

Anabolic Activity of a Soy Extract and Three Major Isoflavones in C2C12 Myotubes

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ABSTRACT

Isoflavones have been reported to stimulate muscle growth. The aim of this *in vitro* study was to examine anabolic activity and associated molecular mechanisms of a soy extract (SoyEx), isoflavone aglycones, and a mixture simulating the composition of SoyEx in C2C12 myotubes. C2C12 cells were differentiated into myotubes. The effects of SoyEx, genistein, daidzein, glycitein, and the mixture of genistein-daidzein-glycitein (Mix) on myotube diameter and number were determined. In addition, the expression of genes and proteins associated with anabolic activity was analyzed. Treatment with SoyEx, genistein, and Mix led to a significant increase of myotube diameter and an increase of the number of myotubes per area compared to the control cell. The increase of diameter by SoyEx was antagonized by an antiestrogen, not by an antiandrogen. Furthermore, gene expressions of insulin growth factor (IGF)-1 and its receptor (IGF-1R), as well as protein expression of myosin heavy chain (MHC), were significantly increased by SoyEx, genistein, and Mix. The effects induced by genistein and Mix were comparable to SoyEx. In conclusion, SoyEx displays an anabolic activity in C2C12 myotubes by binding to ER and modulating IGF-1 and MHC expression. Our studies with isoflavone aglycones and Mix indicate that the isoflavone aglycone with the highest anabolic bioactivity in SoyEx is genistein.

Introduction

Skeletal muscle accounts for the most mass of an individual and daily energy consumption. It is able to respond and adapt to changing environmental stimuli. Endocrine factors influence muscle growth and development throughout life. Increasing evidence shows that menopause is associated with a decline in muscle mass and muscle strength [1], indicating a correlation between muscle mass and estrogen status in females [2]. For these reasons, HRT is regarded as a therapeutic strategy for women who are suffering the loss of muscle mass and muscle function. However, HRT has a complex pattern of risks and benefits [3, 4]. Isoflavones are naturally occurring diphenolic compounds (► **Fig. 1 A**)

and are discussed as an alternative for HRT because of their similar chemical structure to E2. Soy (*Glycine max* [L.] Merr., Leguminosae) is the main source for isoflavones [5]. Aubertin-Leheudre et al. [6] showed that lean body mass and muscle mass of obese-sarcopenic postmenopausal women were significantly increased after supplementation of 70 mg isoflavones per day for 24 wk. Besides genistein, daidzein, and glycitein, which are the principal isoflavones found in soy foods, there are also other chemicals identified in soy products, such as genistin, daidzin, glycitin. However, genistein has been shown to exhibit greater biological activity than its beta-glucoside conjugate genistin [7, 8].

Skeletal muscle is composed of multinucleated fibers that are formed after myoblast differentiation and fusion together. The fi-

ABBREVIATIONS

AR	androgen receptor
DHT	dihydrotestosterone
E2	17 β -estradiol
ER	estrogen receptor
HRT	hormone replacement therapy
IGF	insulin-like growth factor
MHC	myosin heavy chain
Mix	a mixture of genistein-daidzein-glycitein
mTOR	mammalian target of rapamycin
MuRF1	muscle RING finger-containing protein 1
PKB	protein kinase B
PI3K	phosphatidylinositol 3-kinase
SoyEx	a soy extract

bers are analogous to myotubes *in vitro*. C2C12 cells, a myoblast cell line derived from murine satellite cells, have been used extensively as an *in vitro* model to study both muscle differentiation and hypertrophy [9]. Differentiated myotubes of C2C12 cells exhibit a hypertrophic response to growth factors like IGF-1 and E2, characterized by changes of diameters in myotubes and an activation of protein synthesis [9]. Previously, our group demonstrated that ER β is involved in the hypertrophy of C2C12 myotubes induced by the phytoecdysteroid ecdysterone [10]. Another *in vivo* study in our group showed that genistein is able to affect muscle growth through ER β signaling pathway, showing an increased expression of MHC [11]. MHC determines muscle fiber type and contractile speed based on ATPase properties in skeletal muscle. In mammals, several isoforms of MHC proteins are expressed in the sarcomere, displaying distinct contractile properties depending on the composition of muscle fibers. An *in vivo* study showed that MHC composition was shifted from fast to slow MHC isoform in soleus and extensor digitorum longus by ovariectomy in rats [12]. In C2C12 neo-formed myotubes, MHC is a major structural protein and acts as a differentiation marker [13]. Additionally, MyoD, which belongs to a family of proteins known as myogenic regulatory factors, is a protein that plays a major role in regulating muscle differentiation. Moreover, IGF-1 is among the best-characterized factors involved in muscle hypertrophy. IGF-1 directly interacts with its own receptor, IGF-1R, followed by activating the PI3K/Akt pathway [14].

In this study, our major aim was to analyze the effect of SoyEx on hypertrophy of C2C12 myotubes. We compared the biological activity of SoyEx to the activities of its three major isoflavone compositions (genistein, daidzein, glycitein) and Mix simulating the composition in SoyEx. Moreover, the effects of SoyEx and isoflavones on molecular mechanisms related to the hypertrophy of C2C12 myotubes were investigated.

Results

SoyEx contained about 5.3 mg/g genistein, 10.4 mg/g daidzein, and 3.2 mg/g glycitein (data were shown in our previous paper) [15]. Other isoflavone derivatives (i.e., genistin, 6''-O-acetyl-gen-

istin, daidzin, 6''-O-acetyl-daidzin, glycitin, 6''-O-acetyl-glycitin) were also detected (data were shown in our previous paper) [15]. A representative HPLC-UV chromatogram of SoyEx showing the identified isoflavone peaks is illustrated in ► Fig. 1 B.

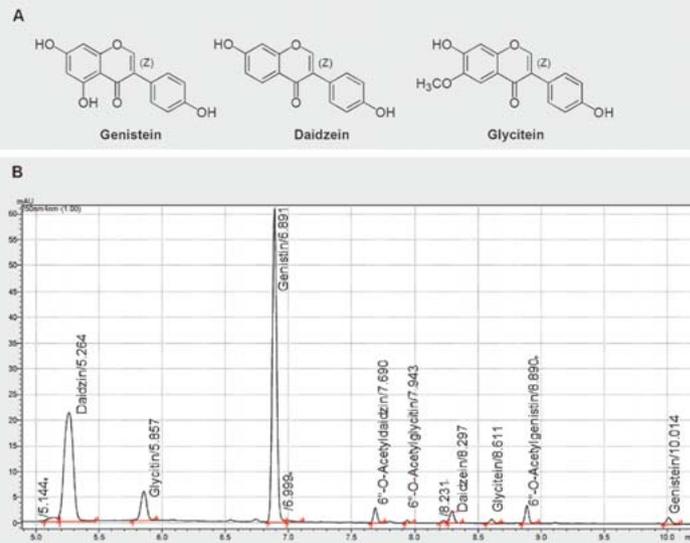
The effect of 48 h treatment of SoyEx on the diameter of myotubes is shown in ► Fig. 2. As shown in ► Fig. 2 A, different dilutions of SoyEx (0.0005–50 μ g/mL) resulted in a bell-shaped dose-response curve, inducing a significant increase in myotube diameter at a concentration of 0.05 μ g/mL. The increase was similar to that induced by E2 at a concentration of 10 nM (► Fig. 2 A, C). Further, the diameters of C2C12 myotubes treated with genistein (0.001 μ M), daidzein (0.0021 μ M), and glycitein (0.0006 μ M), which were at the same concentrations as determined in 0.05 μ g/mL SoyEx, were measured (► Fig. 2 B). The Mix was also composed of genistein, daidzein, and glycitein, which have exactly the same concentrations as in 0.05 μ g/mL SoyEx. Treatment of genistein and Mix led to significant increases of the diameters, which was comparable to the effect induced by SoyEx.

The effect of the respective substances on the number of myotubes in a defined area is shown in ► Fig. 3. ► Fig. 3 A and B show the same area of a six-well plate before and after 48 h treatment with 0.05 μ g/mL of SoyEx. It is clearly visible that the treatment with SoyEx resulted in an increase of the myotube diameter; however, the number of myotubes in this area did not change after 48 h treatment. Comparing the number of myotubes per area among the treatment groups (► Fig. 3 C) revealed treatment with all substances resulted in a significantly higher number of myotubes in the treated groups compared to the control group.

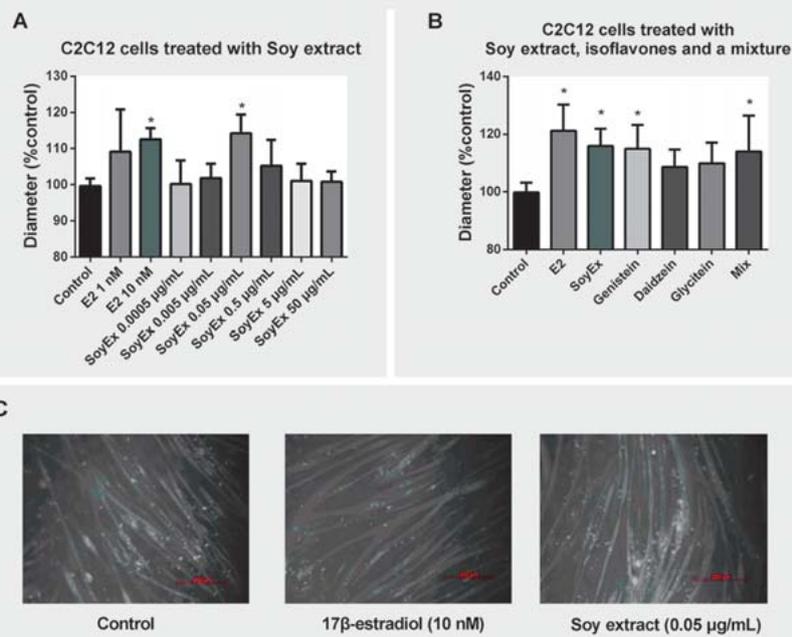
The relevance of ER and AR in the anabolic effect of SoyEx is shown in ► Fig. 4. As shown in ► Fig. 4 A, the increased diameter induced by the treatment of either E2 or SoyEx for 48 h was antagonized by an antiestrogen ZK191703. In contrast, a selective antagonist of AR flutamide significantly antagonized the myotube hypertrophy induced by DHT, whereas it was not able to decrease the diameter when cotreated with SoyEx (► Fig. 4 B).

The mRNA expressions of IGF-1, IGF-1R, and MuRF1 in C2C12 myotubes after 48 h treatment are shown in ► Fig. 5. IGF-1 expression (► Fig. 5 A) was significantly increased by E2, SoyEx, genistein, or Mix. There were no significant differences found between either E2 and SoyEx, between SoyEx and genistein, or between genistein and Mix. Also, IGF-1R expression (► Fig. 5 B) was increased by E2, SoyEx, genistein, or Mix in C2C12 myotubes, which was similar as regulated with IGF-1. However, the expression of MuRF1 (► Fig. 5 C) was not affected by any of the treatments.

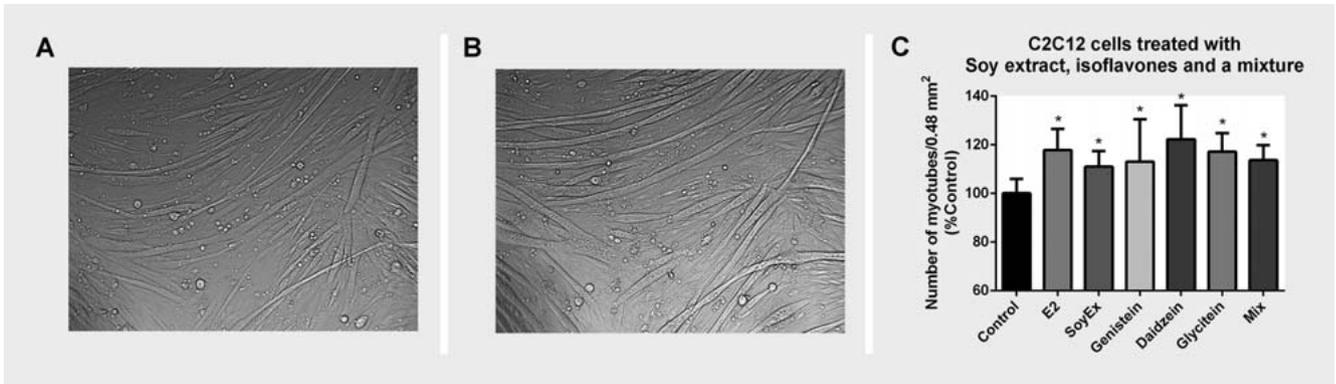
The protein expressions of MHC and MyoD in C2C12 myotubes after 48 h treatment are shown in ► Fig. 6. As shown in ► Fig. 6 A, E2, SoyEx, genistein, daidzein, and Mix significantly increased MHC protein expression and the highest expression was detected in the E2 group. The effect of genistein was similar to SoyEx. Moreover, MyoD protein expression (► Fig. 6 B) was not affected by any of the treatments.



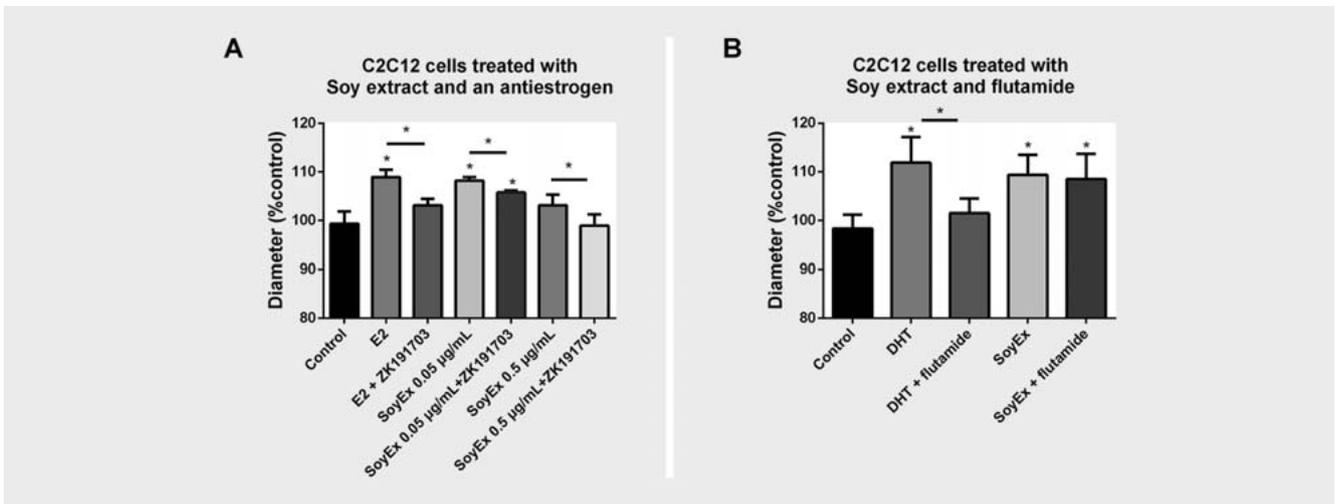
► **Fig. 1** Chemical structure and a detailed chemical characterization SoyEx. A The chemical structure of genistein, daidzein, and glycitein. B A representative HPLC-UV chromatogram (250 nm) of SoyEx showing the identified isoflavone peaks.



► **Fig. 2** Diameter measurement of C2C12 myotubes treated with increasing doses of SoyEx (A) and genistein (0.001 µM), daidzein (0.0021 µM), and glycitein (0.0006 µM) at concentrations the same as determined in 0.05 µg/mL SoyEx, as well as Mix (0.001 µM, 0.0021 µM, 0.0006 µM) (B). C Differences of the diameters in control, E2 (10 nM), and SoyEx (0.05 µg/mL) groups. The diameter of control group was set as 1. * $p \leq 0.05$ vs. control.



► **Fig. 3** Measurement of C2C12 myotube number in specific areas. A, B The same area on a 6-well plate before and 48 h after the treatment with SoyEx (0.05 µg/mL). C The number of myotubes in a 0.48 mm² area after 48 h incubation with the respective substances. E2 = 10 nM, SoyEx = 0.05 µg/mL, genistein = 0.001 µM, daidzein = 0.0021 µM, glycitein = 0.0006 µM, Mix = (0.001 µM, 0.0021 µM, 0.0006 µM). The number of control group was set as 1. * p ≤ 0.05 vs. control.



► **Fig. 4** Investigation of the role of AR (A) and ER (B) in affecting the increases of diameters of C2C12 myotubes that treated with SoyEx. E2 = 10 nM, SoyEx = 0.05 µg/mL, ZK191703 = 1 µM, flutamide = 1 µM. * p ≤ 0.05 vs. control.

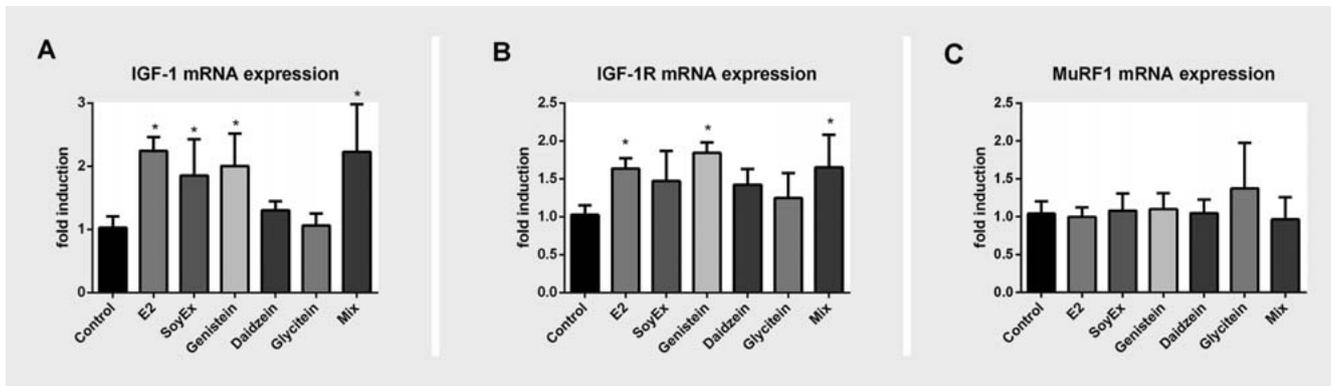
Discussion

Skeletal muscle growth, also known as hypertrophy, is characterized as an increase in muscle mass and myofiber size, which can be induced by anabolic hormonal substances (i.e., DHT and E2) [10]. C2C12 myoblastoma cells differentiated into myotubes is an accepted cell culture model to study anabolic activity [9]. Previously, studies of our group using a model of C2C12 myotubes demonstrated that ecdysterone affects muscle growth via ER-mediated mechanisms [10]. In this study, we could demonstrate a dose-dependent anabolic activity of SoyEx on C2C12 myotubes, which is quantitatively comparable to the effects observed for E2 at 10 nM (► **Fig. 2A**). Information about anabolic effects of SoyEx on skeletal muscle cells is limited. In rat L6 skeletal muscle cells, genistein promoted cells proliferation at doses ≤ 1 µM, which is in agreement with our data – demonstrating a stimulation of protein synthesis in C2C12 myotubes at the same dose [16]. Our data are also

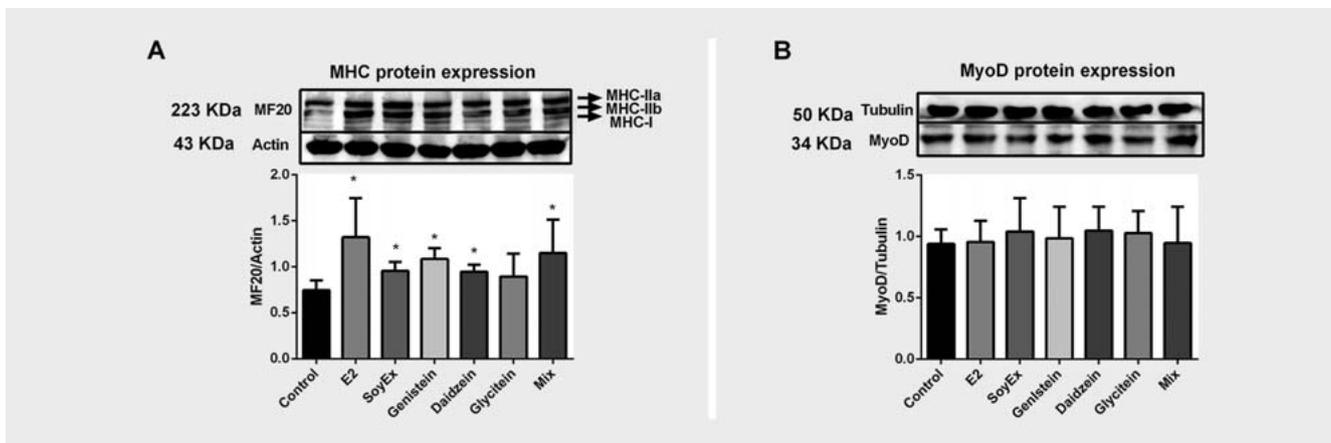
in line with our previous *in vivo* studies demonstrating that isoflavones increased soleus muscle weight in ovariectomized female rats [17, 18].

Interestingly, our investigations also demonstrate that treatment with E2, SoyEx, isoflavones, and Mix resulted in a higher number of myotubes per area compared to the control cells (► **Fig. 3C**). In all groups, the same number of cells was seeded and a comparable number of myotubes was differentiated; therefore, we speculate that in the untreated control group, myotubes may die by mechanisms related to cell death, such as apoptosis and autophagy, but this needs to be investigated in future studies in detail.

To further investigate the molecular mechanisms of soy isoflavone activity and to identify steroid hormone receptors serving as a target for SoyEx action, a co-incubation experiment with SoyEx and the antiestrogen ZK191703, or SoyEx and the antiandrogen flutamide, was performed. It is well known that C2C12 cells ex-



► **Fig. 5** Effects of SoyEx, genistein, daidzein, glycitein, and Mix on relative mRNA expressions of IGF-1 (A), IGF-1R (B), and MuRF1 (C) in C2C12 myotubes. E2 = 10 nM, SoyEx = 0.05 µg/mL, genistein = 0.001 µM, daidzein = 0.0021 µM, glycitein = 0.0006 µM, Mix = (0.001 µM, 0.0021 µM, 0.0006 µM). * $p \leq 0.05$ vs. control.



► **Fig. 6** Effects of SoyEx, genistein, daidzein, glycitein, and Mix on protein expressions of MHC (A) and MyoD (B) in C2C12 myotubes. The MHC isoforms were indicated in the figure based on their molecular weights. E2 = 10 nM, SoyEx = 0.05 µg/mL, genistein = 0.001 µM, daidzein = 0.0021 µM, glycitein = 0.0006 µM, Mix = (0.001 µM, 0.0021 µM, 0.0006 µM). * $p \leq 0.05$ vs. control.

press both AR and ER [19,20]. In ► **Fig. 4**, it is shown that the hypertrophic effect of SoyEx can be antagonized by the antiestrogen but not by the antiandrogen, indicating the anabolic effect of SoyEx is mediated via an interaction with the ER signaling pathway rather than the AR pathway. Some studies pointed out that genistein inhibited cell growth by suppressing AR transcription in prostate cancer cell lines [21]. However, based on our results, as well as those of many other studies that have already shown that genistein binds to ER [22], we conclude that AR is not involved in promoting myotube growth induced by SoyEx.

Genistein, daidzein, and glycitein are the major isoflavone aglycones found, but isoflavone glycosides (genistin, daidzin, glycitin) were also detected in our SoyEx. In the intestine, isoflavone glycosides are first hydrolyzed and converted to corresponding bioactive aglycones [23]. It was found that the aglycones are absorbed faster and in higher amounts than their glycosides after dietary isoflavone intake in humans [24]; therefore, the anabolic effects of isoflavone aglycones and their combinations on C2C12 myotubes were examined. The isoflavone aglycone mixtures contain

the same amounts as analytically determined in 0.05 µg/mL of SoyEx (► **Fig. 2B**). Previously, in a study of our group, Mix did not reach the same potency as SoyEx to stimulate the proliferation of MCF-7 breast cancer cells [15]. However, in this study we found that our isoflavone aglycone mixture simulation SoyEx has a comparable anabolic potency on C2C12 myotubes *in vitro*. Moreover, the results showed that genistein is the most potent aglycone in SoyEx with respect to anabolic activity.

The regulation of skeletal muscle mass depends on the balance between protein synthesis and degradation. The IGF-1/PI3K/Akt/PKB/mTOR pathway acts as a positive regulator of muscle growth [25]. Many studies have reported an important role of IGF-1 in muscle hypertrophy [26]. In addition, muscle-specific inactivation of IGF-1R impairs muscle growth due to reduced muscle fiber number and size [27]. In this study, both IGF-1 and IGF-1R mRNA expressions were detected being upregulated by SoyEx. Meanwhile, genistein increased IGF-1 and IGF-1R expressions, and the effects can be comparable to E2, SoyEx, and Mix, indicating a strong potency of anabolic activity of genistein in soy products

and among the isoflavone aglycones. The mRNA results of IGF-1 and IGF-1R were consistent with our data of hypertrophy. IGF-1 has been reported to stimulate muscle growth by decreasing MuRF1 [28]. MuRF1 is a muscle-specific ubiquitin ligase and regarded as an important regulator for muscle atrophy. In our study, MuRF1 gene expression was not affected by SoyEx or genistein. The explanation could be that muscle hypertrophy is independent of MuRF1 expression, which was demonstrated in another study [29].

MyoD and MHC play important roles during the process of myogenesis in C2C12 cells [30], and they are associated with myostatin expression and localization, which is known as a major negative regulator of muscle growth [31]. Our analysis of the expression of these two proteins in C2C12 myotubes showed that MHC was affected by SoyEx, whereas MyoD was not. A possible explanation is that we analyzed myotubes in which MHC as a structural protein is part of the process of hypertrophy, whereas MyoD, which is mainly expressed during the process of differentiation, is here not of relevance anymore [30]. Several isoforms of MHC proteins are expressed in C2C12 myotubes. Among them, MHC-II is strongly expressed, whereas a relatively small amount of MHC-I is expressed [31]. According to the molecular weights of MHC isoforms indicated in ► Fig. 6A, MHC-IIb might be the isoform modulated in this study. However, this needs to be further confirmed. Our previous study showed that MHC protein expressions of both soleus and gastrocnemius muscles were increased by E2 or genistein in an ovariectomized rat model [11]. In this study, the distinct effects of the compositions in isoflavone aglycones on MHC expression in C2C12 myotubes were also investigated. The MHC expression pattern was similarly regulated as IGF-1 gene expression, which further proved the main role of genistein in SoyEx on stimulation of muscle growth. The effect was mediated by increasing MHC protein expression. Daidzein and glycitein also increased MHC protein expressions; however, the effects were weaker than those of genistein. This result was consistent with the data of IGF-1 mRNA expression and diameter measurement.

In summary, isoflavone aglycones from SoyEx induce anabolic effects on C2C12 myotubes by binding to ER and increasing IGF-1 and MHC expression. Among the tested aglycones, genistein seems to have the strongest anabolic bioactivity. Altogether, this study provides some evidence that SoyEx exerts beneficial effects on muscle growth.

Materials and Methods

Substance and chemicals

SoyEx was obtained from Novasoy 650 (ADM) and stored in the Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut Karlsruhe with the lot number 0601311. Genistein, daidzein, and glycitein (purities $\geq 98\%$) were purchased from LC Laboratories. E2 (purity $\geq 98\%$), DHT (purity $\geq 97.5\%$), and a selective antagonist of AR flutamide (purity $\geq 98\%$) were purchased from Sigma-Aldrich. The antiestrogen ZK191703 (purity $\geq 98\%$) was provided by Bayer Pharma AG. DMSO, EtOH, MeOH (purities $\geq 99.9\%$), and glutaraldehyde (25%) were purchased from Merck.

DMEM and medium components were all purchased from Gibco Life Technologies.

Characterization of isoflavone derivatives in SoyEx

Qualitative and quantitative content of isoflavone derivatives in the purchased SoyEx was measured by LC-DAD analysis and performed as described previously [15,32]. In detail, SoyEx of approximately 50 mg was weighed out accurately and vortexed for 30 s in exactly 40 mL 65% (v/v) MeOH. Next, samples were incubated in an overhead rotation shaker for 1 h at room temperature. The suspensions were centrifuged at 8600 \times g for 5 min and filtered using filters with 15 mm diameter and 0.45 μ m pore size. The filtrates were diluted 50 times with 65% (v/v) MeOH before analysis. The LC-DAD analyses were performed on a Shimadzu LC system equipped with a controller (CBM-20A), a degasser (DGPU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC), and a diode array detector (SPD-M20A). The LC system was controlled by the software LC Solution (version 1.24). Separation of the isoflavone derivatives was performed on a Phenomenex Kinetex PFP column (3.0 mm internal diameter, 100 mm length, 2.6 μ m pore size) with an oven temperature of 35°C. Solvent A was 0.1% (v/v) formic acid in bidest. water, and solvent B was acetonitrile (LC grade). The flow rate was 0.7 mL/min, and the injection volume was 10 μ L. The LC gradient started with an initial period of 3 min at 10% B, increasing linearly to 45% B at 12 min, and finally to 100% at 12.5–15.5 min, re-equilibrating the system in a 7.5 min post-run (10% B). Eluent was monitored between 200 and 500 nm using DAD. Peaks were integrated at 250 nm. To quantify the isoflavone derivatives, external standard calibration curves for each compound in the range of 0.08–50 μ M were prepared. The identity of each compound was confirmed by the retention time and the UV-Vis spectra. The limit of quantification and lowest calibration point was 0.8 pmol on column for all target analytes.

C2C12 hypertrophy cell culture model

C2C12 cells (American Type Culture Collection) were grown in phenol-red-free DMEM supplemented with 10% FBS, 4 nM glutamine, 1.5 g/L sodium bicarbonate, 100 mM sodium pyruvate, and 100 units/mL penicillin/streptomycin. Differentiation toward myotubes was induced at confluence by shifting the proliferation medium to differentiation medium (DMEM with 2% horse serum). During the whole experiment, the cells were stored at atmospheric conditions of 5% CO₂ and 37°C.

Differentiated myotubes were incubated for 48 h in the presence of SoyEx (0.0005–50 μ g/mL), E₂ (1 or 10 nM), ZK191703 (1 μ M), DHT (1 μ M), flutamide (1 μ M), genistein (0.001 μ M), daidzein (0.0021 μ M), glycitein (0.0006 μ M), or control (0.1% DMSO only). For the antagonization study, cells were treated with combinations of E₂ (10 nM) -ZK191703 (1 μ M), SoyEx (0.05 μ g/ml) -ZK191703 (1 μ M), DHT-flutamide (1 μ M each), and SoyEx (0.05 μ g/mL) -flutamide (1 μ M). Moreover, the myotubes were also treated with Mix (0.001 μ M–0.0021 μ M).

Measurement of myotube diameters

Cells were fixed with glutaraldehyde for 1 h immediately after 48 h incubation with the respective substances. Afterward, the myo-

tubes were photographed using a fluorescence microscope (Axiovert 200 M, Zeiss). The glutaraldehyde induced autofluorescence after the treatment of SoyEx, E2, ZK191703, DHT, flutamide, and isoflavones. Myotube diameters were measured every 10–20 μm along the length of the segments of myotubes in the photos and the average data for each myotube were calculated. Minimal 50 myotubes per group of all forms were measured by using the Axiovision LE software.

Measurement of myotube number in defined areas

Before and after treatment with the respective substances, pictures of alive cells were taken with a microscope (Axiovert 200 M, Zeiss). In order to avoid that irregular myotube growth would affect the results, pictures were taken of marked spots before chemical treatment, which could be compared to the pictures of the same spot after the treatment. At the bottom of each well, two dots were drawn with a colored marker. For each group, more than seven pictures were taken. The dots were surrounded by circles in order to find them more easily under the microscope. In the field of view of the microscope, the shadow of the circle and the dot could be detected.

Real-time PCR experiments

Total RNA was extracted from C2C12 myotubes using the standard TRIzol method (Life Technologies). The quality of RNA was checked by agarose gel electrophoresis and the RNA was quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific). cDNA synthesis and real-time PCR was performed as previously described [33]. The PCR program was as follows: 95°C for 3 min for 1 cycle, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, and 1 cycle of 95°C for 1 min, 58°C for 30 s, 95°C for 30 s. Fluorescence was quantified during the 59°C annealing step and product formation was confirmed by melting curve analysis (59–95°C). Relative mRNA amounts of target genes were calculated after normalization to a housekeeping gene (GAPDH). The following oligonucleotide primers (Life Technologies) were used for amplification: GAPDH [34], fwd: 5'-ACCCAGAAGACTGTG-GATGG-3', rev: 5'-TTCAGCTCTGGGATGACCTT-3'; IGF-1, fwd: 5'-AGCTGCAAAGGAGAAGGAAAGGAAG-3', rev: 5'-GGTGATGTGG-CATTTCCTGCT-3'; IGF-1R, fwd: 5'-GGACAACCTGCCCTGATATGC-3', rev: 5'-CTCCATC TCCAGCTCTCTG-3'; MuRF1 [28], fwd: 5'-AGGACAACCTCGTGCCTACAAG-3', rev: 5'-ACAACCTGTGCCGCAAGT G-3'.

Western blotting

Total protein was extracted from C2C12 myotubes using lysis buffer (50 mM Tris pH 8.0, 2 mM CaCl_2 , 80 mM NaCl, 1% Triton-X 100) containing protease inhibitor phenylmethanesulfonyl fluoride (10 mM). Measurement of protein concentration, gel electrophoresis, and protein transfer were performed as previously described [33]. Proteins were detected using primary antibodies of mouse anti-MHC (MF 20, 223 kDa, Developmental Studies Hybridoma Bank), mouse anti-MyoD (D7F2, 34 kDa Developmental Studies Hybridoma Bank), mouse anti-actin (MAB1501, an alpha actin in skeletal muscle, 43 kDa, Millipore GmbH) and mouse anti-alpha-Tubulin (12G10, 50 kDa, Developmental Studies Hybridoma Bank) overnight at 4°C. Afterward, membranes

were incubated for 1 h with peroxidase-conjugated secondary antibodies (rabbit anti-mouse antibody, HRP, Dako). Visualization of the blot signals was obtained by chemiluminescent reaction of the substrate luminol (Lumi-LightPlus Western Blotting Substrate, Roche) and the following detection by a fluorescent detection system (FluorChem, Alpha Innotech). ImageJ software (NIH) was used for densitometric determination of protein expression. Protein expression of MHC and MyoD was respectively related to the reference protein actin, alpha-Tubulin.

Statistical analysis

The substances were compared only to control group or between two groups as indicated in ► Fig. 4. The hypotheses of statistical analysis were not for multiple testing and existed not for all pairwise comparisons. All data were expressed as means \pm standard deviation (SD). Statistical significance of differences was calculated using Kruskal-Wallis test with a subsequent Mann-Whitney U-test (GraphPad Prism version 5). Statistical significance was established at $p \leq 0.05$.

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Conflict of Interest

The authors have declared no conflicts of interest.

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