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Decreased expression of the IGF-II gene during porcine adipose cell differentiation

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1 **Abstract**

2 IGF-I and IGF-II are known to regulate cell development and recent data suggest a possible
3 role of IGF-II on adipose tissue development. This study was undertaken to examine the IGF
4 system gene expression in porcine differentiating adipocytes. Both adipocytes and stromal-
5 vascular (s/v) cells were isolated from subcutaneous adipose tissue collected from 7-day-old
6 piglets. S/v cells were cultured in chemically defined medium. Compared with isolated
7 adipocytes, IGF-II and IGFBP-5 mRNA levels were very high in freshly isolated s/v cells,
8 whereas IGF-I mRNA levels were lower in s/v cells than in adipocytes. Between day 0 and
9 day 6 of culture, IGF-II and IGFBP-5 gene expression decreased whereas expression levels of
10 late markers of adipocyte differentiation were up-regulated. Cell differentiation was also
11 associated with an increase in the expression of IGF-I, insulin and IGF receptor genes. The
12 current findings suggest that IGF-I and IGF-II have different effects on porcine adipose cell
13 development.

14

15 *Key words:* IGF-I; IGF-II; IGFBP; preadipocyte; differentiation; pig

16

1 **1. Introduction**

2 Insulin-like growth factor-II (IGF-II) is a single chain polypeptide that belongs to the
3 family of insulin-related peptides, which includes also insulin and IGF-I (Jones and
4 Clemmons, 1995; O'Dell and Day, 1998). IGFs interact with two types of cell surface
5 receptors, type I and type II, that differ in their amino acid sequence, secondary structure and
6 ligand-binding specificity; and they also interact with six high-affinity IGF binding proteins
7 (IGFBP-1 to IGFBP-6 ; Jones and Clemmons, 1995 ; Firth and Baxter, 2002). These factors
8 are potent growth regulators that exert biological effects in an endo-, para- and/or autocrine
9 manner (Jones and Clemmons, 1995). Whereas IGF-II is known to play a key role in placental
10 and fetal growth (Constância et al., 2002; Fowden, 2003), its action during postnatal life is
11 much less documented (O'Dell and Day, 1998; Chao and D'Amore, 2008). An increasing
12 number of studies argue for a possible role of IGF-II on adipose tissue development in
13 mammals other than rodents.

14 Indirect evidence for effects of IGF-II on adipose tissue growth and development is
15 provided by studies of *igf2* gene polymorphism, with *igf2* genotype influencing fat deposition
16 in the pig (Nezer et al., 1999). In other studies, adipose tissue and isolated adipocytes have
17 been shown to express IGF-II and other members of the IGF system in the pig (Hausman et
18 al., 2002; Hausman et al., 2006; Gardan et al., 2006; 2008). Furthermore, we have found a
19 higher IGF-II mRNA level in isolated intramuscular adipocytes than in subcutaneous or
20 perirenal adipocytes in growing pigs (Gardan et al., 2006). Because intramuscular fat is the
21 latest adipose tissue to develop in the pig (Lee and Kauffman, 1974; Hauser et al., 1997), we
22 hypothesized that IGF-II gene expression may vary with the stage of development of the
23 adipose cells. However, the expression pattern of IGF-II in relation with other components of
24 the IGF system in differentiating adipose cells is still unknown. Therefore, the present study

1 was undertaken to examine the expression of some genes of the IGF system in freshly isolated
2 cells and during differentiation of stromal-vascular (s/v) cells in primary culture.

3

4

5 **2. Materials and methods**

6

7 *2.1. Animals and sample collection*

8 Studies were conducted in compliance with the French guidelines for care and use of
9 animals in research. Male crossbred piglets (Pietrain x (Large White x Landrace)) were killed
10 after an overnight fast by exsanguination after electrical stunning at 7 days of age (2.6 ± 0.3
11 kg body weight, $n = 6$). Subcutaneous dorsal adipose tissue was aseptically removed
12 immediately after death. Samples of 6 g were used for cell preparation.

13

14 *2.2. Cell isolation*

15 Cells were isolated by collagenase digestion as described by De Clercq et al. (1997).
16 Briefly, subcutaneous adipose tissue was minced and digested in a HEPES phosphate buffer
17 (2 mL/g of tissue) containing 2% BSA and 2 mg/mL collagenase II and XI (800 U/mg;
18 Sigma, St-Quentin Fallavier, France) in a shaking water bath for 45 min at 37°C. Flask
19 contents were filtered through nylon meshes (200 μm). A five-fold excess of buffer
20 containing no collagenase was added, before cells were centrifuged at 400 g for 10 min to
21 separate floating adipocytes from the pellet of s/v cells. Floating adipocytes were collected in
22 DMEM at 37°C, and then immediately processed for mRNA extraction. The pellet containing
23 adipocyte progenitors is successively filtrated through a 100- μm and 25- μm nylon mesh. An
24 aliquot (20 μL) of cell suspension was stained with Trypan blue stain, and counted on a

1 hemocytometer. A fraction of s/v cells was then processed for mRNA extraction, whereas the
2 other fraction was cultured.

3

4 *2.3. Cell culture*

5 S/v cells were seeded into 24-well plates (2 cm²/well) at a density of 7.5 x 10⁴
6 cells/cm² for morphological measurements, or in 6-well plates at a similar density for gene
7 expression analyses. Cells were maintained in a humidified 5% CO₂ atmosphere for 24 h at
8 37°C. Plating medium consisted of Dulbecco's Modified Eagle's Medium (DMEM,
9 Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS), 100
10 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone, 0.25 µg/mL nystatine, 4
11 mM L-glutamine, 2.7 nM insulin, 100 nM cortisol. Cells were then cultured in a serum-free
12 medium (DMEM/Ham's Nutrient Mixture F-12 (1:1) (Invitrogen), 100 U/mL penicillin, 100
13 µg/mL streptomycin, 0.25 µg/mL fungizone, 0.25 µg/mL nystatine, 50 mM β-
14 mercaptoethanol, 0.1 nM ascorbic acid, 2.5 mM L-glutamine, 10 µg/ml transferrin, 100 nM
15 cortisol, 0.2 nM triiodothyronine). Media were supplemented with either insulin, and/or IGF-I
16 or IGF-II (GroPep, Adelaïde, Australia, 10 nM). The media were then changed every other
17 day. Cell differentiation was assessed by oil red-O and Hemalun Mayer staining (Gerfault et
18 al., 1999). Cell diameters were also determined using a coulter counter (Coulter Multisizer,
19 Beckmann) as previously described (Etherton, 1980). Cells with a diameter below 5 µm were
20 not considered.

21

22 *2.4. Malic enzyme assay*

23 Serum-free media were replaced with a 0.25 M sucrose solution and cells were then
24 collected. After sonication, homogenates were stored at -70°C until use. Activity of malic
25 enzyme (EC 1.1.1.40) was then measured (Hsu and Lardy, 1969). Substrate quantities were

1 optimized (100 μ L) to ensure linearity of the reactions. Protein concentrations of
2 homogenates were determined using the bicinchoninic acid assay with BSA as standard.
3 Malic enzyme activity was expressed as nmoles NADPH produced/min/mg protein.

4

5 *2.5. Real-time RT-PCR*

6 Total RNA was extracted by the guanidium thiocyanate method (Chomczynski and
7 Sacchi, 1987) with modifications for pig adipocytes (Louveau et al., 1991). Treated-DNAse
8 total RNA (2 μ g) was reverse-transcribed using random hexamer primers and murine
9 Moloney leukemia virus reverse transcriptase, according to the manufacturer's instructions.
10 Primers were selected from porcine sequences as previously described (Gardan et al., 2006;
11 Table 1). Real-time quantitative PCR analyses were performed in a final volume of 12.5 μ L
12 starting with 5 ng of reverse transcribed RNA and both sense and antisense primers (500 nM
13 for each gene), using SYBR[®] Green I PCR core reagents in an ABI PRISM 7000 Sequence
14 Detection System instrument (Applied Biosystems). Forty cycles of amplification were
15 performed with each cycle consisting of denaturation at 95°C for 15 s, and annealing and
16 extension at 59°C for 1 min. Cycle threshold (C_T) values are means of triplicate
17 measurements. Absence of contamination from either genomic DNA amplification or primer
18 dimer formation was ensured using controls without reverse transcriptase or with no DNA
19 template and no reverse transcriptase. A melting curve analysis was also performed, which
20 resulted in single-product specific melting temperatures. Endogenous 18S ribosomal RNA
21 amplifications were used for each sample to normalize the expression of the selected genes. A
22 pool of adipocyte cDNA was used as an inter-plate calibrator for each gene. As PCR
23 efficiencies for selected genes and 18S gene were close to 1, the relative quantification for a
24 target gene in comparison to 18S in a given sample in reference to the calibrator, was
25 calculated according to the following formula (Pfaffl, 2001):

1 $\text{ratio} = 2^{-\Delta C_T^{\text{target (sample - calibrator)}}} / 2^{-\Delta C_T^{18S \text{ (sample - calibrator)}}}$.

2

3 *2.6. Statistical analyses*

4 Data were processed by analysis of variance using the General Linear Model
5 procedure of SAS (SAS Inst., Inc., Cary, NC, USA). Differences were considered to be
6 significant at $P < 0.05$. The mean separation procedure used was a multiple T-test
7 comparison. All data are presented as the mean + S.E.M. from at least three independent
8 experiments.

9

10

11 **3. Results**

12 *3.1. Differentiation of s/v cells into mature adipocytes*

13 Cells became confluent at approximately day 4 of culture. During the following days,
14 cells acquired a round shape and numerous lipid droplets of variable size filled up cytoplasm
15 (Fig. 1). On day 9 of culture, most of the cells were completely filled with multiple lipid
16 droplets. In addition, mean cell diameter continuously increased during the process of
17 differentiation (Fig. 1). The gene expressions of PPAR γ , malic enzyme and insulin receptor,
18 markers of adipose differentiation, were assessed (Fig. 2). These parameters increased during
19 differentiation even though the difference between D3 and D6 of culture was not significant.
20 A significant activity of malic enzyme, a later marker enzyme of adipose differentiation, was
21 also detected from day 6 onwards (Fig. 3). The comparison of freshly isolated cells further
22 supports in vitro data with higher PPAR γ ($P < 0.05$) and malic enzyme ($P < 0.1$) mRNA levels
23 in adipocytes than in s/v cells.

24

25 *3.2. Effects of IGF-I and IGF-II on the differentiation of s/v cells*

1 The addition of IGF-I or IGF-II to the insulin-supplemented medium did not markedly
2 affect malic enzyme activity at day 6 and day 9 of culture (Fig. 3). On the opposite, when
3 IGF-I or IGF-II were added in media in the absence of insulin, malic enzyme activity was
4 much lower ($P < 0.05$) than the same activity measured in insulin-supplemented medium with
5 or without IGFs at each time of culture.

6 7 *3.3. Gene expressions of the IGF system in freshly isolated cells and in cultured cells*

8 Expressions of genes related to the IGF family were determined in freshly isolated
9 adipocytes and in s/v cells (D0), and in 3- and 6-day-cultured s/v cells (Fig. 4). Different gene
10 expression patterns can be identified. Levels of IGF-I and IGFBP-3 mRNA were higher ($P <$
11 0.05) in adipocytes than in s/v cells at D0. During cell culture, both IGF-I and IGFBP-3
12 mRNA levels increased but the increase was significant ($P < 0.05$) only for IGF-I. In contrast,
13 levels of IGF-II and IGFBP-5 mRNA were lower ($P < 0.05$) in adipocytes than in s/v cells at
14 D0. During cell culture, both IGF-II and IGFBP-5 mRNA levels declined but the decrease
15 was significant ($P < 0.05$) only for IGF-II. Expression pattern for IGF-IR, IGF-IIR and
16 IGFBP-2 genes were similar with lower mRNA levels in adipocytes than in s/v cells at D0.
17 For IGFBP-4 gene expression, there was neither significant difference between freshly
18 isolated cells nor between cultured cells.

19 20 21 **4. Discussion**

22 Expressions of some genes of the IGF system were evaluated in both freshly isolated
23 adipose cells and cultured s/v cells to increase common knowledge on this system with
24 respect to adipose tissue development. Indeed, the possible role of IGF-II on adipocyte
25 differentiation has been poorly investigated in the literature, despite an increasing evidence
26 for a role of this factor on body fat deposition in pigs. The present study clearly shows that

1 IGF-I, IGF-II, IGF receptor, IGFBP-2, -3, -4 and -5 genes were expressed in freshly isolated
2 s/v cells, differentiating adipose cells, and lipid-filled adipocytes. The expression patterns
3 during differentiation differed between the genes examined, with IGF-I and IGFBP-3 genes
4 exhibiting clearly an increased expression and both IGF-II and IGFBP-5 showing a clear
5 decreased expression during culture. The comparisons of gene expressions in freshly isolated
6 s/v cells with that in adipocytes further support these observations.

7 The presence of IGF-I mRNA in preadipocytes and/or adipocytes is consistent with
8 previous findings in pigs (Gaskins et al., 1990), rats (Peter et al., 1993) and humans (Wabitsch
9 et al., 2000). The detection of IGF-II, IGFBP-2,-3 and -5 mRNA in the current study agrees
10 with data obtained in neonatal pig adipose tissue and fetal s/v cells using gene microarrays
11 (Hausman et al., 2006). In contrast, Boney et al (1994) failed to detect IGF-II mRNA by
12 Northern blot analysis in primary culture of mouse preadipocytes as well as in 3T3-L1
13 preadipocyte cells. Even though one cannot exclude the lack of sensitivity of Northern blot
14 analysis, these findings reflect likely the difference between rodents and other mammals.
15 Expressed genes in the current study are very likely secreted, because analyses of conditioned
16 media from cultured porcine s/v cells also lead to the identification of IGF-I, IGF-II and
17 several IGFBPs (Gaskins et al., 1990; Chen et al., 1996; Hausman et al., 2006). Likewise,
18 human preadipocytes and adipocytes have been shown to secrete IGF-I, IGF-II and at least
19 IGFBP-3 (Wabitsch et al., 2000). In the current study, both IGF-I and IGF-II receptor gene
20 expressions were also identified in porcine adipocytes, freshly isolated s/v cells and cultured
21 cells. The presence of IGF-I receptor mRNA in pre-adipocytes concurs with previous findings
22 in pigs (Richardson *et al.*, 1994; Gerfault et al., 1999) and rabbit (Nougues et al., 1993). The
23 expression of IGF-I and IGF-II receptors in mature adipocytes has been examined less
24 extensively, and available data from the literature are sometimes controversial. Even though
25 Richardson et al. (1994) failed to detect IGF-I receptors on porcine fat cells using in situ

1 binding, we have previously shown the presence of IGF-I receptor mRNA in adipocytes
2 (Gerfault et al., 1999; Gardan et al., 2006), and IGF-I binding sites in adipose tissue
3 membranes (Louveau and Le Dividich, 2002). In human adipocytes, the presence of IGF-I
4 receptors has been also demonstrated (Kern et al., 1989). It is also possible that adipocytes
5 express insulin receptor/IGF-I receptor hybrids as described in rabbit fat (Baillyes et al., 1997).

6 The current study provides novel information on the expression patterns of genes of
7 the IGF system during porcine adipose cell differentiation. Whereas IGF-I and IGFBP-3
8 exhibited an increased expression, a second group of genes involving IGF-II and IGFBP-5
9 had a decreased expression during differentiation. The finding of an increase in both IGF-I
10 and IGFBP-3 mRNA levels during differentiation agrees with data reported in human
11 (Wabitsch et al., 2000). This supports a significant role of IGF-I in adipocyte differentiation
12 as described in cells in primary culture as well as in different adipose cell lines (Wabitsch,
13 1995; Grégoire et al., 1998; Louveau and Gondret, 2006; Rosen and MacDougald, 2006). The
14 finding that both IGF-I and insulin receptors exhibited a similar pattern of expression did not
15 allow us to distinguish the action of IGF-I from that of insulin. Because IGF-I and insulin did
16 not have the same ability to increase malic enzyme activity in cells after 6 days of culture, we
17 suggest that IGF-I may exert its action through insulin receptors. An action through
18 insulin/IGF-I hybrid receptors is also possible.

19 With respect to the expression pattern of IGF-II and IGFBP-5 genes, much less is
20 known on adipose cells. In the literature, the number of genes that have been shown to be
21 down-regulated during adipose conversion is quite low compared with the number of up-
22 regulated genes (Fève, 2005). It remains to be investigated whether high expression of IGF-II
23 and IGFBP-5 genes in s/v cells at the beginning of cell culture may contribute to the
24 maintenance of the preadipose state, or could delay the differentiation process. IGFBP-5 has
25 been previously described to inhibit IGF actions, to potentiate IGF actions, or to have

1 independent IGF effects (Beattie et al., 2006). Interestingly, a parallel decrease in IGF-II and
2 IGFBP-5 mRNA levels has been also described during differentiation of porcine embryonic
3 myogenic cells (Xi et al., 2007). In addition, IGFBP-5 has been shown to block differentiation
4 of a murine muscle cell line by inhibiting IGF actions (Mukherjee et al., 2008). Therefore,
5 adipose cell differentiation may be inhibited by a high expression in IGF-II and in IGFBP-5
6 genes as reported for muscle cells.

7 In summary, the current study provides new insights in the regulation of adipocyte
8 differentiation by IGF-II. The findings that the expression patterns of IGF-I and IGFBP-3
9 genes differ from that of IGF-II and IGFBP-5 genes suggest that IGF-I and IGF-II play a
10 distinct role on the development of adipose cells. IGF-II may be a new target to delay or to
11 inhibit cell differentiation and to reduce adipose tissue development in non rodent models.

12

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15

1 **Figure legends**

2

3 Fig. 1. Adipose cells during differentiation in primary culture. Cells were first cultured in a
4 plating medium and were then cultured from day 1 to day 9 in a defined medium
5 supplemented with insulin (10 nM). (A) Photomicrographs of cultured stromal-vascular (s/v)
6 cells at D3 and D6 of culture (original magnification x200). (B) Mean diameters (μm) were
7 measured at D0, D3, D6 and D9 as described in Materials and methods. Data represent means
8 \pm S.E.M. of experiments performed in duplicate ($n = 4$).

9

10 Fig. 2. Transcript levels of markers of adipose cell differentiation in freshly isolated cells and
11 in stromal-vascular (s/v) cells in primary culture. Adipocytes and s/v cells were collected on
12 the day of slaughtering (D0). S/v cells were cultured in a defined medium supplemented with
13 insulin (10 nM) and collected on day 3 (D3) and day 6 (D6) of culture for mRNA extraction.
14 Levels of mRNA were quantified by real-time PCR as described in Materials and methods.
15 Values are means \pm SEM ($n = 6$ for adipocytes (Ad-D0) and s/v cells (S/v-D0); $n = 3$ for s/v
16 cells in culture (S/v-D3 and S/v-D6). Within a group of cells (freshly isolated vs culture),
17 values differed at * $P < 0.05$, † $P < 0.1$.

18

19 Fig. 3. Malic enzyme activity in stromal-vascular (s/v) cells in primary culture. Cells were
20 cultured from day 1 to day 9 in a defined medium supplemented with insulin (ins) and/or IGF-
21 I or IGF-II (10 nM). Malic enzyme activity was measured on D6 and D9 of culture as
22 described in Materials and methods and expressed as nmol NADPH per mg proteins. Values
23 are means \pm S.E.M. of experiments performed in triplicate ($n = 4$). Means with different
24 superscript letters are different ($P < 0.05$).

25

1 Fig. 4. Transcript levels of IGF-I, IGF-II, IGF-BPs and IGF receptors in freshly isolated cells
2 and in stromal-vascular (s/v) in primary culture. Adipocytes and s/v cells were collected on
3 the day of slaughtering (D0). S/v cells were cultured in a defined medium supplemented with
4 insulin (10 nM) and collected on day 3 (D3) and day 6 (D6) of differentiation for mRNA
5 extraction. Levels of mRNA were quantified by real-time PCR as described in Materials and
6 methods. Values are means \pm SEM (n = 6 for adipocytes (Ad-D0) and s/v cells (S/v-D0); n =
7 3 for s/v cells in culture (S/v-D3 and S/v-D6). Within a group of cells (freshly isolated vs
8 culture), values differed at * P < 0.05, † P < 0.1.

9

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1 Table 1. Primers used for analysis of gene expression by real-time RT-PCR.

Gene ¹	Accession number	Forward primer (5' → 3')	Reverse primer (5' → 3')
IGF-I	M31175	GCTGGACCTGAGACCCTCTGT	TACCCTGTGGGCTTGTTGAAAT
IGF-II	X56094	AGGGCATCCAAACCACAAAC	GGGTTCAATTTTTGGTATGTAACCTG
IGFBP-2	AF120326	GCACCTGTACTCCTTGACATC	CGCTGCCCATTTCAGAGACAT
IGFBP-3	AF085482	CATCCCCAACTGCGACAAG	ATCCACGCACCAGCAGAAG
IGFBP-4	BX921877	ATCGAGGCCATCCAGGAAA	CCCCGATGACCTTCATCTTG
IGFBP-5	NM_214099	CGTGGACAAGTACGGGATGA	CGAAGCTGTGGCACTGGAA
IGF-I R	U58370	CAACCTCCGGCCTTTTACTTT	CAGGAATGTCATCTGCTCCTTCT
IGF-II R	CB469268	TGCCCGGTGAAGAGCAA	TTGTCCCACACACGATAATGT
Insulin R	AF102858	CAGCGATGTATTTCCATGTTCTGT	GCGTTTCCCTCGTACACCAT
ME	X93016	TGGTGACTGATGGAGAACGTATTC	CAGGATGACAGGCAGACATTCTT
PPAR γ	AF103946	ATTCCCGAGAGCTGATCCAA	TGGAACCCCGAGGCTTTAT

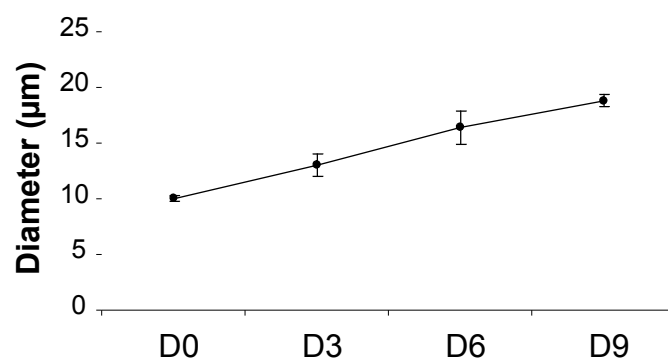
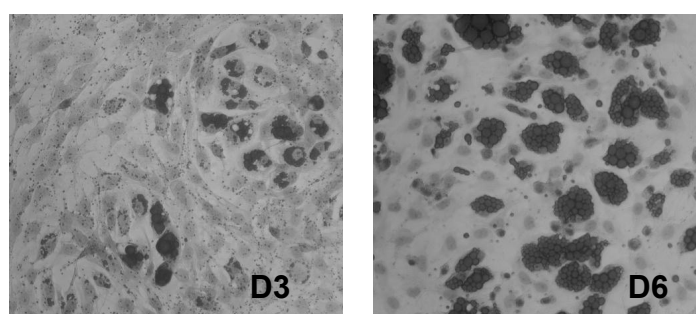
2

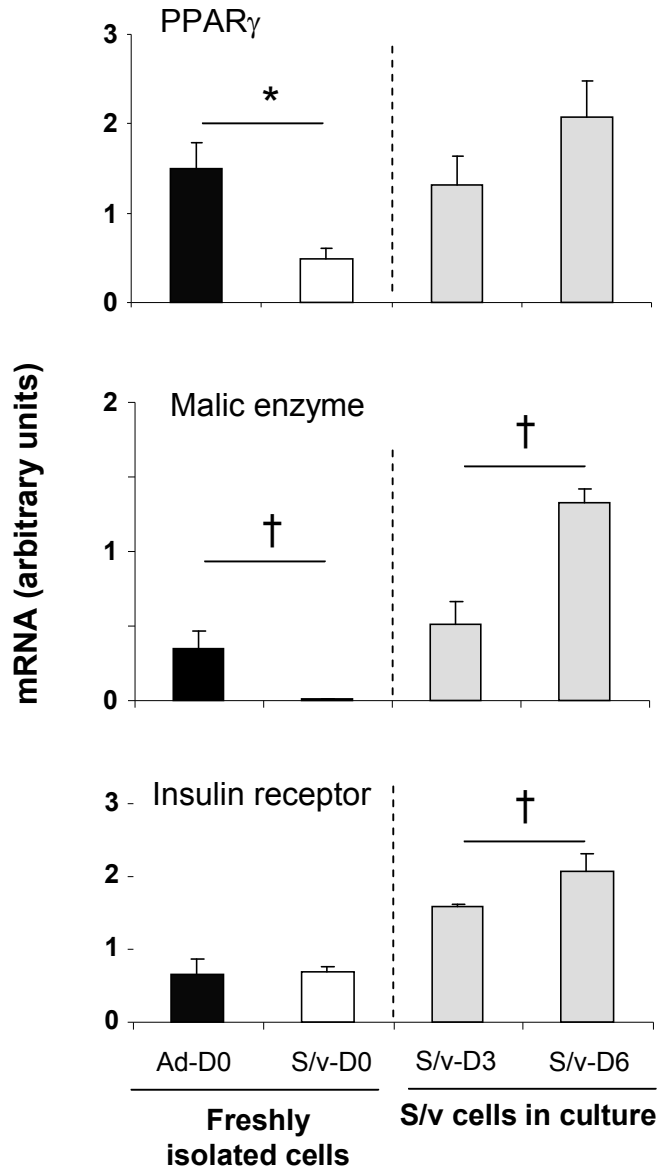
3 ¹IGF-I and IGF-II, insulin-like growth factor-I and -II; IGFBP, insulin-like growth
4 factor binding protein; R, receptor; ME, malic enzyme; PPAR- γ , peroxisome proliferator-
5 activated receptor gamma.

6

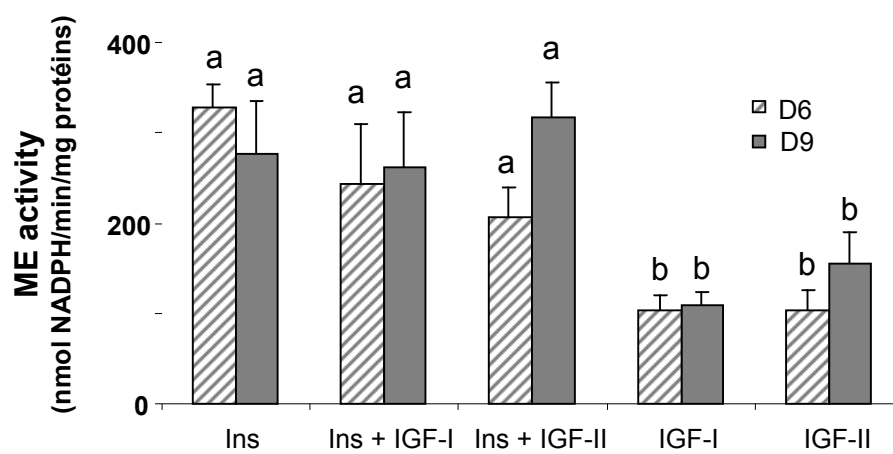
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Fig. 1



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Fig. 2

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Fig. 3



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Fig. 4

