S-Equol Enantioselectively Activates cAMP-Protein Kinase A Signaling and Reduces Alloxan-Induced Cell Death in INS-1 Pancreatic β-Cells

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Summary S-Equol is enantioselectively produced from the isoflavone daidzein by gut microflora and is absorbed by the body. An increase of pancreatic β-cell death is directly associated with defects in insulin secretion and an increased risk of type 2 diabetes mellitus. In the present study, we demonstrate that only the S-enantiomer has suppressive effects against alloxan-induced oxidative stress in INS-1 pancreatic β-cells. S-Equol reduced alloxan-induced cell death in a dose-dependent manner, whereas R-equol had no effects. In contrast, no significant differences were observed between the enantiomers in estrogenic activity. The cytoprotective effects of S-equol were stronger than those of its precursor daidzein and were blocked by the protein synthesis inhibitor cycloheximide. The cytoprotection was diminished when cells were incubated with a protein kinase A (PKA) inhibitor. S-Equol increased intracellular cAMP levels in an enantioselective manner. S-Equol, but not R-equol, induced phosphorylation of cAMP-response element-binding protein at Ser 133, and induced cAMP-response element-mediated transcription, both of which were diminished in the presence of H89. Taken together, these results show that S-equol enantioselectively increases the survival of INS-1 cells presumably through activating PKA signaling. Thus, S-equol might have applications as an anti-type 2 diabetic agent.

Key Words equol, cAMP, protein kinase A signaling, enantioselectivity, pancreatic β-cells

S-Equol (7-hydroxy-3′(4′-hydroxyphenyl)-chroman) is enantioselectively produced from daidzein, a soy isoflavone, by gut microflora and is absorbed by the body (1). S-Equol directly binds to and acts as an agonist for estrogen receptors α and β (ERα and ERβ) more strongly than its precursor daidzein (2–4). The free radical scavenging activity of S-equol is also greater than that of daidzein (5). S-Equol increased intracellular cAMP levels in an enantioselective manner (6). Comparison of the behavior of the S- and R-enantiomers will help to understand the function of S-equol. However, physicochemical differences of the enantiomers have not yet been studied except with respect to their estrogenic properties (3).

Type 2 diabetes mellitus (T2DM) is a major public health problem worldwide. When blood sugar levels are elevated, insulin, which is produced and secreted by pancreatic β-cells, stimulates glucose uptake in peripheral tissues such as skeletal muscle and adipose tissue and suppresses gluconeogenesis in the liver (7). Decreased insulin secretion and insulin sensitivity are associated with abnormally high levels of blood glucose. Reduction of β-cell mass is observed in T2DM patients (8–10) and has been directly linked to defects in insulin secretion (11). Oxidative stress is one of the major causes of β-cell death (12) because the antioxidative capacities of β-cells are quite low compared to those of other tissues (13). Loss of balance between the generation and elimination of reactive oxygen species induces oxidative damage. Alloxan, a cytotoxic glucose analog, causes oxidative-stress-induced β-cell death by being preferentially imported into β-cells through glucose transporter 2 (14), and is thus used to experimentally induce diabetes. Alloxan is also used to examine how β-cells defend against oxidative stress.

Protein kinase A (PKA) signaling plays important roles in β-cell survival (15). PKA signaling is activated by an
increase of intracellular cAMP levels, which stimulates cAMP response element (CRE)-mediated transcription and protects β-cells from oxidative stress-induced cell death (16, 17). In fact, glucagon-like peptide-1 receptor, which is involved in increased cAMP-PKA signaling, is a drug target in T2DM (18). In the present study, we demonstrated that S-equol, but not R-equol, suppressed alloxan-induced β-cell death, presumably through activating PKA signaling.

MATERIALS AND METHODS

Cell culture. INS-1 rat pancreatic β-cells were cultured in RPMI 1640 medium containing 11.1 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM 2-mercaptoethanol supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (19). Cells were maintained at 37°C in a 5% CO₂/95% air atmosphere at 98% humidity. When assays were performed, cells were spread in medium supplemented with 5.5 mM glucose and 2% fetal bovine serum. Steroid-free medium was prepared as described previously (20) and used for analyzing the estrogenic effects.

AlamarBlue cell viability assay. INS-1 cells that had been grown in 48-well plates were preincubated with S-equol, R-equol, and daidzein for 24 h, and then cells were co-incubated with 3 mM alloxan for an additional 24 h. When using inhibitors, cells were preincubated with inhibitors for 30 min prior to S-equol treatment. To quantify the mitochondrial metabolic activity, cells were further incubated in fresh medium containing 5% AlamarBlue (Trek Diagnostic Systems, Cleveland, OH), followed by incubation for a further 4 h under light shielding. Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) was used to measure fluorescence intensity using 544 nm excitation and 590 nm emission wave-lengths. Survival rate was calculated by dividing the fluorescence intensity in the presence of alloxan by the fluorescence intensity in the absence of alloxan.

Plasmids. For construction of p4xCRE-TATA-Luc, annealed oligonucleotides containing four tandem repeats of cAMP response element (CRE, sense: 5′-CTAGC-AGCCTAGCTAGAGAGCTAGCTAGAGCTGTA-GCTCAGAGGCCTAGCTAGAGAG-3′ and antisense: 5′-GATCTTCGCTAGCTAGAGGTCTCTTGCTAGCTAGGC-TCTGAGCTAGGCTCAGGCGCTAGGCT-3′) were inserted into pGL4.14 vector (Promega Corp., Madison, WI). Subsequently, an adenovirus E1b TATA sequence (sense: 5′-AGCTTAGGTATATATAGGAAG-3′ and antisense: 5′-TCTATATATATATACACTAGT-3′) was inserted into the resulting vector. Human ERα expression vector (pCAGGS-ERα) and reporter vector for ER (p3xERE-TATA-Luc) were described previously (20). The native form of human ERβ was expressed by pCAGGS-ERβ which was constructed from pCAGGS-HA-ERβ (21).

Luciferase reporter assay. INS-1 cells that had been cultured in steroid-free medium or medium supplemented with 2% fetal bovine serum and 5.5 mM glucose were used in luciferase reporter assays. To determine ER activities, cells were transiently transfected with pCAGGS-ERα or pCAGGS-ERβ, p3xERE-TATA-Luc, and pGL4.74[hRluc/TK] using GenePORTER reagent (Gene Therapy Systems, San Diego, CA) for 5 h. To determine CRE-mediated transcriptional activities, cells were transiently transfected with p4xCRE-TATA-Luc and pGL4.74[hRluc/TK] using GenePORTER for 5 h. Cells were further incubated in fresh medium for 19 h. After preincubation with 1 μM ICI 182,780 or 3 μM H89 for 30 min, cells were treated with 10 μM S-equol, 10 μM R-equol, 10 μM daidzein, or 10 nM 17β-estradiol (E2) for an additional 24 h. Luciferase reporter activities were determined as described previously (20). Relative light units (RLU) are defined as the relative value of firefly luciferase activity after normalizing to Renilla luciferase activity.

Western blotting. INS-1 cells that had been cultured in the presence of 3 μM H89 for 30 min were incubated with 10 μM S-equol or R-equol for 5 min. Cells were lysed in 50 mm Tris-HCl, pH 7.5, buffer containing 150 mM NaCl, 0.5% Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 10 mM sodium molybdate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin, and sonicated. Cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-cAMP-response element-binding protein (CREB, 48H2, Cell Signaling Technology, Beverly, MA), anti-pCREB (Ser133, Signalway antibody, College Park, MD), and anti-β-actin (137CT26.1.1. Abgent, San Diego, CA) antibodies. After immunoreaction with horse radish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), the immunoreactive bands were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA) and were detected with an LAS4000 imager (Fujifilm, Tokyo, Japan).

Intracellular cAMP levels. INS-1 cells were incubated in the presence of 10 μM S-equol or R-equol for 5 min. Intracellular cAMP levels were measured using ELISA according to the manufacturer’s protocol (Cayman Chemical, Ann Arbor, MI).

Statistical analysis. Data were evaluated by Student’s t-test or one- or two-way analysis of variance with Tukey’s or Dunnett’s post hoc test. Statistical analysis was performed with JMP statistical software version 8.0.1 (SAS Institute, Cary, NC). Data are expressed as means±SD, and statistical differences are indicated when p<0.05.

RESULTS

We examined the effects of S-equol on alloxan-induced pancreatic β-cell death by comparing them with the effects of R-equol. INS-1 β-cells were preincubated in the presence of various concentrations of S-equol or R-equol for 24 h. After incubation with or without alloxan for an additional 24 h, survival rate was determined. S-equol raised the survival rate against alloxan-induced cell death in a dose-dependent manner, whereas R-equol had no effect (Fig. 1A). The effect of S-equol was significant at concentrations above 1 μM.
The cytoprotective effect of S-equol was stronger than that of its precursor daidzein at the same concentration (Fig. 1B). Without preincubation, S-equol did not protect INS-1 cells against alloxan, and the protective effect of S-equol was induced by preincubation for over 6 h (Fig. 2A). The cytoprotective effect of S-equol on alloxan-induced cell death was diminished by the protein synthesis inhibitor cycloheximide (CHX) (Fig. 2B). These results suggest that de novo protein synthesis is necessary for the antioxidative action of S-equol in INS-1 cells.

To determine whether the estrogenic activity of S-equol is responsible for the suppression of alloxan-induced cell death, INS-1 cells were transfected with ERα or ERβ expression vector and luciferase reporter vector, followed by incubation with S-equol, R-equol, or E2. S-Equol, R-equol, and E2 induced ERα and ERβ activities to the same extent (Fig. 3A). These transcriptional activations were completely suppressed by the estrogen receptor antagonist ICI 182,780. In contrast, reduction of alloxan-induced cell death by S-equol was not suppressed by ICI 182,780 (Fig. 3B). In addition, E2 did not show any cytoprotective effects even in the absence of ICI 182,780.

The protective effects of S-equol against alloxan disappeared when INS-1 cells were incubated with H89, an inhibitor of PKA signaling (Fig. 4A). To determine
whether S-equol stimulates PKA signal transduction, we evaluated its effect on the phosphorylation of CREB by PKA. INS-1 cells were exposed to S-equol or R-equol for 5 min, and the cell lysate was analyzed by Western blotting. S-Equol, but not R-equol, increased the phosphorylation of CREB at Ser 133 (Fig. 4B). This effect was diminished when cells were incubated with H89. S-Equol also activated CRE-mediated transcription in an enantioselective manner (Fig. 4C), and this effect was also suppressed by H89. S-Equol stimulated CRE-mediated transcription more strongly than did daidzein (Fig. 4D). Furthermore, cAMP levels were elevated by S-equol but not by R-equol (Fig. 4E).

**DISCUSSION**

The present study shows that S-equol attenuated alloxan-induced INS-1 pancreatic β-cell death. S-Equol induced an increase of intracellular cAMP levels, phosphorylation of CREB at Ser 133, and CRE-mediated transcription in INS-1 cells. Inhibition of PKA signaling by H89 diminished not only the protective effects of S-equol against alloxan-induced cell death but also the phosphorylation of CREB and CRE-mediated transcription. These results are consistent with previous studies that showed that PKA signaling plays an important role in oxidative stress-induced β-cell survival (16, 17). Taken together, our data indicate that S-equol attenuated alloxan-induced β-cell death, presumably by activating PKA signaling. The finding that de novo protein synthesis is required for the cytoprotective effect of S-equol (Fig. 2B) suggests that CRE-mediated gene expression is important for the cytoprotection. A novel finding of this study is that S-equol exerts its biological activities by increasing cAMP levels.

S-Equol protected against alloxan-induced β-cell death in an enantioselective manner. In contrast, S-equol and R-equol enhanced the transcriptional activities of ERα and ERβ to the same extent in INS-1 cells. The abilities of S-equol and R-equol to transactivate ERs seem to depend on the cell type (3), even though S-equol has about 4-fold lower affinity for binding to ERα and about 10-fold higher affinity for binding to ERβ than R-equol in vitro (4). Comparing the properties of enantiomers is essential to understanding their function. So far few studies have compared the properties of S- and R-equol. R-Equol, but not S-equol, suppresses the development of a dimethylbenzanthracene-induced mammary tumor (22), and racemic equol is better than S-equol at preventing DNA damage in breast cancer cells (23). Thus, the present results are the first to show that S-equol possesses enantioselective effects. These findings will help to uncover the characteristics of S-equol.

The cytoprotective function of S-equol in INS-1 cells was observed at concentrations above 1 μM in this study.
S-Equol Action in INS-1 β-Cells

The circulating half-life of S-equol in plasma, approximately 8 h, is comparatively long among polyphenols (24), and about half of the circulating S-equol exists as free aglycone (25). Serum S-equol concentrations are elevated to about 5 μM at 2 h after oral administration of 30 mg S-equol (24). In some Japanese S-equol producers, the S-equol concentration is over 1 μM (26). Therefore, the effective dose observed in this study is physiologically achievable.

There is increasing evidence that soy food has antidiabetic effects. Equol was recently found to have an anti-hyperglycemic effect in T2DM model mice (7), although hyperglycemic effect in T2DM model mice (36) and are believed to result from the estrogenic activity through estrogen receptors alpha and beta. The present results show that S-equol is another soy derivative that might have applications in preventing T2DM through increasing the viability of pancreatic β-cells. So far, soy foods have been found to have beneficial effects against T2DM in Asian populations (32, 33) but not in Caucasian or African populations or the multiethnic population of Hawaii (34, 35). On the other hand, S-equol producers are more common in Asian countries (50–60%), compared with Western countries (25–30%) (1). Together, these results suggest that an individual’s ability to produce S-equol is the key to benefiting from the anti-T2DM effects of soy foods.

Recently, the preventive effects of S-equol against post-menopausal disorders have become widely accepted (36), and are believed to result from the estrogenic action of S-equol. In the present study, we show that S-equol activates PKA signaling in a way that is independent of its estrogenic activity. This work provides new insight into the health benefits of S-equol.

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