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Dose-Dependent Effects of Genistein and Daidzein on Protein Metabolism in Porcine Myotube Cultures

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This study was conducted to investigate whether the isoflavones genistein and daidzein, which are components of soy-based diets, and the estrogen 17β-estradiol affect differentiation and protein metabolism of porcine skeletal muscle cells in vitro. Serum-free porcine myotube cultures expressing the estrogen receptors ERα and ERβ were treated with various concentrations of genistein, daidzein, or 17β-estradiol for 26 h. The degree of differentiation by creatine phosphokinase activity was not altered by treatment. At 100 μmol/L both genistein and daidzein caused decreases in protein amount due to cell loss. In addition, 100 μmol/L genistein reduced protein synthesis rate of the surviving cells ($P < 0.05$) measured as [^{3}H]-phenylalanine incorporation. Interestingly, genistein (0.1 μmol/L), daidzein (10, 100 μmol/L), and 17β-estradiol (0.1, 1 nmol/L) slightly reduced protein degradation ($P < 0.05$). The results suggest that both genistein and daidzein affect protein metabolism in a dose-dependent manner and that estrogenic actions may play a role in decreasing protein degradation in porcine skeletal muscle.

KEYWORDS: Isoflavone; differentiation; skeletal muscle; porcine myoblast; estrogen receptor

INTRODUCTION

The role of estrogens and estrogen-like compounds, such as isoflavonic phytoestrogens, in pig skeletal muscle growth is largely unknown. As prominent ingredients of soy foods, isoflavonic phytoestrogens such as genistein and daidzein are resorbed in the gut (*1, 2*). The bioactive compounds are transported by the blood in aglycanic or glycosylated forms and thereby reach other tissues (*1, 3*). Nevertheless, the distribution and tissue concentrations of isoflavones are little investigated hitherto (*4*).

Isoflavones are supposed to exhibit multibiological properties in diverse tissues (*3*). First, they were shown to exert estrogenic or antiestrogenic effects acting as estrogen receptor agonists or antagonists, respectively, and thereby influencing cellular metabolism (*5, 6*). In this context, isoflavones have been discussed to improve growth performance and/or body composition in pigs (*5, 7*−*9*). However, we have recently shown that the isoflavonic phytoestrogens genistein and daidzein at concentrations of >10 μmol/L act as toxins and inhibitors of in vitro porcine myoblast growth (*10*). Second, isoflavonic phytoestrogens, especially genistein, are indicated to have inhibitory effects on protein tyrosine kinases (*5, 11*). Third, genistein has been shown to influence cell cycle progression in various growing cancer cell lines by blocking the cells during the G2/M phase (*12*−*14*) and is therefore a significant inhibitor of in vitro cell growth (*14, 15*). At high concentrations genistein may also induce apoptosis and cause DNA damage by directly influencing topoisomerase II activity (*16, 17*).

To our knowledge, results on the influence of dietary isoflavones on cell growth and metabolism were mostly obtained from proliferating cell cultures. Nonetheless, there are indications from one in vitro experiment using a differentiating rat muscle cell line (L8) suggesting that genistein inhibits myotube formation in a dose-dependent manner (*15*). In addition, genistein decreased protein accumulation due to an inhibition of protein synthesis with a highly pronounced effect at 100 μmol/L, whereas protein degradation was unaffected.

The impact of isoflavones on the differentiation of porcine skeletal muscle and whether this is related to estrogen-like actions has received no attention so far. Therefore, the aim of this study was to investigate the effects of various concentrations of genistein, daidzein, and 17β-estradiol on the in vitro differentiation and protein metabolism of porcine myotube cultures. The applied dosages corresponded to regular isoflavone concentrations of soy-based diets and included isoflavone concentrations that have been measured in blood plasma after the consumption of soy-containing forage crops (*18*).

MATERIALS AND METHODS

Cell Culture. The establishment of a myoblast cell pool from porcine semimembranosus muscle of newborn German Landrace piglets has

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been carried out as described in detail by Mau et al. (*19, 20*). For each individual replicate within the experiments on protein metabolism and differentiation, satellite cells from the obtained pool were seeded in six 24-well plates (BD Biociences, Heidelberg, Germany) coated with growth factor-reduced matrigel (1:50; BD Biosciences, Bedford, MA) at about 5×10^3 cells per well and grown for 96 h in growth medium 1 with DMEM (Invitrogen, Karlsruhe, Germany) plus 10% fetal bovine serum (FBS; Invitrogen) and 10% horse serum (HS; Sigma-Aldrich, Taufkirchen, Germany) until the cells reached 70−80% confluence. Subsequently, the cells were grown to confluence in growth medium 2 with DMEM plus 10% FBS and 1 μmol/L insulin (Sigma-Aldrich) for 24 h. Thereafter, excess serum was washed out and confluent cultures were differentiated during 72 h in serum- and phenol red-free differentiation medium with MEMα (PAN Biotec GmbH, Aidenbach, Germany):MCDB 110 (Sigma-Aldrich) (4:1) plus 1 μmol/L cytosine arabinoside (Sigma-Aldrich) supplemented according to the method of Doumit et al. (*21*). From the second day of cultivation onward medium was changed daily. During the last 26 h of each experiment the myotube cultures were exposed to various concentrations of genistein (0.1, 1, 10, 20, 100 μmol/L), daidzein (0.1, 1, 10, 100 μmol/L), or 17β-estradiol (0.1, 1 nmol/L) or remained untreated (control). Protein synthesis and degradation were measured by the 6 h incorporation or 26 h release, respectively, of L-[2,6-^{3}H]-phenylalanine (56.0 Ci/mM, batch 94, GE Healthcare, Munich, Germany) in separate experiments after the addition of 148 kBq (4 μCi) per well according to the methods of Ballard (*22*) and Harper et al. (*23*) as described previously in detail (*24*). The effects of the substances on protein synthesis or protein degradation were tested in independent experiments with two replicates each using a total of 24 (2×12) wells for each concentration. The effects on the creatine phosphokinase (CPK) activity as a marker of differentiation were tested in another experiment with three replicates using a total of 36 (3×12) wells for each concentration. The protein content per well was measured in all replicates and therefore available for 84 (7×12) wells. To study the estrogen receptor expression, satellite cells from the established pool were seeded at a titer of 2.5×10^4 cells on matrigel-coated 35 mm culture dishes and grown and differentiated over 8 days as described above.

Measurement of Protein Synthesis and Degradation and CPK Activity. To measure protein synthesis the culture supernatants were discarded and the cells were washed with Dulbecco's PBS (pH 7.5; 137 mmol/L NaCl, 2.7 mmol/L KCl, 3.2 mmol/L $Na_2HPO_4 \cdot 2H_2O$, 1 μg/mL phenol red) and two successive aliquots of 5% trichloroacetic acid (TCA). The monolayer cells were then dissolved with 0.4 mL of 0.5 M NaOH for 2 h at 37 °C. The activity of [^{3}H] (dpm; decays per minute) was measured in a suspension of 100 μL of sample in 0.5 M NaOH and 4.5 mL scintillator (Rotiszint eco-plus, Carl Roth GmbH, Hamburg, Germany) using the LKB 1219 Rackbeta Liquid Scintillation Counter (Perkin-Elmer LAS GmbH, Rodgau-Jügesheim, Germany). To determine protein degradation rate additionally 500 μL of the TCA-soluble cell culture supernatant and 200 μL of the insoluble supernatant in 0.5 M NaOH were measured. Protein amount was determined according to the modified method of Peterson (*25*). Protein synthesis was calculated as dpm/μg of protein, and protein degradation was expressed as the percentage of acid-soluble [^{3}H] activity. To measure CPK activity as marker of skeletal myoblast differentiation cells were added 200 μL of citric phosphate buffer (pH 6.75; 0.1 mol/L citric acid, 0.2 mol/L $Na_2HPO_4 \cdot 2H_2O$) and frozen at −70 °C for 30 min, followed by gentle thawing for 15 min at 37 °C. This step was repeated twice, and plates were subsequently centrifuged at 1300*g* (4 °C) for 10 min. According to the manufacturer's instructions 20 μL of each supernatant was mixed with 240 μL of CPK reagent (Pointe Scientific, Inc., Canton, MI) in a 96-well plate. CPK activity was measured as the change in optical density at 340 nm for 30 min at 37 °C against CPK reagent without sample using the Spectramax 250 photometer (Molecular Devices Corp., Sunnyvale, CA). The specific CPK activity was calculated as IU/mg of protein. All chemicals, if not otherwise stated, were obtained from Carl Roth GmbH.

Protein Expression of Estrogen Receptors (ER) α and *β*. The cells were harvested with a rubber policeman in 0.2 mL ice-cold Tris−EDTA−buffer (TE; 25 mmol/L Tris, 2.5 mmol/L EDTA, 1 mmol/L EGTA, 0.25 mol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 5 mmol/L β-glycerophosphate, 0.1% Nonidet P40; pH 7.4) supplemented with proteinase/phosphatase inhibitors (2 mmol/L DTT, 17.4 mg/mL DMSO, 1 mmol/L PMSF, benzamidin, Na_3VO_4, leupeptin, pepstatin) and homogenized for 1 h at 4 °C. After centrifugation (1300*g*, 10 min, 4 °C), the supernatants were collected and 10 μL of each sample was subsequently used to determine the protein amount (*23*). Thereafter, supernatants were precipitated overnight at −20 °C with 4 volumes of acetone. The next day, the samples were thawed, centrifuged (14000*g*, 20 min, 4 °C), and air-dried for 5 min. The protein pellets were added adequate amounts of sample buffer (125 mmol/L Tris, 15% glycerol, 10% β-mercaptoethanol, 5% SDS, 0.5% bromophenol blue; pH 6.8), and 50 μg protein of each sample was loaded onto a 5% stacking gel and a 7.5% resolving gel according to the method of Laemmli (*26*). After running gel electrophoresis for 2−3 h at 125 V, proteins were transferred to a PVDF membrane. After blocking of the membrane with 5% milk-TBST (Tris-buffered saline with 0.1% Tween-20; 20 mmol/L Tris, 0.137 mol/L sodium chloride, 0.1% Tween-20; pH 7.4) for 1 h at room temperature, primary antibodies (monoclonal mouse anti-ERα-IgG C-311, sc-787; Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:500; polyclonal rabbit anti-ERβ-IgG PA1-310B, Affinity Bioreagents, Dianova, Hamburg, 1:500) were added for overnight incubation at 4 °C. After three washings for 10 min in TBST, membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit- or -mouse-IgG−HRP, NIF824, NIF825; GE Healthcare, 1:10000) for 1.5 h at room temperature. Receptor-specific protein bands were detected using the ECL Plus reagent system provided by GE Healthcare. All chemicals, if not otherwise stated, were obtained from Sigma-Aldrich or Carl Roth GmbH.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis. For total RNA preparation, the cells were washed with PBS and treated with a guanidine isothiocyanate containing buffer (lysis buffer provided with the RNeasy fibrous mini kit, Qiagen, Hilden, Germany). The cells were harvested from the dishes using a rubber policeman and additionally disrupted with QIAshredder Homogenizers (Qiagen). Subsequently, an equal volume of 70% ethanol was added to the homogenized lysates, and RNA was extracted with the kit mentioned above, as recommended by the supplier. The procedure includes two steps for degradation of contaminating DNA by treating with RNase-free DNase. RNA was quantified in a GeneQuant II instrument (Pharmacia, Freiburg, Germany). Quality of RNA was monitored from randomly selected samples by denaturing agarose (1%) gel electrophoresis.

RT was carried out with 1 μg of total RNA preparation, 500 nM final concentration of reverse primer (ERα, 5′-CAGATGCTCCATGCCTTTGTTACT; ERβ, 5′-CATCTCCAGCAGCAGGTCGTAG) as previously described (*27*) and Moloney mouse leukemia virus reverse transcriptase (M-MLV RT RNase H Minus Point Mutant; Promega, Mannheim, Germany) in 25 μL of incubation buffer, supplemented with deoxy-NTPs (Roche, Mannheim, Germany) and RNasin (Promega), for 60 min at 42 °C. The freshly synthesized cDNA samples were cleaned with the High Pure PCR Product Purification Kit (Roche) and eluted in 50 μL of elution buffer. Only 5 μL of the dilution was used as template for PCR analysis.

PCR was performed with *Taq* DNA polymerase (Qbiogene, Heidelberg, Germany) in 25 μL of incubation buffer supplemented with deoxy-NTPs and intron-spanning primers according to the method of Pfaffl et al. (*28*) (ERα-forward, 5′-AGGGAAGCTCCTATTTGCTCC; ERα-reverse, 5′-CGGTGGATGTGGTCCTTCTCT; ERβ-forward, 5′-GCTTCGTGGAGCTCAGCCTG; ERβ-reverse, 5′-AGGATCATGGCCTTGACACAGA; 0.5 μM each). All primers were purchased by Sigma-Genosys (Steinheim, Germany). For amplification, the following cycling conditions were performed: preincubation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. After amplification, the lengths of the PCR products (ERα, 234 bp; ERβ, 262 bp) were monitored by agarose gel electrophoresis (3%), and the identity of products generated was controlled by sequencing.

Statistical Analysis. Data of each experimental type (protein synthesis, protein degradation, and CK activity) were checked for normal distribution and subjected to analyses of variance using the

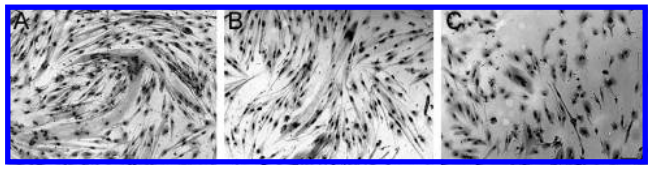

Figure 1. Images of porcine myotube cultures after 8 days of cultivation in growth medium (5 days) and serum-free differentiation medium (3 days) stained by Giemsa dye. (**A**) Myotubes are well exhibited in untreated control cultures and (**B**) after 26 h of exposure to 100 μmol of daidzein (**C**). Cell morphology is untypically changed after 26 h of exposure to 100 μmol of genistein. Bar = 100 μm.

Table 1. Total Protein Amount, Creatine Phosphokinase (CPK) Activity, Protein Synthesis Rate, and Protein Degradation Rate Measured as Incorporation or Release, Respectively, of [2,6-^{3}H]-Phenylalanine in Differentiating Porcine Myotube Cultures after 26 h of Exposure to 17β-Estradiol (E2), Genistein, or Daidzein Compared with Untreated Controls (Least-Squares Means ± SE)

	protein (μg/well)	CPK activity (IU/g of protein)	protein synthesis (dpm/μg of protein)	protein degradation (% dpm acid soluble)
replicates/total *n*	7/79−84	3/31−36	2/22−24	2/22−24
control	33.6 ± 0.48	164 ± 4.9	702.2 ± 14.30	32.0 ± 0.24
E2				
0.1 nmol/L	34.0 ± 0.48	161 ± 4.8	705.4 ± 14.79	30.8 ± 0.24[a]
1 nmol/L	33.8 ± 0.47	158 ± 4.9	710.4 ± 14.53	30.8 ± 0.24[a]
genistein				
0.1 μmol/L	32.6 ± 0.47	163 ± 4.9	701.6 ± 14.53	30.8 ± 0.25[a]
1 μmol/L	32.9 ± 0.47	157 ± 5.1	709.2 ± 14.76	31.3 ± 0.25
10 μmol/L	33.1 ± 0.47	158 ± 4.9	686.5 ± 14.53	31.2 ± 0.25
20 μmol/L	31.9 ± 0.48[a]	155 ± 4.9	709.3 ± 14.78	31.6 ± 0.25
100 μmol/L	30.2 ± 0.48[a]	164 ± 5.1	608.8 ± 14.76[a]	32.1 ± 0.25
daidzein				
0.1 μmol/L	33.4 ± 0.47	167 ± 4.8	687.8 ± 14.30	31.4 ± 0.24
1 μmol/L	32.7 ± 0.47	168 ± 5.0	687.5 ± 14.53	31.1 ± 0.25[b]
10 μmol/L	33.8 ± 0.48	154 ± 4.9	684.4 ± 14.30	30.8 ± 0.24[a]
100 μmol/L	31.8 ± 0.47[a]	163 ± 4.9	666.0 ± 15.32	30.8 ± 0.25[a]

[a] $P < 0.05$. [b] $P = 0.10$ for the difference to untreated control.

MIXED procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC) with replicate and treatment and their interaction as fixed factors and the plate as random factor, if applicable. Data given in the table are least-squares means. Comparisons between least-squares means were performed using the Tukey post hoc test. A value of $P < 0.05$ was set as the limit of statistical significance.

RESULTS

Differentiation and Protein Metabolism. After 8 days of cultivation in growth medium and serum-free differentiation medium, large myotubes were apparent in the cultures derived from porcine satellite cells (**Figure 1A**). The total protein content in the wells was significantly decreased to 90% in response to 26 h of exposure to 20 and 100 μmol/L genistein as well as 100 μmol/L daidzein ($P < 0.05$), which is indicative of cell loss, but remained unchanged with either 17β-estradiol or isoflavones at lower concentrations (**Table 1**). In the case of 100 μmol of genistein, the monolayers showed an untypical morphological appearance in that greater areas of the well were uncovered and the myotubes were shrunk to globular shape (**Figure 1C**). In response to 100 μmol/L daidzein, some uncovered areas were also present, but myotubes were still recognizable as typical myotubes (**Figure 1B**).

The degree of myogenic differentiation in terms of specific CPK activity was not altered in the myotube cultures in response to 26 h of exposure to genistein, daidzein, or 17β-estradiol at any of the applied concentrations, not even at the highest isoflavone concentrations (**Table 1**). However, both isoflavones and/or 17β-estradiol affected protein metabolism in the myotube cultures in a dose-dependent manner. Protein synthesis rate was influenced exclusively by genistein (**Table 1**), which caused a reduction to 87% of the untreated control at a concentration of 100 μmol/L ($P < 0.05$). Daidzein had no significant effects on protein synthesis. Likewise, 17β-estradiol did not affect protein synthesis, neither at physiological (0.1 nmol/L) nor at supraphysiological (1 nmol/L) concentrations. In contrast, protein degradation was slightly reduced by 17β-estradiol (0.1, 1 nmol/L) after 26 h of exposure, and both genistein (0.1 μmol/L) and daidzein (10, 100 μmol/L) caused a decline in protein degradation rate of 2−4% (**Table 1**). Likewise, daidzein at a concentration of 1 μmol/L tended to reduce protein degradation in differentiating porcine skeletal muscle cells ($P = 0.10$). In contrast, 1, 10, 20, and 100 μmol/L genistein or 0.1 μmol/L daidzein did not influence the cellular protein degradation rate.

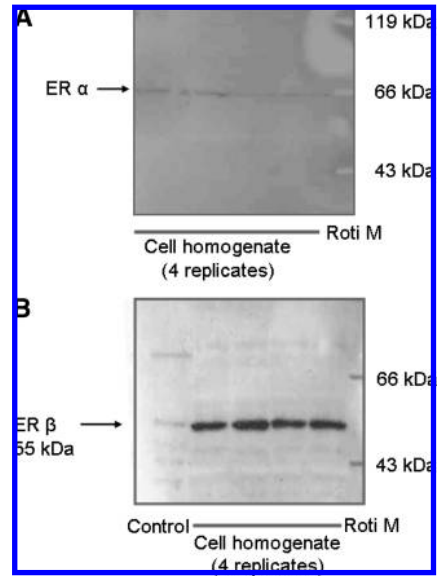

Figure 2. Western blot analysis for estrogen receptor (ER)α and ERβ of cell homogenates from porcine myotube cultures grown and differentiated for 8 days. Extracts were resolved with 50 μg of protein per sample in a 10% SDS-PAGE. (**A**) The major band of ERα has been detected by the mouse monoclonal anti-ERα antibody C-311 as a faint band at 66 kDa. (**B**) The band of ERβ detected with the polyclonal anti-ERβ PA1-310B was visible at 55 kDa comigrating with the main band of the positive control of human cells (HC)-16. The numbers on the right indicate the molecular weights according to the molecular weight standard Roti Mark.

Expression of the Estrogen Receptors α and β. To verify that estrogenic actions by isoflavones or 17β-estradiol can be mediated by the estrogen receptors in our myotube cultures, the expression of the estrogen receptor α (ERα) and estrogen receptor β (ERβ) was measured in untreated control cultures at the protein and transcript level by Western blotting and RT-PCR, respectively. The monoclonal anti-ERα-specific antibody C-311 faintly detected the ERα protein at 66 kDa (**Figure 2A**). Using the polyclonal anti-ERβ-specific antibody PA1-310B, the ERβ protein was detected as a single band of 55 kDa migrating between the 66 and 43 kDa bands of the molecular weight standard, and it comigrated with the 55 kDa band of the HC-16 lysate used as a positive control for the ERβ protein (**Figure 2B**). The presence of ERα- and ERβ-specific mRNA in the porcine myotube cultures is represented by the agarose gel

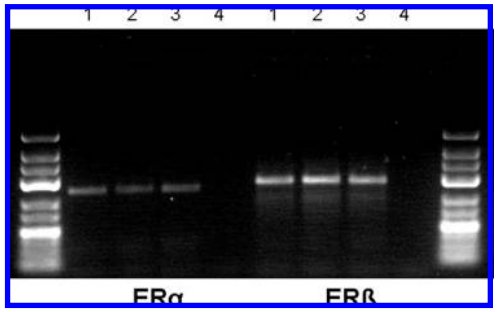

Figure 3. Reverse transcription (RT)-PCR experiment after the amplification of the estrogen receptor (ER)α and ERβ transcripts from porcine myotube cultures grown and differentiated for 8 days. The PCR fragments (ERα 234 bp; ERβ 262 bp) of three differentiation experiments (lanes 1−3) were separated on a 3% agarose gel stained with ethidium bromide. Negative controls (lane 4) were carried with the PCR reactions to exclude contaminations of the PCR reagents. DNA molecular weight marker (Gene ruler low range; Fermentas) was provided in the first and in the last lane.

shown in **Figure 3**. The results on the protein and mRNA expression of ERα and ERβ are identical to those obtained with proliferating myoblasts of the same cell line (*26*).

DISCUSSION

The effects of isoflavones and estrogens on differentiation and protein metabolism of skeletal muscle cells are scarcely investigated and to our knowledge are completely missing for the pig. From the results of our study on the influence of genistein and daidzein on proliferating porcine myoblasts (*10*) we suggested isoflavones to have ambivalent and concentration-dependent effects on porcine skeletal muscle cell differentiation and protein accumulation in vitro. In detail, concentrations of genistein or daidzein achievable in serum after the consumption of typical soy-based human and animal diets could promote myogenic differentiation and/or protein accretion. This would be consistent with in vivo studies showing that daidzein supplementation at typical concentrations to sows increased growth performance of pig fetuses (*29*) or increased lean accretion in male pigs (castrates) during postnatal growth (*7*). On the other hand, we supposed higher isoflavone concentrations (>10 μmol/L) to have detrimental effects on differentiation and protein accretion of porcine skeletal muscle cells.

As a result of this study, specific CPK activity as a marker of muscle cell differentiation was not affected by genistein, daidzein, or 17β-estradiol at any of the concentrations tested, although in the case of 100 μmol/L genistein the morphology of the myotubes appeared untypical. This indicates that neither estrogens nor isoflavones affect the degree of differentiation of porcine skeletal muscle cells under the experimental conditions used in our study. At first glance this is in contrast to findings of Hashimoto et al. (*30*) and Woo et al. (*31*), who reported genistein to reduce myotube formation and transcription of muscle-specific proteins such as myogenin in rodent myoblasts. However, results obtained with L8 rat myoblast cultures (*15*) reveal that a more thorough interpretation of the results concerning the effects of genistein on skeletal muscle differentiation is necessary. Thus, the inhibitory effect of genistein on differentiation seems to depend on the developmental status of the cell culture. When genistein was added to the medium during the first 24 h in low-serum medium to initiate differentiation, myotube formation was clearly reduced, whereas later addition of genistein did not change the degree of myoblast fusion in comparison with the untreated cultures (*15*). In conformity with these findings, neither genistein nor daidzein altered the degree of differentiation (CPK activity) in our cell culture, where the isoflavones have been added 2 days after differentiation had been initiated.

However, for the first time we could show that genistein, daidzein, and 17β-estradiol affect protein metabolism in differentiating porcine skeletal muscle cells in a dose-dependent manner. For example, 100 μmol/L genistein clearly reduced protein synthesis rate (to 87%), which was accompanied by a reduction in total protein amount, whereas neither daidzein nor 17β-estradiol influenced protein synthesis rate. Ji et al. (*15*) showed previously that 24 h of exposure to genistein at a concentration of 10 and 100 μmol/L decreased protein synthesis rate in cultured L8 myoblasts even to ca. 80% and 42% of the control, respectively. In addition, genistein or genistin consistently decreased protein accumulation relative to control cells at 50 μmol/L. Decreases in protein synthesis rate (by 8%) were also found with the supraphysiological (nearly 10000-fold) concentration of 1 μmol/L estradiol benzoate in L6 myotube cultures (*32*). Nevertheless, the basic mechanisms underlying the observed isoflavonic effects on protein synthesis in differentiating rat and porcine myotubes need still to be investigated. We have recently shown that high-concentrated isoflavones disturb transcriptional processes of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) systems in our porcine myoblast and myotube cultures (*33*). High concentrations of estrogens and isoflavones seem to impair basic cellular functions and finally act as toxins. Accordingly, in our study the cell morphology was untypically changed after exposure to 100 μmol/L genistein, and concentrations of 20 and 100 μmol/L genistein and 100 μmol/L daidzein reduced the total protein amount in the myotube cultures. The latter was obviously caused by cell death as has been demonstrated previously for proliferating porcine myoblasts of the same cell line in response to highly concentrated genistein or daidzein (*10*).

Interestingly, genistein (0.1 μmol), daidzein (10 μmol/L), and 17β-estradiol (0.1, 1 nmol/L) reduced protein degradation in the porcine myotube cultures without affecting the total protein amount after 26 h of exposure. Over longer time periods, 17β-estradiol and low concentrations of genistein and daidzein may therefore have the potential to enhance net protein accretion in porcine skeletal muscle by inhibiting protein degradation. Because the estrogen receptors ERα and ERβ were expressed in the differentiating porcine myotube culture with both 17β-estradiol and isoflavones reducing protein degradation, we suggest that isoflavones inhibit protein degradation via an ER-mediated mechanism. Our results are consistent with previous findings (*15*) showing that degradation of total protein in rat myotubes has not been altered in response to genistein at concentrations of 1, 10, and 100 μmol/L, as we observed a decrease in protein degradation by genistein at 0.1 μmol/L only. However, these authors also found that slow myosin degradation tended to be slowed with 1 μmol/L genistein. In summary, genistein and daidzein at low concentrations may act as estrogen receptor agonists in myotubes, thereby inhibiting protein degradation. Daidzein at 100 μmol/L also decreased the protein degradation rate in our myotube cultures. This, however, was accompanied by a reduction in total protein amount, which is most probably due to toxic effects of that high isoflavone concentration. No effects on protein degradation in L6 myotubes were observed using estradiol benzoate at 1 μmol/L (*32*), a concentration that is nonphysiologically high, wherefore these findings are not directly comparable with our results.

In conclusion, the degree of myogenic differentiation in porcine myotube cultures is not affected by estrogens and isoflavones once the initiation of differentiation is finished. However, both estrogens and isoflavones are able to inhibit protein degradation rate in porcine myotubes in a dose-dependent manner. Even though estrogens are commonly not considered to promote muscle growth in pigs (*34*), both 17β-estradiol and low concentrations of genistein and daidzein may have the potential to increase protein accumulation in porcine skeletal muscle by lowering protein degradation via the estrogenic pathway. In this way isoflavones in concentrations achievable in serum with typical soy-based diets may have beneficial effects on protein accretion in porcine skeletal muscle. On the other hand, high isoflavone concentrations (20, 100 μmol/L) are expected to exert detrimental effects on survival and protein metabolism of differentiating porcine muscle cells. Knowledge about these dose-dependently ambivalent actions of the isoflavones on cellular growth and future research on this issue is important in terms of sustainable animal nutrition as worldwide soy covers two-thirds of the protein requirements in animal feed (*35*). Moreover, because the pig is often used as a model for human physiology and metabolism with respect to dietary isoflavones (*36*−*38*), the results of this study are not only significant for applications in animal nutrition but may also serve to evaluate isoflavonic effects of soy-based infant formulas on the development of newborn children, which is worthy of future research.

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