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Isoflavones Promote Mitochondrial Biogenesis

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ABSTRACT

Mitochondrial damage is often both the cause and outcome of cell injury resulting from a variety of toxic insults, hypoxia, or trauma. Increasing mitochondrial biogenesis after renal proximal tubular cell (RPTC) injury accelerated the recovery of mitochondrial and cellular functions (*Biochem Biophys Res Commun* **355:**734–739, 2007). However, few pharmacological agents are known to increase mitochondrial biogenesis. We report that daidzein, genistein, biochanin A, formononetin, 3-(2′,4′-dichlorophenyl)-7-hydroxy-4*H*-chromen-4-one (DCHC), 7-hydroxy-4*H*-chromen-4-one (7-C), 4′7-dimethoxyisoflavone (4′,7-D), and 5,7,4′-trimethoxyisoflavone (5,7,4′-T) increased peroxisome proliferator-activated receptor γ coactivator (PGC)-1α expression and resulted in mitochondrial biogenesis as indicated by increased expression of ATP synthase β and ND6, and 1.5-fold increases in respiration and ATP in RPTC. Inhibition of estrogen receptors with ICI182780 (fulvestrant) had no effect on daidzein-induced mitochondrial biogenesis. The isoflavone derivatives showed differential effects on the activation and expression of sirtuin (SIRT)1, a deacetylase and activator of PGC-1α. Daidzein and formononetin induced the expression of SIRT1 in RPTC and the activation of recombinant SIRT1, whereas DCHC and 7-C only induced the activation of recombinant SIRT1. In contrast, genistein, biochanin A, 4′,7-D, and 5,7,4′-T only increased SIRT1 expression in RPTC. We have identified a series of substituted isoflavones that produce mitochondrial biogenesis through PGC1α and increased SIRT1 activity and/or expression, independently of the estrogen receptor. Furthermore, different structural components are responsible for the activities of isoflavones: the hydroxyl group at position 7 is required SIRT1 activation, a hydroxyl group at position 5 blocks SIRT1 activation, and the loss of the phenyl ring at position 3 or the 4′-hydroxy or -methoxy substituent blocks increased SIRT1 expression.

Mitochondrial dysfunction is a common consequence and cause of ischemia/reperfusion, trauma, and drug/toxicant induced organ injury (Dhar-Mascareño et al., 2005). Mitochondrial abnormalities also have been associated with the progression of a variety of pathologies such as multiple sclerosis, Alzheimer's disease, Huntington's disease, and cancer (Kalman and Leist, 2003; Andrews et al., 2005; Baloyannis, 2006; Devi et al., 2006; Kroemer, 2006; Solans et al., 2006). Therefore, developing therapeutics to improve mitochondrial function and/or number is an attractive strategy for preventing cell death, preserving organ function, and treating a number of pathologies (Rasbach and Schnellmann, 2007a).

Peroxisome proliferator-activated receptor γ coactivator (PGC)-1α has been characterized as a master regulator of mitochondrial biogenesis (Puigserver et al., 1998; Vega et al., 2000; Zhou et al., 2006). The overexpression of PGC-1α results in a robust increase in mitochondrial number, cellular respiration, and intracellular ATP concentrations in a variety of cell types (Wu et al., 1999; Lehman et al., 2000; Rasbach and Schnellmann, 2007b).

Decreased PGC-1α expression increases oxidative stress, and it is associated with neurodegeneration, Huntington's disease, renal failure, and congestive heart failure, suggesting that the maintenance of PGC-1α expression and mitochondrial function is critical for organ function and survival (Portilla et al., 2002; Garnier et al., 2003; Cui et al., 2006; St-Pierre et al., 2006). PGC-1α also has been shown to be up-regulated during oxidant injury, and it mediates the recovery of mitochondrial function associated with oxidant-induced mitochondrial autophagy (St-Pierre et al., 2006; Rasbach and Schnellmann, 2007b). Furthermore, up-regulation of PGC-1α expression through the adenoviral delivery of a PGC-1α construct and mitochondrial biogenesis after oxidant

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ABBREVIATIONS: PGC, peroxisome proliferator-activated receptor γ coactivator; MAPK, mitogen-activated protein kinase; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPTC, renal proximal tubular cell(s); PBS, phosphate-buffered saline; DCHC, 3-(2′,4′-dichlorophenyl)-7-hydroxy-4*H*-chromen-4-one; 7-C, 7-hydroxy-4*H*-chromen-4-one; 4′,7-D, 4′7-dimethoxyisoflavone; 5,7,4′-T, 5,7,4′-trimethoxyisoflavone.

injury accelerated the recovery of mitochondrial and cellular functions, suggesting that PGC-1α is an important target for promoting the recovery of organ function after an oxidative insult (Rasbach and Schnellmann, 2007a). Interestingly, mitochondrial biogenesis before oxidant injury was neither cytoprotective nor accelerated the recovery of mitochondrial function (Rasbach and Schnellmann, 2007a).

The regulation of PGC-1α expression is under the control of several signaling pathways involving nitric oxide, calcium/calmodulin-dependent protein kinases, calcineurin A, AMP-activated protein kinase, and p38 MAPK (Barger et al., 2001; Zong et al., 2002; Handschin et al., 2003; Suwa et al., 2003, 2006; Yu et al., 2003; Fan et al., 2004; Ojuka, 2004; Schaeffer et al., 2004; Akimoto et al., 2005; Pelletier et al., 2005; Borniquel et al., 2006; Rasbach and Schnellmann, 2007b). The transcriptional activity of PGC-1α can be increased by post-translational modifications, including p38 MAPK-dependent phosphorylation of PGC-1α to promote the dissociation of the negative regulator p160 myb-binding protein (Knutti et al., 2001; Fan et al., 2004) and subsequent nuclear localization (Puigserver et al., 1998).

In addition to modification by p38 MAPK, PGC-1α maintains a functional interaction with the NAD-dependent deacetylase SIRT1 (Nemoto et al., 2005). SIRT1 deacetylates specific lysine residues on PGC-1α, increasing both PGC-1α expression and activity (Nemoto et al., 2005; Rodgers et al., 2005). Activation of SIRT1 also promotes longevity, in part, by regulating energy expenditure though the regulation of PGC-1α expression during periods of caloric restriction (Corton and Brown-Borg, 2005; Guarente and Picard, 2005; Leibiger and Berggren, 2006). An activator of SIRT1, resveratrol, was recently reported to promote mitochondrial biogenesis and to protect from metabolic disease through the SIRT1-dependent deacetylation of PGC-1α (Lagouge et al., 2006). Therefore, small molecules that increase SIRT1 activity may be useful therapeutically to promote and maintain PGC-1α expression and mitochondrial biogenesis.

Phytoestrogens containing an isoflavone core are commonly derived from a variety of plant tissues. Daidzein, genistein, and their metabolites are the most prevalent phytoestrogens found in soybean, and they are thought to be the chemical entities responsible for the estrogenic effects of soy (Axelson et al., 1984). Daidzein and genistein also are formed in vivo through the conversion of the isoflavones biochanin A and formononetin by intestinal glucosidases (Rowland et al., 2003; Usui, 2006). Although these compounds do possess limited estrogenic activity, isoflavones preferentially bind to ERβ over ERα, limiting their estrogenic activity to tissues expressing ERβ (Kuiper et al., 1997). Similar to resveratrol, both daidzein and genistein have been shown to be protective against metabolic disease by enhancing lipid and glucose metabolism (Ae Park et al., 2006). Furthermore, it has been suggested that some xenoestrogens may effect the mitochondrial gene expression profile (Shioda et al., 2006). However, it has not been determined whether these isoflavones produce mitochondrial biogenesis. It should be noted that very few pharmacological agents initiate mitochondrial biogenesis. We have identified mitochondrial biogenesis as a novel estrogen receptor-independent action of a series of related isoflavones, and we provide evidence that the actions are probably occurring through PGC-1α and increased SIRT1 activity and/or expression.

Materials and Methods

Reagents. ICI182780 (fulvestrant) was obtained from Cell Signaling Technology Inc. (Danvers, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to ATP synthase β subunit, ND6, and GAPDH were purchased from Abcam Inc. (Cambridge, MA), Invitrogen (Carlsbad, CA), and Fitzgerald Antibodies (Concord, MA), respectively. PGC-1α (H300) and COX III antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Myc antibody was purchased from Cell Signaling Technology Inc. All secondary antibodies were purchased from Pierce Chemical (Rockford, IL). Anti-PGC-1 was used at 1:300, whereas all other antibodies were used at 1:1000. Isoflavones were purchased from Sigma-Aldrich. 4′,7-Dimethoxyisoflavone was purchased from Alfa Aesar (Heysham, Lancaser, UK) and 5,7,4′-trimethoxyisoflavone was purchased from Indofine Chemical Company (Hillsboro, NJ).

Isolation and Culture of Renal Proximal Tubules. Female New Zealand White rabbits (2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method, and cells were grown in 35-mm tissue culture dishes under improved conditions as described previously (Nowak and Schnellmann, 1996). The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), 5 ng/ml selenium, 5 μg/ml human transferrin, 10 nM bovine insulin, and 50 μM L-ascorbic acid-2-phosphate were added daily to fresh culture medium.

Confluent RPTC were used for all experiments. RPTC monolayers were treated with various compounds or diluent (dimethyl sulfoxide) for 48 h. Fresh media were added, and compounds were replaced at 24-h intervals.

Basal Oxygen Consumption. RPTC bathed in 37°C culture medium were gently detached from culture dishes with a rubber policeman, and then they were transferred to a 37°C QO_2 chamber 48 h after the initial exposure to the compounds. Basal and uncoupled (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) RPTC QO_2 was measured polarographically using a Clark-type electrode as described previously (Nowak and Schnellmann, 1996).

Adenoviral Overexpression of PGC-1α. PGC-1α was overexpressed in RPTC using adenovirally encoded PGC-1α as described previously (Rasbach and Schnellmann, 2007a,b).

Cell Number. Measurement of monolayer protein content over time was used to estimate cell number. RPTC monolayers were washed with PBS, solubilized in Triton buffer (0.05% Triton X-100, 100 mM Tris-base, and 150 mM NaCl, pH 7.5), and sonicated for 60 s. Protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce Chemical).

Preparation of Cell Lysates and Immunoblot Analysis. After treatment, RPTC were washed twice with PBS without Ca^{2+} and Mg^{2+}, and they were harvested in cell lysis buffer from BioVision (Mountain View, CA). Before immunoprecipitation or immunoblot analysis, all cells were disrupted by sonication for 30 s. Immunoprecipitations were performed by incubating lysates (1 mg of total protein) overnight with protein G-coated agarose beads to preclear nonspecific proteins. Lysates were then incubated with an anti-myc antibody for 12 h followed by a 3-h incubation with protein G-coated agarose beads. Samples were boiled and prepared for electrophoresis. Equal amounts of cellular protein lysates were separated by SDS-polyacrylamide gel electrophoresis, and then they were electrophoretically transferred to nitrocellulose membranes. After treatment with 5% skim milk or bovine serum albumin at 4°C overnight, membranes were incubated with various antibodies for 2 h, and then they were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Bound antibodies were visu-

alized by chemiluminescence detection on an imaging system (Alpha Innotech, San Leandro, CA).

Measurement of ATP. ATP was measured via luciferase-mediated bioluminescence as described previously (Lundin et al., 1986). In brief, RPTC were washed three times with ice-cold PBS and subsequently lysed on ice with Triton/glycylglycine lysis buffer. Homogenates were immediately centrifuged at 14,000*g* for 5 min at 4°C. Supernatants were collected, and they were kept on ice. Each sample was combined with an equal amount of luciferase reagent (ATP Bioluminescence Assay kit CLS II; Roche Diagnostics (Indianapolis, IN), and samples were imaged immediately using an Alpha Innotech imaging system.

Measurement of SIRT1 Activity. SIRT1 activity was measured as described in the SIRT1 Fluorimetric Activity Assay/Drug Discovery kit (BIOMOL Research Laboratories, Plymouth Meeting, PA). In brief, an acetylated peptide fragment derived from p53, known to be deacetylated by SIRT1, fluoresces upon deacetylation. Recombinant SIRT1 was preincubated with potential activators or inhibitors of the enzyme for 10 min. The acetylated p53-based substrate was then added, and the reaction was allowed to proceed for 45 min. The reaction was quenched by the addition of nicotinamide, and fluorescence was measured in the white-bottomed, reduced volume 96-well plate included as part of the kit.

Statistical Analysis. Data are presented as means ± S.E., and they were subjected to one- or two-way analysis of variance as appropriate. Multiple means were compared using Student-Newman-Keuls test, with $p < 0.05$ considered to be a statistically significant difference between values. Renal proximal tubules isolated from an individual rabbit represent a single experiment ($n = 1$). Experiments were performed with two to five plates of cells, and experiments were repeated until an n of at least three was reached.

Results

Daidzein, genistein, biochanin A, formononetin, DCHC, 7-C, 4′,7-D, and 5,7,4′-T are structurally similar, with the differences being 1) the presence of a hydrogen, hydroxyl, or methoxy group at position 5, 2) the presence of a hydroxyl or methoxy group at position 7, 3) the presence of a substituted phenyl ring at position 3, 4) the presence of a hydroxyl or methoxy group at position 4′, and 5) the presence of chlorines at positions 2′ and 4′ (Fig. 1).

Treatment of confluent RPTC with daidzein, genistein, biochanin A, formononetin, DCHC, 7-C, 4′,7-D, or 5,7,4′-T (10 μM) for 48 h increased the expression of PGC-1α and the mitochondrial proteins ATP synthase β and ND6 (Fig. 2). Protein expression was not increased consistently with 24-h exposure times or isoflavone concentrations of 5 μM (data not shown). The up-regulation of the mitochondrial biogenesis regulator PGC-1α and the nuclear-encoded mitochondrial protein ATP synthase β and mitochondrial-encoded ND6 (Fig. 2) reveals that the substituted isoflavones promote the biogenesis of mitochondria.

Cellular respiration was measured to determine whether the increase in mitochondrial protein expression resulted in increased mitochondrial function. Treatment with all substituted isoflavones equally increased basal respiration approximately 1.7-fold and uncoupled respiration approximately 1.5-fold (Fig. 3). All substituted isoflavones also equally increased ATP levels approximately 1.9-fold (Fig. 3). The data

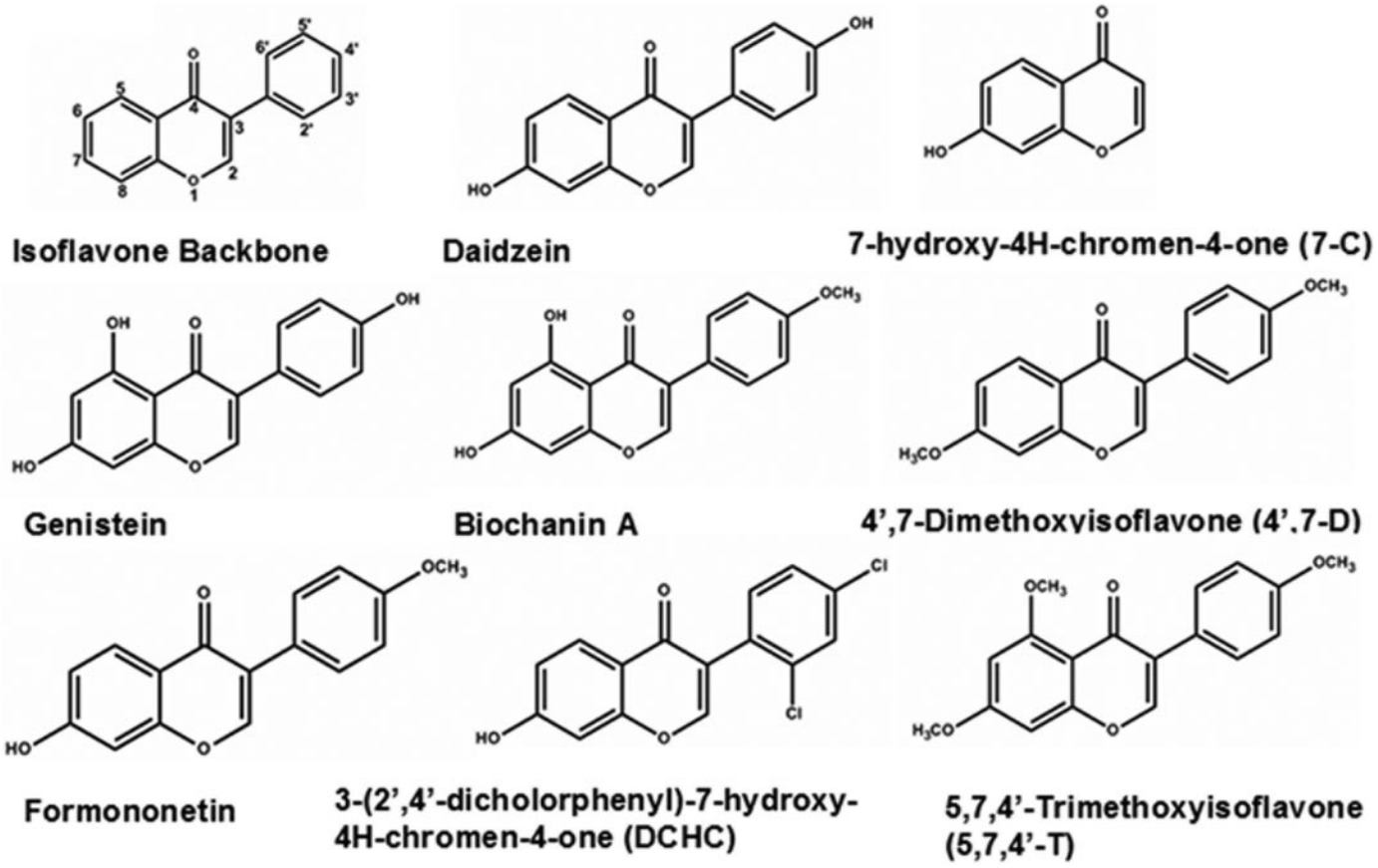

Fig. 1. Structures of mitochondrial biogenesis activators. Structures of labeled isoflavone backbone, daidzein (4′,7-dihydroxyisoflavone), genistein (5,7,4′-trihydroxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), formononetin (7-hydroxy-4′methoxyisoflavone), DCHC, 7-hydroxy-4*H*-chromen-4-one, 4′7-dimethoxyisoflavone, and 5,7,4′-trimethoxyisoflavone.

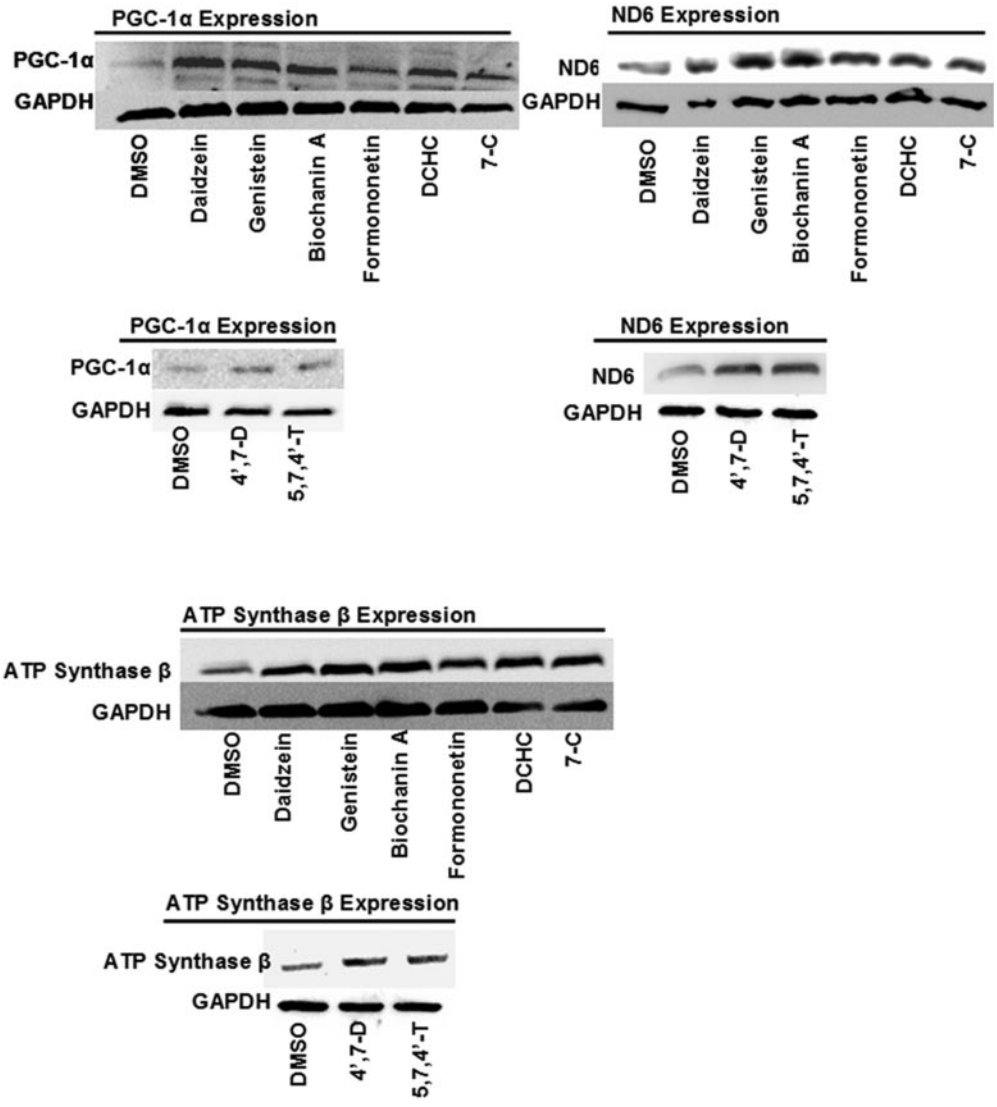

Fig. 2. Substituted isoflavones promote the expression of mitochondrial protein markers. RPTC were treated with 10 μM daidzein, genistein, biochanin A, formononetin, DCHC, 7-C, 4′,7-D, or 5,7,4′-T for 48 h, and then PGC-1α, ATP synthase β, and ND6 levels were determined by immunoblot analysis. GAPDH was used as a loading control. Blots are representative of at least three separate experiments.

reveal that the increased mitochondrial biogenesis produced by these substituted isoflavones also resulted in increased mitochondrial function.

To determine whether isoflavone-induced mitochondrial biogenesis was mediated by the estrogen receptor, RPTC were preincubated with the ERα/ERβ antagonist ICI182780 (1 μM) for 45 min before the addition of daidzein, and the expression of mitochondrial proteins was measured 48 h later. Inhibition of the estrogen receptors did not block the daidzein induced increases in mitochondrial biogenesis (Fig. 4), suggesting that isoflavone-induced mitochondrial biogenesis is independent of the estrogenic properties of this class of compounds.

Mitochondrial biogenesis has been identified as a compensatory response to cellular injury. To determine whether cell number changed during the 48-h exposure to substituted isoflavones, the amount of cellular protein per dish, a marker of cell number, was measured in the presence and absence of daidzein (10 μM) or diluent after 48 h. Monolayer protein content did not change in the presence of daidzein, providing evidence that daidzein is not toxic (data not shown).

The activity of SIRT1 can be increased directly through allosteric interactions with small molecule activators (Borra et al., 2005). Because SIRT1 has been implicated in polyphenol-mediated mitochondrial biogenesis (Lagouge et al., 2006; da-Silva et al., 2007), the effect of the substituted isoflavones on SIRT1 was explored. Daidzein, formononetin, DCHC, and 7-C activated recombinant SIRT1 in a concentration-dependent manner, as demonstrated by increases in the SIRT1-dependent deacetylation of lysine residues contained within a short peptide fragment of human p53 (Fig. 5). In contrast, neither genistein, biochanin A, 7,4-D, nor 5,7,4′-T activated SIRT1 at concentrations up to 100 μM (Fig. 5).

To determine whether molecules capable of increasing SIRT1 activity promote the deacetylation of PGC-1α in RPTC, PGC-1α was overexpressed in RPTC and the cells were treated with 7-C (10 μM) for 48 h. Nicotinamide (2 mM) was included to increase PGC-1α acetylation. Lysine residues in adenovirally overexpressed PGC-1α were deacetylated in RPTC treated with 7-C (Fig. 6).

Because SIRT1 activity is also dependent upon the relative abundance of SIRT1, the effects of the substituted isoflavones on SIRT1 expression were determined. RPTC treated for 48 h with daidzein, genistein, biochanin A, formononetin, 7,4′-D, or 5,7,4′-T increased the expression of SIRT1; how-

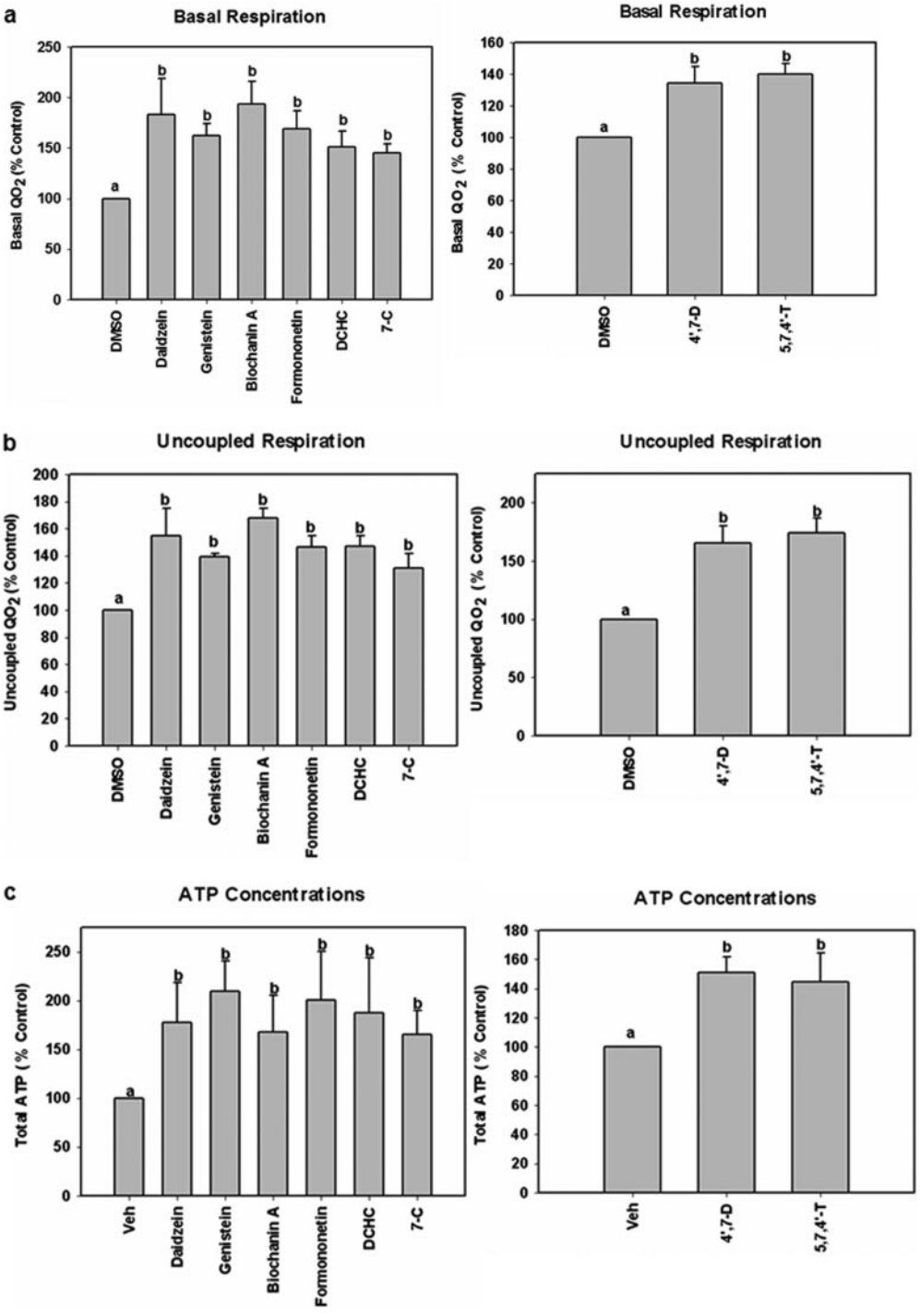

Fig. 3. Treatment with substituted isoflavones increases mitochondrial function. RPTC were treated with 10 μM daidzein, genistein, biochanin A, formononetin, DCHC, 7-C, 4′,7-D, or 5,6,4′-T for 48 h, and basal (a) and uncoupled (b) oxygen consumption (QO_2) and ATP (c) levels were measured at 48 h. Data are presented as means ± S.E., $n = 3$ to 5 for each group. Different subscripts are significantly different from each other; $p < 0.05$.

ever, treatment with DCHC or 7-C did not promote increases in SIRT1 (Fig. 7). These data reveal that distinct structural determinants of the substituted isoflavones regulate SIRT1 through activation and increased expression.

Discussion

Whereas mitochondrial dysfunction has been known to play a significant role in the progression of cell death and organ failure (Toback, 1992; Weinberg et al., 2000; Garnier et al., 2003), very little is known regarding the mechanism underlying the restoration of mitochondrial function associated with recovery from cellular injury (Nowak et al., 1998). Recently, the up-regulation of PGC-1α has been described as a compensatory response to oxidant injury and lipopolysaccharide exposure, and it has been associated with the recovery of mitochondrial function after acute cell injury (Suliman et al., 2004; St-Pierre et al., 2006; Rasbach and Schnellmann, 2007b). In addition, overexpression of PGC-1α subsequent to oxidant injury has been shown to accelerate the recovery of mitochondrial and cellular functions, suggesting that PGC-1α-mediated mitochondrial biogenesis may play a significant role in the recovery from ischemic or toxicant-induced injury (Rasbach and Schnellmann, 2007a).

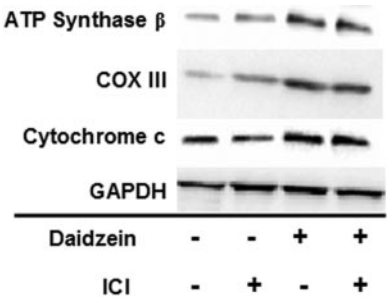

Fig. 4. Isoflavone-induced increases in mitochondrial protein expression are independent of estrogen receptor activation. RPTC were preincubated (45 min) with the estrogen receptor antagonist ICI182780 followed by treatment with 10 μM daidzein for 48 h. ATP synthase β, COX III, and cytochrome c levels were determined by immunoblot analysis. GAPDH was used as a loading control. Blots are representative of at least three representative experiments.

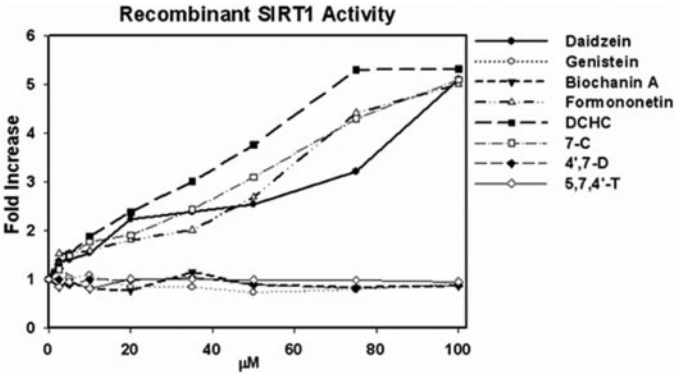

Fig. 5. Substituted isoflavones differentially affect the activity of SIRT1. Treatment with 10 μM daidzein, formononetin, DCHC, and 7-C increases the deacetylation activity of recombinant SIRT1 in a dose-dependent manner, whereas the addition of neither genistein, biochanin A, 4′,7-D, nor 5,7,4′-T increased SIRT1 activity. Data represent the mean of two to three separate experiments.

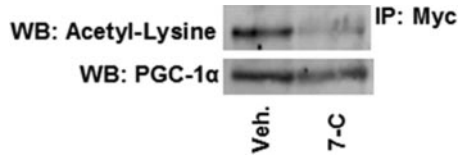

Fig. 6. PGC-1α is deacetylated in the presence of SIRT1 activator 7-C. Treatment of RPTC with the sirtuin deacetylase inhibitor nicotinamide (2 mM) and 7-C (10 μM) for 48 h results in the acetylation of PGC-1α in dimethyl sulfoxide controls; deacetylation is increased by treatment with 7-C.

The SIRT1 member of sirtuin deacetylases has been shown, along with PGC-1α, to mediate the life span extension associated with caloric restriction (Cohen et al., 2004; Chen et al., 2005; Corton and Brown-Borg, 2005; Anastasiou and Krek, 2006). SIRT1 exerts its functions primarily by deacetylating protein substrates, thus altering their activity or function. Recent studies also have shown SIRT1 to interact directly with PGC-1α to mediate gluconeogenesis and mitochondrial biogenesis by deacetylating and activating PGC-1α (Nemoto et al., 2005; Rodgers et al., 2005; Lagouge et al., 2006). The polyphenols resveratrol and kaempferol, both activators of SIRT1, have been shown to increase mitochondrial function and energy expenditure (Howitz et al., 2003; Lagouge et al., 2006; da-Silva et al., 2007).

We have demonstrated that the substituted isoflavones, daidzein, genistein, biochanin A, formononetin, DCHC, 7-C, 7,4′-D, and 5,7,4′-T promote mitochondrial biogenesis by increasing PGC-1α protein expression. The mitochondrial biogenesis is associated with the production of functional mitochondria as demonstrated by increases in basal and uncoupled respiration of RPTC. In addition, exposure to the substituted isoflavones resulted in significant increases in intracellular ATP, suggesting that the increase in mitochondrial biogenesis and respiration is coupled to ATP synthesis. Using an adenovirus-coupled plasmid that results in increased PGC-1α expression, we observed similar increases in mitochondrial biogenesis and mitochondrial function in these cells (Rasbach and Schnellmann, 2007a,b).

Because isoflavones and isoflavone-like compounds possess estrogenic activity, we sought to investigate the role of estrogen receptors in isoflavone-induced mitochondrial biogenesis. Daidzein possesses relatively low estrogenic activity relative to the other phytoestrogens (Kuiper et al., 1997), and it was expected that the observed mitochondrial biogenesis was not mediated by estrogen receptor activation. To test this hypothesis, RPTC were preincubated with the selective estrogen receptor inhibitor ICI182780 before treatment with daidzein. Blockade of ERα/ERβ did not inhibit the daidzein-induced increases in mitochondrial protein expression, suggesting that the increases mitochondrial biogenesis were controlled through another mechanism.

Because increases in SIRT1 expression and/or SIRT1 activity have been associated with increases in mitochondrial function, we examined the effect of isoflavone derivatives on recombinant SIRT1 activity. Daidzein, formononetin, DCHC, and 7-C activated SIRT1 at low micromolar concentrations (5–10 μM), and they continued to increase SIRT1 activity at 100 μM. In contrast, no SIRT1 activation was observed with genistein, biochanin A, 7,4′-D, or 5,7,4′-T. Resveratrol binding to SIRT1 results in a conformational change in the enzyme that promotes an increase in the binding affinity of SIRT1 substrates (Borra et al., 2005); although the binding of the substituted isoflavones to SIRT1 has not been examined, the presence of the 5-hydroxyl group in genistein and biochanin A blocks the ability of these compounds to activate SIRT1, whereas the absence of the 5-hydroxyl group in daidzein and formonenetin promotes SIRT1 deaceylation activity. Substitution of a methoxy group for a hydroxyl group at the 7-position, as seen in 7,4′D and 5,7,5′-T, blocked SIRT1 activation, suggesting that a free hydroxyl group is necessary at the 7-position to promote SIRT1 deacetylase activity. It is interesting to note that the flavone apigenin (5,7,4′-trihydroxyflavone) does increase SIRT1 activity, although it has a hydroxyl group in the 5-position (Howitz et al., 2003). Thus, shifting the phenyl group from the 3-position of isoflavones to the 2-position of flavones decreases the importance of the 5-position, and it allows the activation of SIRT1 in the presence of a hydroxyl group at position 5. Removing the phenyl ring at position 3 while maintaining the hydroxyl group at position 7, compound 7-C, is sufficient to activate SIRT enzymatic activity. This is the first report to identify 7-C as the basic isoflavone pharmacophore necessary to promote the activation of SIRT1 deacetylase activity.

Because neither genistein, biochanin A, 7,4′-D, nor 5,7,4′-T activated recombinant SIRT1, but they still promoted mitochondrial biogenesis, the effect of these compounds on SIRT1 expression was determined. Dadzein, genistein, biochanin A,

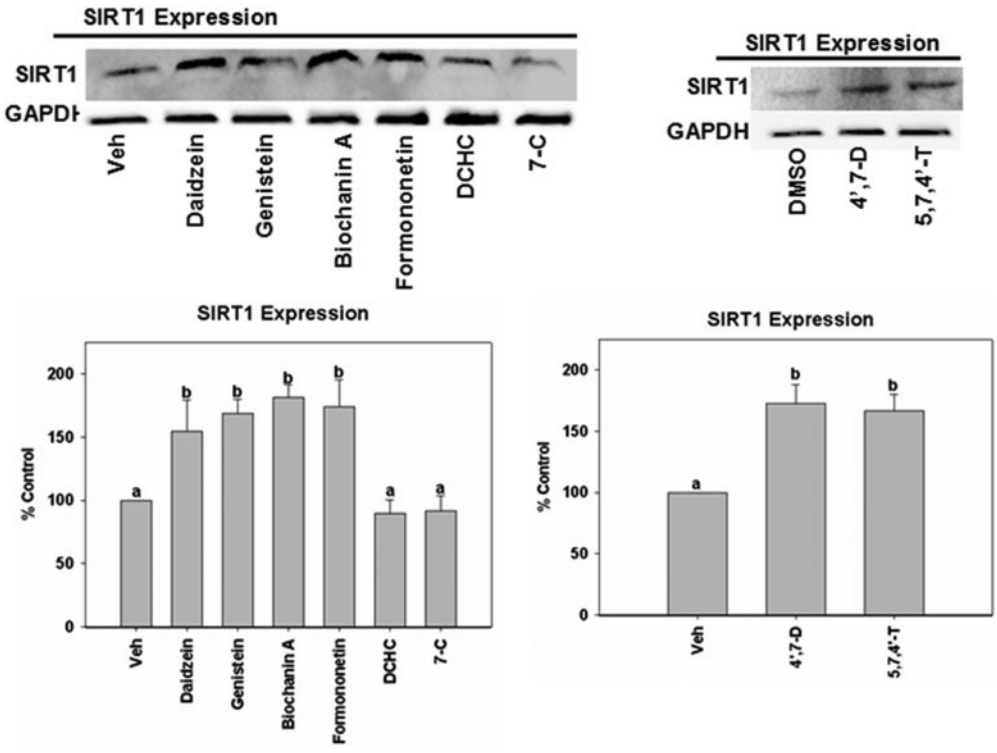

Fig. 7. Substituted isoflavones differentially affect the expression of SIRT1. A 48-h incubation of RPTC with daidzein, genistein, biochanin A, formononetin, 4′,7-D, and 5,7,4′-T promotes the expression of SIRT1, whereas DCHC and 7-C do not induce SIRT1 expression. Data represent the mean of three separate experiments. Different subscripts are significantly different from each other; $p < 0.05$.

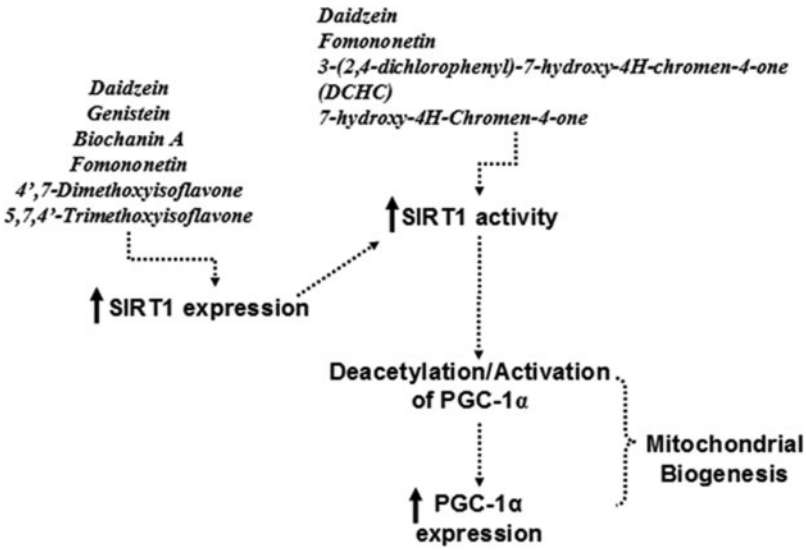

Fig. 8. Increases in SIRT1 activity/expression may promote increases in PGC-1α activity/expression and activate the mitochondrial biogenesis program.

formononetin, 7,4′D, and 5,7,4′-T increased the expression of SIRT1 protein, whereas DCHC and 7-C did not have any effect. We suggest that the phenyl ring at position 3 is required for increased SIRT1 expression. Although it does not matter whether the 4′-position is hydroxylated or methoxylated for increased SIRT1 expression to occur, the substitution of the phenyl ring with chlorines at the 4′- and 6′-position blocked SIRT1 protein expression.

In conclusion, we identified numerous compounds that promote the biogenesis of functional mitochondria, independently of the estrogen receptor, and probably through an SIRT1-PGC-1α-dependent manner (Fig. 8). Furthermore, we have elucidated unique structural components of isoflavone derivatives that promote increases in SIRT1 activity and/or SIRT1 expression. It is interesting to note that compounds that only activate SIRT1 or increase SIRT1 expression increase mitochondrial biogenesis, and they function to the same extent as compounds that both activate and increase the expression of SIRT1. Ultimately, this information may be used to develop and optimize new therapeutics to treat metabolic disorders, cell injury, and conditions associated with mitochondrial dysfunction.

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