intake of genistein (Gen) improves pancreatic β-cell proliferation, survival, and mass in STZ-induced diabetic mice. Panels A and B, Pancreatic sections from control (C) or STZ diabetic mice fed control or Gen diet were stained with an antibody against insulin. The β-cell mass was determined as described in *Materials and Methods*. Panels C and D, Pancreatic sections were double immunostained for BrdU and insulin. The number of BrdU- and insulin-positive cells as identified by the *arrows* from merged images was counted. Panels E and F, Pancreatic sections were stained using TUNEL procedure and costained for insulin. TUNEL- and insulin-positive cells were identified by the *arrows* in merged sections (panel E). The number of apoptotic β-cells in each islet was counted and expressed as percentage of total insulin-positive cells (panel F). Data are expressed as mean ± se (n = 12 mice). *, $P < 0.05$ *vs.* healthy control (C); #, $P < 0.05$ *vs.* STZ alone-treated mice

Discussion

Recent studies have shown that genistein may have antidiabetic potential (3, 4, 5, 6, 7). However, the mechanism of this effect is not understood. Here we provide evidence that genistein stimulates both clonal and human islet β-cell proliferation through the cAMP/PKA- and ERK1/2-dependent mechanisms. Recent studies demonstrated that loss of β-cell mass and function is central to the development of both type 1 diabetes and T2D (21, 22). Therefore, induction of β-cell proliferation is one of the essential strategies to prevent diabetes (21, 56). Our

data from animal studies showing that genistein can act as a growth factor for β-cells *in vivo* provide a novel mechanism for the observed antidiabetic effect of this compound.

Genistein is a widely used dietary supplement. The reported plasma concentrations of genistein in both humans (15) and rodents (17) through dietary supplementation are usually within the range of 1–5 μm. To consider the potential biological relevance of the observed effects of genistein on β-cell proliferation, we used genistein concentrations that are comparable with the physiologically achievable levels through dietary means. Whereas there is a trend for genistein to stimulate β-cell proliferation at 0.1 μm, a significant effect was observed at 1 μm, with the maximal effect achieved at 5 μm genistein. The results observed in INS1 cells were confirmed with isolated human islets, suggesting that physiologically relevant concentrations of genistein may have antidiabetic implications by directly acting as a β-cell growth factor. In addition, these data may provide a molecular basis for some of the antidiabetic effects of genistein observed recently in human and animal studies (3, 4, 5).

The result from comparing the effect of genistein on β-cell proliferation with a cohort of structurally related flavonoids demonstrated a highly specific genistein effect. Although the specific chemical structure responsible for the β-cell proliferative effect of genistein remains to be determined, the hydroxyl group at the 5C position on the A ring may be crucial for the unique effect of genistein because equol and 17β-estradiol, which lack a hydroxyl group at 5C, failed to induce β-cell proliferation. In addition, we showed that replacing the hydroxyl group at either 7C position with a glucose molecule (genistin) or at 4C position with a methyl group (biochanin A) had no significant effect on genistein activity, suggesting that these structure components may not be important for genistein action.

Genistein has well-known weak estrogenic effects by binding to ERs. However, we provided evidence that the observed proliferative effect of genistein on β-cells is not related to its potential estrogenic effect. Interestingly, recent studies reported that genistein can activate an orphan G protein-coupled receptor 30 (GPR30) (58). Whereas the physiological role of GPR30 is still unknown, female GPR30 knockout mice display hyperglycemia and impaired glucose tolerance (59). Therefore, it is intriguing to speculate that GPR30 may play a role mediating genistein effect.

Activated ERK1/2 plays a pivotal role in environmentally stimulated cellular responses, including cellular proliferation, growth, and differentiation. We observed that genistein induced a rapid ERK1/2 phosphorylation, which was sustained for at least 6 h. Such a prolonged ERK1/2 activation may be necessary for growth factors to drive β-cell proliferation (60). ERK1/2 can be activated by various different mechanisms (34). In this report, we found that genistein-stimulated ERK1/2 phosphorylation was dependent on PKA activation in β-cells, suggesting that ERK1/2 is located downstream of PKA, which is generally cAMP dependent. Cell growth and division are regulated by an array of D-type cyclins, cyclin-dependent protein kinases, and inhibitors of cyclin-dependent protein kinases. In the islets, cyclin D1 and D2 are essential for β-cell growth

(48), and cyclin D1 alone can induce β-cell proliferation in rat and human islets (61). Whereas the mechanisms that link genistein to the cell cycle machinery are still unclear, we found that genistein elevated cyclin D1 protein expression. Previous studies demonstrated that cyclin D1 is a nuclear target of activated ERK1/2 (62). In addition, cAMP-responsive element sites (CREs) are present within cyclin D1 promoters, which are trans-activated by CRE-binding protein to regulate cyclin D1 gene expression (63). In β-cells, both PKA and ERK1/2 can induce CRE-binding protein phosphorylation at serine 133, which is required for CRE-regulated transcription (64). Therefore, it is tempting to speculate that genistein may stimulate cell cycle progress at least partially through regulating cyclin D1 expression via activation of the PKA and ERK1/2 cascades, although we cannot exclude the possibility that genistein also modulates other nuclear molecules involved in β-cell proliferation.

Using a diabetic mouse model induced by multiple low doses of STZ, which causes mild to moderate level of diabetes mediated by a destruction of islet β-cells (40), we provided evidence that dietary supplementation of genistein could ameliorate diabetes and improve glucose tolerance, which are likely a result of improved functional β-cell mass by genistein treatment. Consistently, diabetic mice fed genistein displayed about 2-fold increase in circulating insulin levels compared with those in the control group, which, however, could be partially due to improved insulin secretion from existing β-cells by genistein, as observed in our previous studies (33). We further found that diabetic mice fed genistein exhibited greater islet β-cell proliferation and reduced levels of β-cell apoptosis caused by STZ, which should primarily contribute to improved β-cell mass observed in genistein-treated mice. However, the relative contributions of proliferative and antiapoptotic effects of genistein to the improved β-cell mass in diabetic mice cannot be established in this study. Whereas we provide sufficient evidence that genistein directly induces pancreatic β-cell proliferation *ex vivo*, suggesting that genistein improvement of pancreatic β-cell mass *in vivo* might be partially attributable to its growth-promoting effect on β-cells, there is a possibility that observed mitogenic effect of genistein on β-cells in diabetic mice may simply represent enhanced repair of STZ-induced damage to the DNA or a protective effect on replicating β-cells. Further studies are needed to determine whether genistein improvement of pancreatic β-cell mass is primarily due to the promotion of compensatory growth of β-cells or protection of proliferating β-cells after islet injury.

Because blood lipids could be risk factors for the pathogenesis of diabetes, the secondary action whereby genistein improves lipid profiles of diabetic animals may contribute to the overall antidiabetic effects of this agent. We considered this possibility and measured plasma lipid levels. However, we did not find that genistein lowered plasma lipid levels or improved insulin sensitivity. Oxidative stress may play a potential role in the initiation of diabetes (8). STZ may induce oxidative stress in diabetic mice, which may contribute to the diabetogenic effect of this agent. Whereas genistein at physiologically relevant concentrations (<5 μm) is a poor ROS scavenger (65), its biological effects are frequently attributed to a presumably antioxidant activity. We considered the possibility that the antidiabetic effect of genistein might partially be

due to a reduced tissue damage caused by STZ-induced oxidative stress and therefore evaluated hepatic antioxidant defense system in mice treated or untreated with genistein. Although the activities of some of these enzymes were significantly increased in diabetic mice compared with normal mice, which may reflect an adaptive mechanism in response to elevated oxidative stress, genistein intake had no further effects on these enzymes, further confirming that the antidiabetic effect of was likely a result of improved functional β-cells in diabetic mice.

The glucose analog STZ is reported to be transported into β-cells by the glucose transporter 2 (GLUT2) for exerting its apoptotic effect (66). Although we did not study the effect of genistein on GLUT2 protein expression in mouse islets, our recent studies found no such effect in cultured β-cells (67), suggesting that improvement of islet β-cell mass and survival by genistein may not be due to modulation of GLUT2 expression, thereby preventing STZ influx in β-cells. Some studies showed that STZ increased peripheral lymphocytic infiltration into islets, thereby producing insulitis (40, 68), which may contribute to STZ-induced β-cell apoptosis and diabetes. However, we observed no effect of genistein on mononuclear cell infiltration into the islets. In fact, we detected hardly any infiltrated immune cells in these mice (data not shown), consistent with previous finding that this mouse strain is resistant to STZ-induced insulitis (57).

In summary, we have identified for the first time that genistein may be a putative β-cell growth factor by targeting the cAMP and ERK1/2 signaling pathways. Our animal studies showed that genistein is capable of preserving islet β-cell mass and alleviating diabetes. Loss of functional β-cell mass through apoptosis is central to the development of both type 1 diabetes and T2D and islet β-cell proliferation is a very important component of β-cell adaptation to increased apoptosis and insulin resistance and therefore holds promise as a treatment for this disease. In this context, these findings may potentially lead to the development of novel, natural agents for diabetes prevention and treatment.

Disclosure Summary: The authors have nothing to disclose.

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Abbreviations: AdPKI, Adenovirus containing complete sequence of endogenous PKA inhibitor cDNA; BrdU, bromodeoxyuridine; CRE, cAMP-responsive element; ER, estrogen receptor; GLUT2, glucose transporter 2; GPR30, G protein-coupled receptor 30; GPx, GSH peroxidase; GR, GSH reductase; GSH, glutathione; GST, GSH S-transferase; HAEC, human aortic endothelial cell; ICI, ICI 182,780; MEK, MAPK kinase; MOI, multiplicity of infection; NQO1, nicotinamide adenine dinucleotide phosphate oxidase quinone oxidoreductase 1; PANC1, pancreatic ductal cell; PD, PD 098059; PKA, protein kinase A; ROS, reactive oxygen species;

References

1.**Lavigne C, Marette A, Jacques H** 2000 Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. Am J Physiol Endocrinol Metab 278:E491–E500

2.**Jayagopal V, Albertazzi P, Kilpatrick ES, Howarth EM, Jennings PE, Hepburn DA, Atkin SL** 2002 Beneficial effects of soy phytoestrogen intake in postmenopausal women with type 2 diabetes. *Diabetes Care* 25:1709–1714

3.**Mezei O, Banz WJ, Steger RW, Peluso MR, Winters TA, Shay N** 2003 Soy isoflavones exert antidiabetic and hypolipidemic effects through the PPAR pathways in obese Zucker rats and murine RAW 264.7 cells. *J Nutr* 133:1238–1243

4.**Ae Park S, Choi MS, Cho SY, Seo JS, Jung UJ, Kim MJ, Sung MK, Park YB, Lee MK**2006 Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. *Life Sci* 79:1207–1213

5.**Lee JS** 2006 Effects of soy protein and genistein on blood glucose, antioxidant enzyme activities, and lipid profile in streptozotocin-induced diabetic rats. *Life Sci* 79:1578–1584

6.**Cheng SY, Shaw NS, Tsai KS, Chen CY** 2004 The hypoglycemic effects of soy isoflavones on postmenopausal women. *J Womens Health (Larchmt)* 13:1080–1086

7.**Ali AA, Velasquez MT, Hansen CT, Mohamed AI, Bhathena SJ** 2005 Modulation of carbohydrate metabolism and peptide hormones by soybean isoflavones and probiotics in obesity and diabetes. *J Nutr Biochem* 16:693–699

8.**Evans JL, Goldfine ID, Maddux BA, Grodsky GM** 2002 Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* 23:599–622

9.**Haskins K, Bradley B, Powers K, Fadok V, Flores S, Ling X, Pugazhenthi S, Reusch J, Kench J** 2003 Oxidative stress in type 1 diabetes. *Ann NY Acad Sci* 1005:43–54

10.**Robertson RP** 2004 Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet β cells in diabetes. *J Biol Chem* 279:42351–42354

11.**Lankin VZ, Lisina MO, Arzamastseva NE, Konovalova GG, Nedosugova LV, Kaminnyi AI, Tikhaze AK, Ageev FT, Kukharchuk VV, Belenkov YN** 2005 Oxidative stress in atherosclerosis and diabetes. *Bull Exp Biol Med* 140:41–43

12.**Wei H, Wei L, Frenkel K, Bowen R, Barnes S** 1993 Inhibition of tumor promoter-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. *Nutr Cancer* 20:1–12

13.**Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA** 1997Antioxidant activity of phytoestrogenic isoflavones. *Free Radic Res* 26:63–70

14.**Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S** 1995 Bioavailability of soybean isoflavones depends upon gut microflora in women. *J Nutr* 125:2307–2315

15.**King RA, Bursill DB** 1998 Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr* 67:867–872

16.**Santell RC, Chang YC, Nair MG, Helferich WG** 1997 Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J Nutr*127:263–269

17.**Naaz A, Yellayi S, Zakroczymski MA, Bunick D, Doerge DR, Lubahn DB, Helferich WG, Cooke PS** 2003 The soy isoflavone genistein decreases adipose deposition in mice.*Endocrinology* 144:3315–3320

18.**Steinberg FM, Guthrie NL, Villablanca AC, Kumar K, Murray MJ** 2003 Soy protein with isoflavones has favorable effects on endothelial function that are independent of lipid and antioxidant effects in healthy postmenopausal women. *Am J Clin Nutr* 78:123–130

19.**Patel RP, Boersma BJ, Crawford JH, Hogg N, Kirk M, Kalyanaraman B, Parks DA, Barnes S, Darley-Usmar V** 2001 Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging. *Free Radic Biol Med* 31:1570–1581

20.**Chacko BK, Chandler RT, Mundhekar A, Khoo N, Pruitt HM, Kucik DF, Parks DA, Kevil CG, Barnes S, Patel RP** 2005 Revealing anti-inflammatory mechanisms of soy isoflavones by flow: modulation of leukocyte-endothelial cell interactions. Am J Physiol Heart Circ Physiol 289:H908–H915

21.**Tourrel C, Bailbe D, Lacorne M, Meile MJ, Kergoat M, Portha B** 2002 Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the β-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. *Diabetes*51:1443–1452

22.**Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S** 2002 Reduced β-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients. *Diabetologia* 45:85–96

23.**Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A** 2005 Combination therapy with epidermal growth factor and gastrin increases β-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* 54:2596–2601

24.**Dor Y, Brown J, Martinez OI, Melton DA** 2004 Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429:41–46

25.**Stoffers DA** 2004 The development of β-cell mass: recent progress and potential role of GLP-1. *Horm Metab Res* 36:811–821

26.**Sreenan S, Pick AJ, Levisetti M, Baldwin AC, Pugh W, Polonsky KS** 1999 Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse.*Diabetes* 48:989–996

27.**Wang Q, Brubaker PL** 2002 Glucagon-like peptide-1 treatment delays the onset of diabetes in 8-week-old db/db mice. *Diabetologia* 45:1263–1273

28.**Rolin B, Larsen MO, Gotfredsen CF, Deacon CF, Carr RD, Wilken M, Knudsen LB**2002 The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases β-cell mass in diabetic mice. Am J Physiol Endocrinol Metab 283:E745–E752

29.**Ohno T, Kato N, Ishii C, Shimizu M, Ito Y, Tomono S, Kawazu S** 1993 Genistein augments cyclic adenosine 3′5′-monophosphate (cAMP) accumulation and insulin release in MIN6 cells. *Endocr Res* 19:273–285

30.**Sorenson RL, Brelje TC, Roth C** 1994 Effect of tyrosine kinase inhibitors on islets of Langerhans: evidence for tyrosine kinases in the regulation of insulin secretion.*Endocrinology* 134:1975–1978

31.**Jones PM, Persaud SJ** 1994 Tyrosine kinase inhibitors inhibit glucose-stimulated insulin secretion. *Biochem Soc Trans* 22:209S

32.**Persaud SJ, Harris TE, Burns CJ, Jones PM** 1999 Tyrosine kinases play a permissive role in glucose-induced insulin secretion from adult rat islets. *J Mol Endocrinol* 22:19–28

33.**Liu D, Zhen W, Yang Z, Carter JD, Si H, Reynolds KA** 2006 Genistein acutely stimulates insulin secretion in pancreatic β-cells through a cAMP-dependent protein kinase pathway. *Diabetes* 55:1043–1050

34.**Gomez E, Pritchard C, Herbert TP** 2002 cAMP-dependent protein kinase and Ca2+ influx through L-type voltage-gated calcium channels mediate Raf-independent activation of extracellular regulated kinase in response to glucagon-like peptide-1 in pancreatic β-cells. *J Biol Chem* 277:48146–48151

35.**Wang X, Zhou J, Doyle ME, Egan JM** 2001 Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic β-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism.*Endocrinology* 142:1820–1827

36.**Zhou K, Hao J, Griffey C, Chung H, O'Keefe SF, Chen J, Hogan S** 2007 Antioxidant properties of Fusarium head blight-resistant and -susceptible soft red winter wheat grains grown in Virginia. *J Agric Food Chem* 55:3729–3736

37.**Liu D, Iruthayanathan M, Homan LL, Wang Y, Yang L, Wang Y, Dillon JS** 2008Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms. *Endocrinology* 149:889–898

38.**Lum H, Jaffe HA, Schulz IT, Masood A, RayChaudhury A, Green RD** 1999 Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction. Am J Physiol 277:C580–C588

39.**Adams MR, Golden DL, Williams JK, Franke AA, Register TC, Kaplan JR** 2005 Soy protein containing isoflavones reduces the size of atherosclerotic plaques without affecting coronary artery reactivity in adult male monkeys. *J Nutr* 135:2852–2856

40.**Yang Z, Chen M, Fialkow LB, Ellett JD, Wu R, Nadler JL** 2003 The novel anti-inflammatory compound, lisofylline, prevents diabetes in multiple low-dose streptozotocin-treated mice. Pancreas 26:e99–e104

41.**Jia X, Chen Y, Zidichouski J, Zhang J, Sun C, Wang Y** 2008 Co-administration of berberine and plant stanols synergistically reduces plasma cholesterol in rats.*Atherosclerosis* 201:101–107

42.**Thomas BF, Zeisel SH, Busby MG, Hill JM, Mitchell RA, Scheffler NM, Brown SS, Bloeden LT, Dix KJ, Jeffcoat AR** 2001 Quantitative analysis of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine using reversed-phase high-performance liquid chromatography with ultraviolet detection. *J Chromatogr B Biomed Sci Appl* 760:191–205

43.**Si H, Liu D** 2008 Genistein, a soy phytoestrogen, upregulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats. *J Nutr* 138:297–304

44.**Jia Z, Misra BR, Zhu H, Li Y, Misra HP** 2009 Upregulation of cellular glutathione by 3H-1,2-dithiole-3-thione as a possible treatment strategy for protecting against acrolein-induced neurocytotoxicity. *Neurotoxicology* 30:1–9

45.**Xu G, Stoffers DA, Habener JF, Bonner-Weir S** 1999 Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270–2276

46.**Teta M, Long SY, Wartschow LM, Rankin MM, Kushner JA** 2005 Very slow turnover of β-cells in aged adult mice. *Diabetes* 54:2557–2567

47.**Rabinovitch A, Suarez-Pinzon WL, Shapiro AM, Rajotte RV, Power R** 2002 Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 51:638–645

48.**Kushner JA, Ciemerych MA, Sicinska E, Wartschow LM, Teta M, Long SY, Sicinski P, White MF** 2005 Cyclins D2 and D1 are essential for postnatal pancreatic β-cell growth. *Mol Cell Biol* 25:3752–3762

49.**Sepehr E, Robertson P, Gilani GS, Cooke G, Lau BP** 2006 An accurate and reproducible method for the quantitative analysis of isoflavones and their metabolites in rat plasma using liquid chromatography/mass spectrometry combined with photodiode array detection. *J AOAC Int* 89:1158–1167

50.**Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 138:863–870

51.**Liu D, Si H, Reynolds KA, Zhen W, Jia Z, Dillon JS** 2007 Dehydroepiandrosterone protects vascular endothelial cells against apoptosis through a Gαi protein-dependent activation of phosphatidylinositol 3-kinase/Akt and regulation of antiapoptotic Bcl-2 expression. *Endocrinology* 148:3068–3076

52.**Hussain MA, Porras DL, Rowe MH, West JR, Song WJ, Schreiber WE, Wondisford FE** 2006 Increased pancreatic β-cell proliferation mediated by CREB binding protein gene activation. *Mol Cell Biol* 26:7747–7759

53.**Wang RN, Bouwens L, Klöppel G** 1994 β-Cell proliferation in normal and streptozotocin-treated newborn rats: site, dynamics and capacity. *Diabetologia* 37:1088–1096

54. **Tschen SI, Dhawan S, Gurlo T, Bhushan A** 2009 Age-dependent decline in β-cell proliferation restricts the capacity of β-cell regeneration in mice. *Diabetes* 58:1312–1320

55. **Chen H, Gu X, Su IH, Bottino R, Contreras JL, Tarakhovsky A, Kim SK** 2009 Polycomb protein Ezh2 regulates pancreatic β-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev* 23:975–985

56. **Hennige AM, Burks DJ, Ozcan U, Kulkarni RN, Ye J, Park S, Schubert M, Fisher TL, Dow MA, Leshan R, Zakaria M, Mossa-Basha M, White MF** 2003 Upregulation of insulin receptor substrate-2 in pancreatic β cells prevents diabetes. *J Clin Invest* 112:1521–1532

57. **Leiter EH** 1982 Multiple low-dose streptozotocin-induced hyperglycemia and insulitis in C57BL mice: influence of inbred background, sex, and thymus. *Proc Natl Acad Sci USA* 79:630–634

58. **Thomas P, Dong J** 2006 Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol* 102:175–179

59. **Mårtensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grände PO, Owman C, Rosen CJ, Adamo ML, Lundquist I, Rorsman P, Nilsson BO, Ohlsson C, Olde B, Leeb-Lundberg LM** 2009 Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* 150:687–698

60. **Lingohr MK, Dickson LM, McCuaig JF, Hugl SR, Twardzik DR, Rhodes CJ** 2002 Activation of IRS-2-mediated signal transduction by IGF-1, but not TGF-α or EGF, augments pancreatic β-cell proliferation. *Diabetes* 51:966–976

61. **Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF** 2004 Induction of β-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. *Diabetes* 53:149–159

62. **Kim MJ, Kang JH, Park YG, Ryu GR, Ko SH, Jeong IK, Koh KH, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH** 2006 Exendin-4 induction of cyclin D1 expression in INS-1 β-cells: involvement of cAMP-responsive element. *J Endocrinol* 188:623–633

63. **Kim MJ, Maachi M, Debard C, Loizon E, Clément K, Bruckert E, Hainque B, Capeau J, Vidal H, Bastard JP** 2006 Increased adiponectin receptor-1 expression in adipose tissue of impaired glucose-tolerant obese subjects during weight loss. *Eur J Endocrinol* 155:161–165

64.**Costes S, Broca C, Bertrand G, Lajoix AD, Bataille D, Bockaert J, Dalle S** 2006ERK1/2 control phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic β-cell survival. *Diabetes* 55:2220–2230

65.**Patel RP, Moellering D, Murphy-Ullrich J, Jo H, Beckman JS, Darley-Usmar VM** 2000Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. *Free Radic Biol Med* 28:1780–1794

66.**Wang Z, Gleichmann H** 1998 GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. *Diabetes* 47:50–56

67.**Fu Z, Liu D** 2009 Long-term exposure to genistein improves insulin secretory function of pancreatic β-cells. *Eur J Pharmacol* 616:321–327

68.**Kiesel U, Falkenberg FW, Kolb H** 1983 Genetic control of low-dose streptozotocin-induced autoimmune diabetes in mice. *J Immunol* 130:1719–1722