Short communication

A second look at leptin and adiponectin actions on the growth of primary porcine myoblasts under serum-free conditions

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Abstract

Cross-talk between adipose tissue and skeletal muscle may be mediated in part by adipokines. This study was conducted to elucidate further aspects of a possible role of recombinant adiponectin and leptin in the *in vitro* growth of primary porcine skeletal muscle cells cultured in energetically balanced, growth factor-supplemented, serum-free medium (GF-SFM). Therefore, the effects of these adipokines on cell number (DNA content), DNA synthesis rate, cell death and on key intracellular signalling molecules were investigated. Short-term adiponectin and leptin treatment decreased DNA synthesis, measured as [^{3}H]-thymidine incorporation, as early as after 4-h exposure ($P<0.01$), without alterations in DNA content. Both adipokines attenuated the rate of cell death in terms of lactate dehydrogenase (LDH) activity in the culture medium after 48-h treatment ($P<0.05$). The specific activation of p44/42 MAP kinase (MAPK) was reduced ($P<0.05$) after 15-min incubation with either adipokine. In conclusion, the early decreases in DNA synthesis of primary porcine myoblasts cultured in GF-SFM in response to adiponectin or leptin are related to p44/42 MAPK signalling and adipokine treatment does not impair cell viability.

Keywords: adiponectin, leptin, pig, satellite cell culture, signalling molecules

Abbreviations: Akt: protein kinase B; AMPK: AMP-activated protein kinase; bFGF: basic fibroblast growth factor; BSA: bovine serum albumin; DNA: deoxyribonucleic acid; FBS: fetal bovine serum; GF-SFM: growth factor-supplemented, serum-free medium; GM: growth medium; HS: horse serum; LDH: lactate dehydrogenase; MAPK: mitogen-activated protein kinase; MCDB 110: molecular, cellular, and developmental biology medium 110; MEMα: minimum essential medium α; mTOR: mammalian target of rapamycin; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SM: *semimembranosus*

Introduction

Today, the view of adipose tissue as an active endocrine organ by its ability to secrete adipokines is well-established (Miner 2004, Hauner 2005, Gimeno & Klaman 2005). Interactions of adipokines with skeletal muscle have been reported and are of scientific interest as this tissue plays a major role in regulating metabolic homeostasis (Bonen 2010). Furthermore, gaining detailed insight into the functional cross-talk between adipocytes and muscle cells is an important aim not only in animal science (Bonnet *et al.* 2010) but also in human medical research (Fruehbeck 2008). Until now, most of the investigations on adipokines, such as adiponectin and leptin, focused on their function in energy metabolism of several tissues and cell types (Brochu-Gaudreau *et al.* 2010, Moon *et al.* 2013).

Concerning adipokine-mediated regulation of intracellular signalling pathways, the AMP-activated protein kinase (AMPK) was shown to be activated by globular and full-length adiponectin treatment in skeletal muscle of rats and humans (Tomas *et al.* 2002, Tsao *et al.* 2003, Mullen *et al.* 2009). Besides AMPK, other kinases, such as protein kinase B (Akt), mammalian target of rapamycin (mTOR) and/or p44/42 mitogen-activated protein kinase (MAPK), known to be involved in growth-mediating intracellular signalling pathways, can be phosphorylated after adiponectin treatment of murine muscle cell lines (Yamauchi *et al.* 2001, Wang *et al.* 2007, Fiaschi *et al.* 2009, Fiaschi *et al.* 2010) or human embryonic kidney cells (Lee *et al.* 2008). Similarly, leptin was found to affect skeletal muscle growth of rodents and humans via activation of components of the insulin signalling pathway such as Akt and p44/42 MAPK (Maroni *et al.* 2005, Maroni *et al.* 2009) and by the stimulation of AMPK (Minokoshi *et al.* 2002). As adiponectin and leptin can stimulate antagonistic intracellular pathways, it is difficult to predict the effects of these adipokines on muscle cell growth. This becomes even clearer by looking at inconsistent results of a few studies, most of them performed using rodent muscle, reporting about stimulating (Lamosova and Zeman 2001, Ramsay 2003, Yu *et al.* 2008, Fiaschi *et al.* 2010), inhibitory (Arita *et al.* 2002, Wang *et al.* 2005) or a lack of effects (Carbo *et al.* 2000). As previously shown, long-term adiponectin and leptin treatment over 24 or 48 h appeared not to have direct effects on growth and protein metabolism of proliferating porcine myoblasts (Will *et al.* 2012). However, the presence of growth factors in cell culture medium was suggested to be an important factor for the adipokine effects, especially of adiponectin. To elucidate further aspects of adiponectin and leptin action on the growth of porcine skeletal muscle cells under serum-free culture conditions in growth factor-supplemented serum-free medium (GF-SFM), the effects of these adipokines on cell number (DNA content) and DNA synthesis rate after short-term treatment as well as on cell death and the activation of key signalling molecules involved in energy metabolism and cell growth were investigated.

Material and methods

Muscle cell culture

The right and left *semimembranosus* (SM) muscles of newborn German Landrace piglets (n=34 males) were taken to establish a pool from all cells isolated during several preparation procedures. To isolate myogenic cells, a Percoll gradient was used as described previously (Mau *et al.* 2008a). Thereafter, fluorescence analysis of an aliquot of cells immunostained for desmin resulted in 86 % muscle satellite cells in the pool. The flow cytometry step was performed using an EPICS-Elite flow cytometer (Beckman Coulter, Krefeld, Germany) as described before (Mau *et al.* 2008a, Loehrke *et al.* 1998). Satellite cell-derived myoblast cultures were used.

The guidelines set by the Animal Care Committee of the State Mecklenburg-Western Pomerania, Germany, based on the German Law of Animal Protection were observed during animal husbandry and slaughter.

Sources of adiponectin and leptin

For this study, recombinant porcine adiponectin produced in *E. coli* was used (Ledoux *et al.* 2006). The detailed process of its obtaining was already described by Will *et al.* (2012) and its biological activity was shown in a former study by inducing periovulatory changes in ovarian follicular cells (Ledoux *et al.* 2006). Recombinant porcine leptin produced in *E. coli* was obtained from ProSpec (East Brunswick, NJ, USA). According to the manufacturer, porcine leptin was proved to be biologically active by causing proliferation of BA/F3 cells (murine bone marrow-derived pro-B cell line) stably transfected with the long form of human leptin receptor.

Experiments on DNA synthesis rate

For the experiments on DNA synthesis rate (Table 1), gelatin-coated (0.1 %) 96-well microplates were used. After cautious thawing, satellite cells were seeded at a density of about 5×10^3 cells per well and cultured for two days in minimum essential medium α (MEMα) and molecular, cellular and developmental biology medium 110 (MCDB 110; ratio 4:1) (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Karlsruhe, Germany) and 10 % horse serum (HS; Sigma-Aldrich) plus glutamine (4 mM; Serva, Heidelberg, Germany) and antibiotics (2.5 µg/ml amphotericin B; 100 IU/ml penicillin; 100 µg/ml streptomycin; Sigma-Aldrich) as described in former studies (growth medium, GM; Will *et al.* 2012). On day 3, cells were incubated for 24 h with GF-SFM, a serum-free culture medium supplemented with insulin (1 µM), basic fibroblast growth factor (bFGF; 10 ng/ml), platelet-derived growth factor (PDGF-BB; 5 ng/ml), bovine serum albumin (BSA; 0.5 mg/ml), dexamethasone (0.1 µM), linoleic acid (0.5 µg/ml) and transferrin (100 µg/ml) (Sigma-Aldrich or Invitrogen) according to Doumit *et al.* (1996) before being treated with adiponectin (40 µg/ml) or leptin (20 ng/ml) for 4 h. Cells were labelled with 0.2 µCi [^{3}H]-thymidine (60-90 Ci/mM, batch 123716; Hartmann Analytic, Braunschweig, Germany) during the entire period (4 h) of treatment. As described previously (Rehfeldt & Walther 1997, Will *et al.* 2012), DNA synthesis, as the incorporation of [^{3}H]-thymidine into cellular DNA, and DNA content could be measured in combined assays in monolayers.

Table 1
Design of cell culture experiments on adipokine effects on cellular growth, signalling and cell viability of porcine myoblasts

Experiment	Time of cultivation (d) in		Treatment in GF-SFM		
	GM	GF-SFM	Duration (h)	n	Treatment
DNA synthesis	2	1	4	10	40 µg/ml adiponectin,
Signalling molecules	2	1	0.25, 0.5	3	or 20 ng/ml leptin,
Cell viability	2	0	10, 24, 48	10	or untreated control

GM: growth medium (MEMalpha:MCDB+10 % FBS+10 % HS),   GF-SFM: growth factor-supplemented, serum-free medium

Specific activation of key intracellular signalling molecules

Experiments
For the investigation of the phosphorylation level of AMPKα, Akt, mTOR and p44/42 MAPK in proliferating myoblasts after short-term treatment with adiponectin or leptin (Table 1), satellite cells were seeded in gelatin-coated (0.1 %) 35 mm dishes at a density of 4×10^5 cells per dish. Cells were grown for two days in MEMα and MCDB 110 (ratio 4:1) plus 10 % FBS and 10 % HS plus glutamine and antibiotics as described above. Thereafter, cells were grown in GF-SFM for 24 h. On day 4 of the experiments, cells were treated with adipokines at the same concentrations used for experiments on DNA synthesis rate and cell viability (20 ng/ml leptin, 40 µg/ml adiponectin) for 15 or 30 min.

Protein extraction, SDS-PAGE and Western Blot
The total protein lysate of the cells was extracted and supernatants were collected as described previously (Will *et al.* 2012). After determining the protein content using a method described by Peterson (1977), aliquots were stored at −80 °C until further analysis. After thawing, the preparation of protein aliquots for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (Will *et al.* 2012). Before being separated on 10 % SDS-PAGE gels (2-3 h at 125 V), fifty micrograms of protein samples contained in sample buffer (125 mM Tris-HCl, pH 6.8, 4 % SDS, 20 % glycerol, 0.05 % bromophenol blue, 10 % β-mercaptoethanol) were heated for 5 min at 95 °C. After electrophoresis, proteins were transferred to polyvinylidine fluoride (PVDF) membranes (GE Healthcare Europe GmbH, Munich, Germany; 140 mA for 2 blots, 10 V, 1.5 h). Coomassie blue staining of membranes was used to ensure the efficient protein transfer to the membranes. To verify equal loading of the gels, α tubulin was detected using a mouse monoclonal anti-α tubulin antibody (1:5 000; T5168, Clone B-5-1-2, Sigma-Aldrich). After blocking of the membranes with a solution of 5 % dry milk and Tris-buffered saline for 1 h at room temperature, the incubation with primary antibodies (5 % dry milk and Tris-buffered saline with 0.1 % Tween 20; TBST) was performed overnight at 4 °C. The phosphorylation levels of AMPK (on Thr172, rabbit mAb no. 2535), Akt (on Ser473, rabbit mAb no. 4060), mTOR (on Ser2448, rabbit mAb no. 2971) and p44/42 MAPK (on Thr202/Tyr204, rabbit mAb no. 4370) were studied. With the exception of Akt and pAkt antibodies, which were used at dilutions of 1:2 000 and 1:4 000, respectively, all other antibodies were diluted as recommended by the manufacturer. Additionally, all membranes were incubated with antibodies specific for

the core proteins (AMPKα no. 2603, Akt no. 9272, mTOR no. 2983, p44/42 MAPK no. 4695). All used antibodies were purchased from Cell Signaling Technology (New England Biolabs, Frankfurt, Germany). After treatment with the primary antibody, membranes were washed three times for 10 min in TBST followed by incubation with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:5 000; sc-2313; Santa Cruz Biotechnology) or, for α tubulin with HRP-conjugated sheep anti-mouse IgG (1:10 000 or 1:25 000; NA931-100 µl, GE Healthcare), for 1.5 h at room temperature. For the development of the membranes, the ECL Plus Western Blotting Detection System (RPN 2132; GE Healthcare) and a Chemocam HR-16 imager (INTAS, Göttingen, Germany) were used. The LabImage 1D Software (Kapelan Bio-Imaging GmbH, Leipzig, Germany) was utilized to quantify the intensities of the gained protein bands. According to Rehfeldt *et al.* (2010), signals derived from phosphospecific antibodies were normalized for the expression of their respective core protein to estimate the specific phosphorylation level of the investigated signalling molecules. The analyses were done in triplicates for each protein.

Cell viability
For the experiments on adipokine-mediated effects on cell viability (Table 1), satellite cells were cultured in GF-SFM in 96-well plates as described above. The rates of cell death were determined by measuring lactate dehydrogenase (LDH) activity in cell culture supernatants, as described previously (Mau *et al.* 2008b), after 10-, 24- and 48-h exposure to leptin (20 ng/ml) or adiponectin (40 µg/ml).

Statistics
Analysis of variance was performed using the GLM procedure of SAS (Version 9.2; SAS Institute Inc., Cary, NC, USA) with experiment, treatment and respective interactions and side of the plate as fixed factors for experiments on DNA synthesis rate and DNA content. The considered fixed factors for analysis of LDH activity were treatment and side of the plate. Treatment was the fixed factor for the analysis of the data on specific activation of signalling molecules. Values of $P<0.05$ and $0.05<P<0.10$ were considered to be statistically significant and as a tendency, respectively.

Results

Effects of adiponectin and leptin on DNA synthesis rate and
*cell viability of porcine skeletal muscle cultures*

In addition to 24-h and 48-h exposure to the adipokines (Will *et al.* 2012), the effects of adiponectin or leptin were examined in proliferating porcine myoblast cultures after short-term exposure (4 h) in GF-SFM. Both the addition of adiponectin and leptin recombinant proteins led to a significant decrease of DNA synthesis rate (Figure 1A), while the DNA content remained unchanged (Figure 1B). The effects of adiponectin and leptin on cell viability were examined after 10-, 24-, and 48-h exposure in GF-SFM. Treatment of cells with adiponectin or leptin for 48 h decreased LDH activity to approximately 76 % of the control value (Table 2) indicating that myoblast viability was somewhat increased by either adipokine.
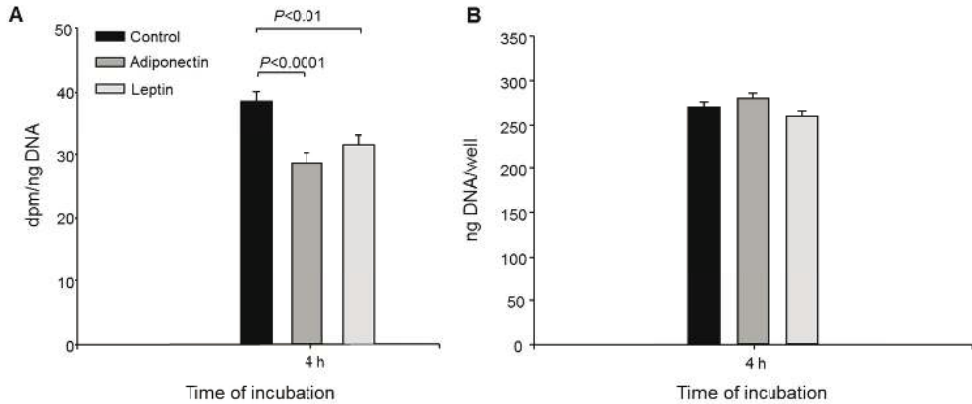
Columns and error bars represent least squares means and SE of dpm/ng DNA and ng DNA/well. The number of observations per treatment and time point was n=10.

Figure 1

DNA synthesis rate (A) and DNA content (B) - taken as equivalent for cell number - of porcine myoblasts measured as incorporation of [³H]-thymidine over 4 h in response to a 4 h exposure to recombinant porcine adiponectin (40 µg/ml) or leptin (20 ng/ml) in growth factor-supplemented, serum-free culture medium
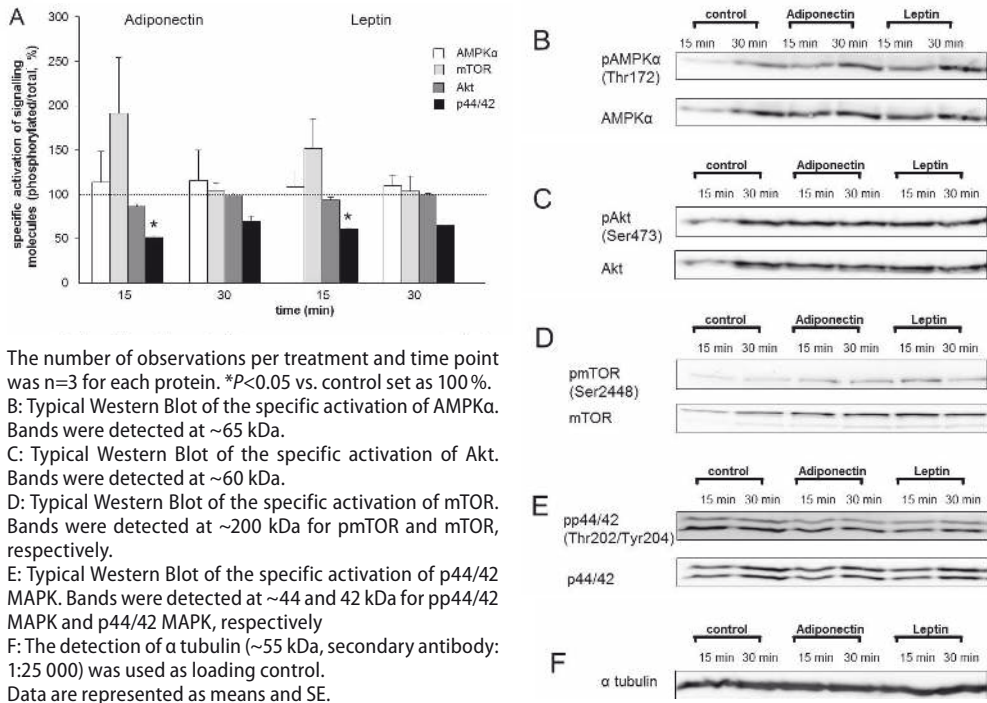
The number of observations per treatment and time point was n=3 for each protein. *$P<0.05$ vs. control set as 100%.
B: Typical Western Blot of the specific activation of AMPKα. Bands were detected at ~65 kDa.
C: Typical Western Blot of the specific activation of Akt. Bands were detected at ~60 kDa.
D: Typical Western Blot of the specific activation of mTOR. Bands were detected at ~200 kDa for pmTOR and mTOR, respectively.
E: Typical Western Blot of the specific activation of p44/42 MAPK. Bands were detected at ~44 and 42 kDa for pp44/42 MAPK and p44/42 MAPK, respectively
F: The detection of α tubulin (~55 kDa, secondary antibody: 1:25 000) was used as loading control.
Data are represented as means and SE.

Figure 2

Activation of various intracellular signalling molecules in porcine myoblasts in untreated cultures (control) or after application of 40 µg/ml adiponectin or 20 ng/ml leptin for 15 or 30 min in growth factor-supplemented, serum-free medium (A)

Table 2
Lactate dehydrogenase (LDH) activity in supernatant as a measure for rates of cell death in proliferating porcine myoblast cultures after treatment with adiponectin (40 µg/ml) or leptin (20 ng/ml) for 10, 24 or 48 h in growth factor-supplemented, serum-free medium

	Treatment	Time of Exposure		
		10 h	24 h	48 h
LDH/DNA (mIU/µg)	Control	0.65±0.06	0.46±0.06	0.84±0.06[a]
(n=10)	Adiponectin	0.66±0.06	0.35±0.06	0.67±0.06[b]
	Leptin	0.64±0.06	0.34±0.06	0.64±0.06[b]

Data are represented as least squares means and SE.   [ab]LS Means within a column not sharing common letters significantly differ ($P<0.05$).

Specific activation of signalling molecules

The effects of adiponectin or leptin on the phosphorylation or on their specific activation, respectively, of AMPK, Akt, mTOR and p44/42 MAPK were examined in proliferating porcine myoblast cultures after exposure times of 15 and 30 min (Figure 2). Adiponectin or leptin alone decreased the phosphorylation levels of p44/42 MAPK after 15-min (adiponectin: −39.6 %±0.9 %, $P=0.03$; leptin: −49.1 %±2.4 % $P=0.01$), but only numerically after 30-min treatment. No significant changes in the phosphorylation levels of AMPK, Akt or mTOR after myoblast exposure to either adiponectin or leptin were observed compared with untreated controls after 15 min and 30 min (Figure 2).

Discussion

In addition to findings reported in our previous study (Will *et al.* 2012), it is shown here that short-term treatment of primary porcine skeletal muscle cells with adiponectin or leptin in GF-SFM led to diminished DNA synthesis rate, whereas DNA content remained unaffected. Furthermore, cell viability remained unchanged after 24-h and was increased by adiponectin and leptin treatment after 48-h exposure. We further provide evidence that p44/42 MAPK signalling is involved in mediating the adipokine effects, i.e. proliferation.

Not only after 48 h (Will *et al.* 2012), but already after 4 h, adiponectin treatment of porcine myoblasts resulted in a significant decrease of DNA synthesis rate, which may indicate a delayed onset of proliferation. This is suggested to result from the inhibition of bFGF, which was identified to be the reason for attenuated DNA synthesis of porcine myoblasts after adiponectin long-term treatment (Will *et al.* 2012). The decrease in the specific activation of p44/42 MAPK after 15- and 30-min exposure to adiponectin, observed in the present study, could also result from an adipokine-mediated attenuation of bFGF action, as p44/42 MAPK is activated by extracellular stimuli like growth factors or hormones (Cobb & Goldsmith 1995). As p44/42 MAPK is an important mediator of cell proliferation, the decline in its specific activation, observed in GF-SFM, may result in a reduced DNA synthesis rate. Further studies on porcine skeletal muscle cells with respect to receptor ligand binding under GF-SFM culture conditions may clarify the adiponectin effects in more detail. Whether the inhibition of p44/42 MAPK in myoblasts by adiponectin is of biological relevance and results in changes in the activities of down-stream targets under the conditions used in this study needs further

elucidation. Short-term leptin effects on signalling as well as DNA synthesis rate were similar to those of adiponectin. These effects, however, are considered to be transient, because after long-term treatment with leptin over 24 h or 48 h, any responses of porcine myoblasts in DNA synthesis rate or DNA content were no longer detectable under identical culture conditions (Will *et al.* 2012). As the event of proliferation occurs as a consequence of DNA synthesis, a decrease in DNA content might be probably not yet apparent after 4-h treatment. Under low serum conditions, the phosphorylation level of p44/42 MAPK behaved differently in response to the adipokines. There was a transient activation of p44/42 MAPK after 15 min, followed by decreases after 60 and 180 min, which was associated with increases in DNA synthesis rate and cell death (Will *et al.* 2013).

In the long range, the presence of adiponectin or leptin in the culture medium seemed to improve cell viability of the myoblasts as LDH activity was lower in medium samples taken at 48-h, but not after 10-h and 24-h treatments. However, under GF-SFM conditions, the rate of cell death was very low at each of three time points examined, nearly 30-50 % in comparison to low serum (1 % FBS) conditions (Will *et al.* 2013). In conclusion, adiponectin and leptin treatment will not enhance the rate of cell death in primary porcine myoblast cultures, if growth factors are present in cell culture medium. In contrast, cell viability was transiently decreased by about 30 % in response to adiponectin or leptin after 24-h treatment under low serum conditions (Will *et al.* 2013). The different responses of porcine myoblasts to the adipokines in GF-SFM compared to low serum conditions suggest that adipokine actions on muscle cells may be modified by endocrine and paracrine actions.

The phosphorylation levels of AMPK, Akt and mTOR were not changed under leptin or adiponectin treatment of cells for 15 or 30 min in GF-SFM, which might be due to a cell type-specific activation of intracellular signalling pathways by adiponectin and leptin. Although various studies have reported on adiponectin-induced activation of AMPK in muscle cells or muscle tissue, this was only in the context of glucose transport or fatty acid oxidation (Yoon *et al.* 2006). Several other studies showed an activation of AMPK and Akt after leptin treatment (Maroni *et al.* 2005, Minokoshi *et al.* 2002). However, nothing is known about intracellular signalling pathways involved in adipokine-mediated effects on cell proliferation of primary porcine myoblasts. Thus, the animal species, the nature of the cells used, culture conditions or the specific preparations of recombinant adiponectin or leptin may be responsible for the lack of activation of these signalling molecules in our study. The growth medium used in this study was energetically balanced. Therefore, adiponectin and leptin might not lead to regulatory cell responses regarding energy metabolism and any short-term effects may be compensated very fast. Moreover, in contrast to other studies using immortalized cell lines, in this study primary skeletal muscle cells were used, which may exhibit different sensitivity to external signals.

In conclusion, short-term adiponectin or leptin treatments resulted in similar decreases in DNA synthesis rate of porcine skeletal muscle cells when cultured under growth factor-supplemented, serum-free conditions. No negative effects were observed on cell viability within 48 h. Phosphorylation levels of AMPK, mTOR and Akt remained unchanged during incubation of primary myoblasts with adiponectin or leptin over a period of 30 min. However, the phosphorylation levels of extracellular-signal regulated kinases-type MAP kinases (p44/42 MAPK) were at least transiently reduced during the initial period of adiponectin or leptin stimulation of cells. Additional research is needed to clarify the lack of effects of adipokine

treatment on other key signalling molecules and to identify factors acting as modulators for adipokine action on muscle cell growth.

Acknowledgements

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