GH and insulin affect fatty acid synthase activity in isolated porcine adipocytes in culture without any modifications of sterol regulatory element binding protein-1 expression

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Abstract

The ability of GH to decrease fatness and insulin-regulated events such as lipogenic enzyme activities is well known in pigs. Nevertheless, the precise mechanism underlying these actions has not been elucidated yet. Expression of the transcription factor sterol regulatory element binding protein (SREBP)-1 has been reported as a key mediator of insulin action in rat hepatocytes and adipose cell lines. The present study aimed to determine whether the regulation of lipogenesis by GH and/or insulin in porcine adipocytes also involved SREBP-1. Isolated adipocytes, obtained from perirenal or s.c. adipose tissue samples of female pigs (51 ± 0.4 kg; $n=17$), were cultured in serum-free medium in the absence or presence of these hormones for

up to 4 days. Glucose incorporation and fatty acid synthase activity were increased by insulin in a dose-dependent manner in adipocytes of both sites. The increase was maximal at 1.7 and 17 nM in s.c. and perirenal adipocytes respectively, suggesting inter-depot differences in the regulation of lipogenesis by insulin. These insulin-stimulated events were decreased by GH (1 nM). No change in SREBP-1 mRNA levels was observed in response to GH and/or insulin. Taken together, these data indicate that the regulation of lipogenesis by insulin and GH appears to not involve changes in SREBP-1 mRNA levels in porcine adipocytes.

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Introduction

Growth hormone (GH) is known to regulate adipose tissue growth and development in several species. A large number of studies have shown that chronic administration of GH to growing pigs causes a dramatic decline in adipose tissue lipid accretion (Etherton & Bauman 1998, Etherton 2000). This effect is due primarily to a decrease in lipogenesis rather than to an increase in lipolysis, and involves a decrease in adipocyte insulin sensitivity. This leads to a marked decrease in insulin-regulated events such as glucose transport and lipogenic enzyme activities as assessed *in vivo* (Dunshea *et al.* 1992) and *in vitro* (e.g. Magri *et al.* 1990, Harris *et al.* 1993, Wang *et al.* 1999). The effect of GH on the regulation of fatty acid synthase (FAS), the key lipogenic enzyme that catalyzes all the reactions implicated in the conversion of acetyl-CoA and malonyl-CoA to palmitic acid, is especially well documented in pigs. Both activity and mRNA levels of FAS (Magri *et al.* 1990, Mildner & Clarke 1991, Harris *et al.* 1993, Donkin *et al.* 1996) are decreased in adipose tissue of GH-treated animals. It has also been shown that GH attenuates the stimulatory effect of insulin on FAS gene transcription in 3T3-F442A adipocytes (Yin *et al.* 1998, 2001). Nevertheless, the mechanism by which GH inter-

feres with insulin action on FAS gene transcription has not been elucidated yet. The GH-dependent impairment of insulin action does not result from changes in insulin binding or insulin receptor kinase activity in porcine adipocytes (Magri *et al.* 1990). It is mediated by neither protein kinase A, protein kinase C nor Janus kinase-2 in 3T3-F442A adipocytes (Yin *et al.* 2001). Further studies are therefore required to identify the molecule(s) involved in this process. One possible candidate is the sterol response element binding protein (SREBP)-1c, a member of the helix-loop-helix-leucine zipper family of transcription factors, which has been reported to play a pivotal role in mediating the effects of insulin on gene expression in liver (Foufelle & Ferré 2002). SREBP-1c is synthesized as a precursor form anchored into membranes of the endoplasmic reticulum and nuclear envelope. After proteolytic cleavage, the mature form constituted by the amino-terminal half of the precursor, enters the nucleus where it can bind both sterol regulatory elements and E-boxes on the target genes (Brown & Goldstein 1997). Adenoviral-mediated overexpression of constitutively active SREBP-1c up-regulates FAS promoter activity and increases FAS mRNA levels in primary cultured rat hepatocytes (Foretz *et al.* 1999a) or 3T3-L1 adipocytes (Kim *et al.* 1998, Le Lay *et al.* 2002, Palmer *et al.* 2002).

Conversely, adenoviral vectors expressing a dominant negative version of SREBP-1c block the effect of insulin on FAS mRNA in primary cultures of rat hepatocytes (Foretz *et al.* 1999b) and 3T3-L1 adipocytes (Le Lay *et al.* 2002). Transcription of SREBP-1c is controlled positively by insulin in isolated rat hepatocytes (Foretz *et al.* 1999b, Azzout-Marniche *et al.* 2000) and in 3T3-L1 adipocytes (Kim *et al.* 1998, Le Lay *et al.* 2002), thus leading to increased amounts of the precursor form and thus, of the nuclear mature form of SREBP-1c in insulin-treated cells (Azzout-Marniche *et al.* 2000). However, a recent study has shown that SREBP-1c gene expression is not modified by insulin in freshly isolated rat adipocytes (Palmer *et al.* 2002). The present study was undertaken to examine the regulation of lipogenesis by insulin and GH in isolated porcine adipocytes maintained in primary culture and to determine whether this regulation involves modification of SREBP-1 expression. Cells obtained from both s.c. and perirenal adipose tissue were studied, as depot-specific gene regulation in adipocytes has been suggested in rats and human (Lefebvre *et al.* 1998, Villafuerte *et al.* 2000).

Material and Methods

Materials

Porcine GH (AFP 11716C) was provided by Dr A F Parlow, National Hormone and Peptide Program (NHPP), National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA. Insulin (Actrapid) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Collagenase A (0.22 U/mg) and protease inhibitors (COMPLETE) were purchased from Roche Applied Science (Meylan, France). Dulbecco's modified Eagle's medium (DMEM), Hepes, antibiotics and RTS labeling reagents (for random priming in the presence of [α - 32 P]deoxycytidine triphosphate) were obtained from Invitrogen (Cergy-Pontoise, France). D-[U- 14 C]glucose (306 mCi/mmol), [α - 32 P]deoxycytidine triphosphate, ECL Western blot detection kits, Hybond C nitrocellulose and Hybond N⁺ membranes were obtained from Amersham Biosciences (Orsay, France). RNase-free DNase was obtained from Ambion (Austin, TX, USA). Highly purified salt-free primers were generated from Prologo (Paris, France). The bicinchoninic acid protein assay kit was obtained from Pierce (Rockford, IL, USA). All other reagents were of analytical grade and were supplied by Sigma (St-Quentin-Fallavier, France) or Eurobio (Les Ulis, France).

Adipose tissue collection, isolation and primary culture of porcine adipocytes

The experiment was conducted in accordance with the national regulation for the human care and use of animals

in research (certificate of authorization to experiment on living animals no.7676 delivered by the French Department of Agriculture to I Louveau).

Adipocytes were obtained by collagenase treatment of perirenal (leaf fat) or s.c. adipose tissue samples from Piétrain $\times$ (Large-White $\times$ Landrace) female pigs (50.9 ± 0.4 kg in body weight; 100 ± 2 days of age; means $\pm$ S.E.M.; $n=17$), as previously described (Etherton & Chung 1981). Briefly, minced adipose tissue in Krebs-Ringer-bicarbonate buffer (3 ml/g of tissue) containing 3% BSA, 10 mM glucose, 1.3 mg/ml collagenase and antibiotics, was shaken (40 r.p.m.) in a sterile polypropylene flask for 60 min at 37 °C. Digestion products were filtered through nylon mesh (200 μ m) and mature adipocytes were allowed to float. Cells were then washed by removing the infranatant using a plastic catheter attached to a syringe and adding 37 °C DMEM, 5.5 mM glucose. After three such washes, adipocytes were placed in DMEM containing 10 mM Hepes, 1% L-glutamine, 140 nM hydrocortisone, and 62.5 U/ml penicillin and 62.5 μ g/ml streptomycin. Cells were cultured to a final concentration of approximately $1-2 \times 10^5$ /ml in a humidified 5% CO₂ atmosphere at 37 °C for up to 4 days, in the absence or presence of GH and/or insulin, as indicated in the Figure legends and the cell media were changed every other day.

Cell number determination

Cell number estimation is based on the measurement of lipid content of an aliquot of cell suspension (Dole & Meinertz 1960) and on the measurement of adipocyte diameters using an image analysis system (Optimas 6.5; Media Cybernetics, Silver-Spring, MD, USA). Before culture, fat cell diameter assessed in all experimental pigs was 50.7 ± 0.7 μ m for perirenal fat and 51.1 ± 1.4 μ m for s.c. fat. Two pigs were also used in a preliminary study to evaluate adipocyte size variation and cell lysis (determined from the number of cells remaining after the lipid layer has been removed) during the whole culture. Fat cell size did not change during the culture (data not shown). Estimation of cell number before and during culture indicates that cell breakage occurred mainly during the first 2 days of culture (e.g. cell breakage was 36 and 40% after 2 and 4 days in culture respectively in perirenal-derived adipocytes; data not shown).

Glucose incorporation into lipids

The lipogenic rate of adipocytes was estimated by quantifying the conversion of [14 C]glucose into lipids as described previously (Foster *et al.* 1988). In brief, cells were incubated with 0.4 μ Ci D-[U- 14 C]glucose at 37 °C for 2 h in the absence or presence of hormones. Incubations were terminated by removing the medium, followed by immediately adding Dole's reagent. Fatty acids were then extracted according to Dole & Meinertz (1960) and

incorporated radioactivity was measured by liquid scintillation counting. Our preliminary experiments indicated that glucose conversion to lipids increased in a linear fashion during a 2 h incubation. Glucose incorporation into lipids was expressed as nanomoles glucose incorporated per 2 h.

FAS activity assay

Adipocytes were homogenized by hand in ice-cold buffer (0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA and protease inhibitors) using a glass–glass homogenizer. The homogenate was centrifuged at 100 000 *g* for 60 min. The supernatant was collected and the FAS (EC 2.3.1.85) assay was performed immediately or after storage at -70°C , as previously described (Guichard *et al.* 1992). The protein concentration of the cytosolic fraction was determined by using the bicinchoninic acid assay with BSA as a standard. FAS activities were expressed as nanomoles of NADPH oxidized/minute and per milligram of protein. Measurements were done in triplicate.

Western blotting

Preparation of nuclear extracts from isolated adipocytes was performed as described by Dugail (2001) and protein content was determined using BSA as standard (Bradford 1976). Adipocyte cytosolic proteins (40 μg) or nuclear extract proteins (20 μg) were electrophoresed under reducing conditions on 8 or 12% SDS–polyacrylamide gels respectively, and were then electro-transferred onto Hybond C nitrocellulose membranes. The blots were blocked for 1 h in PBS–Tween 20 (0.1% w/v) containing 5% non-fat dry milk and were then incubated overnight with a primary antibody. The FAS protein was probed with a rabbit polyclonal antibody against FAS (1:1000) (Abcam Ltd, Cambridge, UK) and membranes were rinsed three times with PBS–Tween 20 (0.1 v/v). The SREBP-1 protein was detected with a mouse monoclonal antibody against amino acids 301–407 of human SREBP-1 (1:200) (NeoMarkers, Fremont, CA, USA). Membranes were rinsed three times with PBS buffer containing 0.1% SDS, 1% Nonidet-P40 and 0.5% deoxycholic acid. Detection of the signals was performed with the ECL Western blot detection kit, with an anti-rabbit for FAS (1:10 000) or an anti-mouse (1:2500) for SREBP-1 horseradish peroxidase-conjugated IgG as second antibodies. Autoradiograms were scanned and quantified with an image processor (Quantity One, V4; Bio-Rad, Hercules, CA, USA).

Total RNA isolation and Northern blot hybridization

Total cellular RNA was extracted from cultured adipocytes by the guanidinium thiocyanate method (Chomczynski & Sacchi 1987) with modification

(Louveau *et al.* 1991). Twenty micrograms of total RNA were denatured in formamide and formaldehyde and subsequently separated in 1% formaldehyde–agarose gel. RNA was then transferred overnight into a Hybond N⁺ membrane. Membranes were hybridized with labeled probes obtained by RTS labeling. A partial cDNA fragment encoding porcine SREBP-1 was used for probe generation, as detailed in Gondret *et al.* (2001). This probe, corresponding to amino acids 241–367 in human SREBP-1a, recognized both 1a and 1c isoforms. Autoradiograms for SREBP-1 were developed after 4 days. Membranes were then re-probed with a cDNA for porcine 18S to assess variation in loading or transfer of RNA samples. Signals were scanned and quantified with an image processor program (Quantity One, V4; Bio-Rad). Results are expressed as relative expression of SREBP-1 to 18S.

Real-time RT-PCR

After DNase treatment, total RNA (1.5 μg) was reverse transcribed using random hexamers and murine Moloney leukemia virus reverse transcriptase, according to the manufacturer's instructions (Amersham Biosciences). The design of the primers for the selected genes (FAS: forward primer, 5'-AGCCTAACTCCTCGCTGCAAT-3'; reverse primer, 5'-TCCTTGAACCGTCTGTGTTC-3'; SREBP-1, forward primer: 5'-CGGACGGCTCACAA TGC-3'; reverse primer, 5'-GCAAGACGGCGGATT TATTC-3') was done using Primer Express software (Applied Biosystems, Warrington, Cheshire, UK), based on porcine sequences (FAS: Genbank AY183428; SREBP-1: Genbank AF102873). Real-time RT-PCR analyses were performed starting with 100 ng of reverse-transcribed total RNA, and both sense and antisense primers (FAS: 500 nM, SREBP-1: 200 nM), in a final volume of 25 μl , using SYBR Green I PCR core reagents in an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystem). Forty cycles of amplification were performed. Absence of contamination from either genomic DNA amplification or primer dimer formation was ensured using controls without reverse transcriptase or with no DNA template. An annealing temperature of 59°C was used for each gene. Specificity of RT-PCR products was documented on ethidium bromide-stained agarose gel electrophoresis and resulted in a single product of the expected size (FAS: 196 bp, SREBP-1: 118 bp). A melting curve analysis was also performed, which resulted in single-product specific melting temperatures as follows: FAS: 87°C ; SREBP-1: 82°C . Endogenous 18S ribosomal RNA amplifications (Human 18S rRNA pre-developed TaqMan kit; Applied Biosystem) were used to normalize the expression of the selected genes. A cDNA sample of freshly isolated perirenal adipocytes was used as an inter-plate calibrator for each gene. To measure real-time PCR efficiencies, serial

Figure 1 Dose-response of insulin-stimulated glucose incorporation in cultured adipocytes isolated from perirenal (P) or s.c. (SC) adipose tissue. Cells were incubated in a defined medium without or with increasing concentrations of insulin (nM) for 2 days. Data represent means $\pm$ S.E.M. of experiments performed in triplicate ($n=4$ for P; $n=5$ for SC). The asterisk (*) indicates a statistically significant difference compared with control cultures without insulin ($P<0.05$).

Figure 2 Dose-response of insulin-stimulated FAS activity in cultured adipocytes isolated from perirenal (P) or s.c. (SC) adipose tissue. Cells were incubated in a defined medium without or with increasing concentrations of insulin (nM) for 2 days. Data represent means $\pm$ S.E.M. of experiments performed in triplicate ($n=4$ for P; $n=6$ for SC). The asterisk (*) indicates a statistically significant difference compared with control cultures without insulin ($P<0.05$).

dilutions of reverse transcribed RNA (0.03–200 ng) were amplified. A line was obtained by plotting cycle threshold (C_T) values as a function of starting cDNA, the slope of which was used in the equation $E=10^{(1/\text{slope})}-1$ to calculate PCR efficiencies (FAS: 0.92; SREBP-1: 0.92; 18S: 1.08). Assuming that efficiencies for target genes and 18S were close to 1, the relative quantification for a target gene in comparison with a reference gene in a given sample was calculated according to the formula presented in Pfaffl (2001):

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

C_T values are means of triplicate measurements. Experiments were repeated twice. Values obtained in hormone-free medium were arbitrarily set to 100, and percent relative expressions of SREBP-1 and FAS genes in hormone-supplemented media were calculated.

Statistical analysis

Results were analyzed using the General Linear Model procedures of SAS (1990). The mean separation procedure used was a multiple *t*-test comparison. All data are presented as means $\pm$ S.E.M.

Results

Initial experiments were conducted to verify that the culture system was suitable to examine hormonal regulation of lipogenesis in pig adipocytes isolated from s.c. and perirenal adipose tissue. In both sites, glucose incorporation into lipids and FAS activity were assessed in response to increasing concentrations of insulin. At the start of

culture, basal FAS activity levels were similar in s.c. and perirenal adipocytes (20.2 ± 3.5 vs 17.4 ± 3.8 nmol/mg protein, $P>0.1$), whereas basal glucose incorporation tended to be higher in s.c. than in perirenal adipocytes (48.4 ± 13.6 vs 16.1 ± 4.8 nmol/2 h, $P=0.08$). Time-course monitoring for changes in glucose incorporation and FAS activity revealed that lipogenesis decreased throughout the culture time in basal conditions for both sites (data not shown). The incubation of s.c. or perirenal adipocytes for 2 days with insulin resulted in a dose-dependent increase in glucose incorporation (Fig. 1) and FAS activity (Fig. 2), compared with cells cultured in the absence of the hormone. The stimulatory effect of insulin on glucose incorporation was significant at concentrations of 0.17 and 1.7 nM for s.c. and perirenal adipocytes respectively. The insulin-dependent increase in FAS activity was significant at concentrations of 1.7 and 17 nM for s.c. and perirenal adipocytes, respectively. Treatment of adipocytes from both sites with higher concentrations of insulin than those cited above led to a similar increment in lipogenesis. After 4 days of culture, a similar insulin-responsiveness profile was observed in s.c. and perirenal adipocytes (data not shown). For further analysis, s.c. and perirenal adipocytes were exposed to an insulin concentration of 1.7 and 17 nM respectively. Freshly isolated adipocytes exposed for 2 h in the presence of insulin displayed higher glucose incorporation levels than cells maintained in the absence of the hormone (Fig. 3). The addition of insulin to cells that had been cultured for 2 days in the absence of the hormone provoked at least a 4-fold increase in glucose incorporation in s.c. and perirenal adipocytes at day 3 (Fig. 3), compared with cells cultured in the absence of insulin over time. Thereafter, in both s.c. and perirenal adipocytes, there was no significant difference in glucose incorporation between cells which had

Figure 3 Effect of insulin addition on time-course incorporation of glucose into lipids in cultured adipocytes isolated from s.c. (SC) or perirenal (P) adipose tissue. Cells were incubated in a defined medium for 2, 3 or 4 days. Insulin (1.7 and 17 nM for adipocytes isolated from SC and P adipose tissue respectively) was added at the start of culture (■) or after 2 days of culture (▲) before glucose incorporation measurement or was not added to the culture medium (○). Data represent means $\pm$ S.E.M. of one experiment performed in triplicate.

been insulin-treated throughout culture and those that had been insulin-treated only for 2 days. Similar profiles were elicited for FAS activity in both sites (data not shown).

Addition of GH for 2 or 4 days to the medium induced a decrease in insulin-stimulated glucose incorporation in s.c. and perirenal adipocytes (Fig. 4). The decline was significant ($P < 0.05$) in s.c. adipocytes after 2 days in culture. Insulin-stimulated FAS activity was also inhibited by GH in both s.c. and perirenal adipocytes (Fig. 4). Nevertheless, whereas the inhibitory effect was seen on both days 2 and 4 for s.c. adipocytes, this effect was seen only on day 2 for perirenal adipocytes. On day 2, modifications observed for catalytic FAS activity were

strongly paralleled by variations in FAS protein and FAS gene expression (Fig. 5). To examine the molecular signal involved in the effects of GH on the insulin-stimulated lipogenesis, we investigated the expression level of the SREBP-1 transcription factor in untreated cells, cells treated with optimal concentration of insulin, or cells exposed to GH (1 nM) and insulin during 2 days of culture. Whatever the donor site, cells which were cultured in the presence or the absence of GH and insulin displayed similar SREBP-1 mRNA levels, when evaluated by Northern blot analysis and by the highly sensitive real-time RT-PCR analysis (Fig. 5). In an additional experiment, we examined SREBP-1 protein expression in cells cultured for 2 days in the presence or absence of the hormones. Nuclear content of the cleaved form of SREBP-1 protein (showing a size closely similar to that of rat SREBP-1c (Azzout-Marniche *et al.* 2000)) was not different between untreated cells, cells treated with insulin alone and cells treated with both insulin and GH (Fig. 6).

Discussion

Although several studies indicate that the GH effects on porcine adipose tissue involve a marked decrease in the activity of several insulin-stimulated lipogenic enzymes including FAS, few studies have examined the mechanism by which GH affects insulin action on lipogenesis in adipocytes. For the first time in meat-producing animals, the present study aimed to determine whether the effects of GH and/or insulin on FAS activity could be mediated by SREBP-1 in isolated adipocytes. Investigating these effects requires a system in which adipocytes can be maintained metabolically active for several days. Indeed, GH exerts a chronic control of adipocyte function. Moreover, one can assume that the half-life of FAS is about 40–50 h in porcine adipocytes as in rodents (Weiss *et al.* 1980). In pigs, *in vitro* studies have concerned only short-term incubation of adipocytes (< 24 h) (Etherton & Chung 1981, Smith *et al.* 1996, Wang *et al.* 1999) or of adipose tissue fragments (< 48 h) (Walton & Etherton 1986). In the current study, we have provided evidence that cultured adipocytes can be used as a model for studying hormonal control of lipogenesis in pigs.

Insulin responsiveness of isolated porcine adipocytes

The present experiments indicate that after at least 2 days in culture, isolated adipocytes maintained the capacity to incorporate glucose into total lipids and displayed FAS activity at rates increasing with insulin concentrations in the medium. Interestingly, mature cells cultured in the absence of insulin, when challenged with the hormone after 2 days, displayed a marked increase in lipogenesis, thus demonstrating the ability to reinstate insulin stimulation from a basal state. The response to insulin was also

Figure 4 Effect of GH on insulin-stimulated glucose incorporation and FAS activity in cultured adipocytes isolated from s.c. (SC) or perirenal (P) adipose tissue. At the start of culture, cells were incubated in a defined-medium without or with insulin (1.7 and 17 nM for adipocytes isolated from SC and P adipose tissue respectively) in the absence or presence of GH (1 nM). Data represent means $\pm$ S.E.M. of experiments performed in triplicate (glucose incorporation: $n=4$ for P; $n=5$ for SC; FAS activity: $n=8$ for P and SC). Within any one day of culture, means with different letters are significantly different ($P<0.05$). Open bars: defined medium; black bars: defined medium supplemented with insulin alone; gray bars: defined medium supplemented with insulin and GH.

observed with freshly isolated adipocytes after short-term acute incubation. Altogether, these observations indicate that the adipocytes were still metabolically active. The magnitude of glucose incorporation found in this study is in the range of levels that have been reported previously in primary cultures of rat (Briquet-Laugier *et al.* 1994) or human adipocytes (Moustaid *et al.* 1996). In the present study, glucose incorporation and FAS activity were regulated by insulin in a dose-dependent manner. An optimum response for FAS activity was reached for a concentration as low as 1.7 nM in s.c. adipocytes, whereas higher concentrations of insulin are often used in isolated adipocytes from other species, such as rat (Briquet-Laugier *et al.* 1994) or human (Moustaid *et al.* 1996). In pigs, adipose tissue and/or adipocyte response to insulin has been often reported to be inconsistent (Mills 1999), with reports of absence of any responsiveness (Rule *et al.* 1987, Benmansour *et al.* 1991), little response (Liu *et al.* 1989, Harris *et al.* 1993) but not related to insulin concentrations

(Mersmann & Hu 1987), or clear stimulation of glucose incorporation into fatty acids and lipogenesis *in vitro* (Walton & Etherton 1986, Smith *et al.* 1996, Mills 1999). One possible explanation for these discrepancies is that an exaggerated basal rate of fatty acid synthesis could mask insulin responses in porcine adipocytes (Mills 1999). The more likely explanation is that the culture medium may interfere with the insulin response. In previous experiments, BSA has been shown to interfere with insulin response in pig adipose tissue slices (Walton & Etherton 1986, Mersmann & Hu 1987). For this reason, a BSA-free cell medium was used (Walton *et al.* 1986).

Donor site effects on insulin sensitivity

We have currently demonstrated that perirenal-derived adipocytes were less sensitive to insulin than s.c.-derived cells, in that the dose response to acute insulin challenge was shifted to the right by an order of magnitude for both

Figure 5 Effect of GH and insulin on FAS expression and SREBP-1 mRNA levels in adipocytes isolated from s.c. (SC) or perirenal (P) adipose tissue. At the start of culture, cells were incubated in a defined-medium without or with insulin (1.7 and 17 nM for adipocytes isolated from SC and P adipose tissue respectively) in the absence or presence of GH (1 nM). Measurements were performed after 2 days of culture. (A) Western blot of FAS protein ($n=4$ in P or SC adipose tissue). (B) FAS mRNA level was determined by real-time RT-PCR and quantified as described in Materials and Methods ($n=2$ in P or SC adipose tissue). (C) SREBP-1 mRNA level was determined by Northern blot analysis and densitometric values on the autoradiogram were quantified by image analysis ($n=5$ in P or SC adipose tissue). (D) SREBP-1 mRNA level was determined by real-time RT-PCR and quantified as described in Materials and Methods ($n=2$ in P or SC adipose tissue). Representative blots are shown, together with the mean quantification of the signals (associated with S.E.M.). Open bars: defined medium; black bars: defined medium supplemented with insulin alone; gray bars: defined medium supplemented with insulin and GH.

Figure 6 An additional experiment on the effect of GH and insulin on SREBP-1 protein. At the start of culture, adipocytes isolated from s.c. (SC) or perirenal (P) adipose tissue were incubated in a defined medium without or with insulin (1.7 and 17 nM for adipocytes isolated from SC and P adipose tissue respectively) in the absence or presence of GH (1 nM). Measurements were performed after 2 days of culture. SREBP-1 protein was detected by Western blot as described in Materials and Methods. Measurements were performed in duplicate.

glucose incorporation and FAS activity. Because cells were cultured under identical environmental conditions, it is likely that the observed difference between the two depots reflects a difference in intrinsic features of cells. These observations support regional differences in metabolic function of adipose tissue that have been reported by several investigators (Arner 1997). There are, for example, known differences in the lipolytic response to adrenergic stimulation between visceral and s.c. adipose tissue in human. The mechanisms underlying difference in lipogenic response in our study remain to be clarified. Differences in insulin sensitivity between donor sites could not be attributed to variation in s.c. and perirenal adipocyte sizes but could involve differences in cell surface insulin receptor number or affinity, as insulin itself regulates adipocyte sensitivity to the biological action of insulin by negative modulation of the number of cell surface insulin receptors (Marshall *et al.* 1984). Difference in the level of expression of insulin signaling molecules could also contribute to the reduced insulin sensitivity of perirenal adipocytes compared with other cells (Lefebvre *et al.* 1998).

Mechanism involved in GH and insulin actions on lipogenesis in isolated porcine adipocytes

In agreement with previous observations in pig adipose tissue (Walton *et al.* 1986) and 3T3-F442A murine adipocytes (Yin *et al.* 2001), we have found that GH antagonized the stimulatory effect of insulin on glucose incorporation into lipids and FAS activity in primary cultures of pig adipocytes isolated from both perirenal and s.c. adipose tissue. Changes in FAS mRNA and protein levels in the presence or absence of these hormones are observed in parallel. Studies performed in various mammalian species have led to the conclusion that FAS is controlled primarily at the level of gene transcription (Semenkovich 1997); however, the mechanism by which GH interacts with insulin action on FAS gene transcription has not been elucidated yet. We speculated that

SREBP-1c was a possible mediator of the action of insulin and GH on lipogenesis in porcine adipocytes. The SREBP-1 gene gives rise to two separate protein isoforms (SREBP-1a and SREBP-1c) through alternative splicing of the first exon (Brown & Goldstein 1997). The cDNA probe we used recognized both 1a and 1c isoforms; however, based on the relative levels of each isoform expression in mammalian tissues (Brown & Goldstein 1997), the observed signal corresponded very probably to SREBP-1c mRNA. The pattern of expression of SREBP-1 we observed in adipocytes, i.e. two bands of approximately 5 kb in length, is similar to that reported for pig adipose tissue (Ding *et al.* 2000, Gondret *et al.* 2001) and for freshly isolated rat adipocytes (Palmer *et al.* 2002).

In a situation of clear insulin stimulation or GH inhibition of FAS gene expression, protein content and catalytic activity, we failed to evidence any significant variation in SREBP-1 mRNA level in perirenal or s.c. isolated mature adipocytes. In line with our results, GH inhibits FAS gene expression at a step distal to the expression of SREBP-1 in differentiating primary rat adipocytes (Hansen *et al.* 1998). The absence of any apparent effect of insulin on SREBP-1 transcription contrasts with the general dogma that SREBP-1 is an important mediator of insulin action. It agrees, however, with recent findings reported in freshly isolated rat adipocytes under conditions where FAS expression is stimulated by insulin (Palmer *et al.* 2002). The basis of these discrepancies is not clear. A possible explanation is that the principal mechanism by which insulin stimulates lipogenesis differs between isolated mature adipocytes and adipocyte cell lines and/or between cell types. Indeed, the regulation of SREBP-1 has been studied mostly in 3T3-L1 adipocytes (Kim *et al.* 1998, Le Lay *et al.* 2002) and in rat isolated hepatocytes (Foretz *et al.* 1999a,b, Azzout-Marniche *et al.* 2000). One can also argue that the time selected for mRNA analysis in the current study (2 or 4 days after insulin addition) was not appropriate to show any effects of insulin on SREBP-1 mRNA, since many transcripts exhibit transient changes in response to a stimulus. However, this hypothesis is unlikely. First, even though a 2 h exposure to insulin is sufficient to induce a significant increase in SREBP-1 mRNA levels (Kim *et al.* 1998) in fully differentiated 3T3-L1 adipocytes, an increase in SREBP-1 expression is still observed after a 48 h exposure to insulin (Le Lay *et al.* 2002). Secondly, Palmer *et al.* (2002) failed to detect any change in SREBP-1 mRNA expression in isolated rat adipocytes despite the use of a shorter incubation time (4–6 h) with insulin. It is important to note that the time in our study was selected because our aim was to study GH effects on lipogenic genes. Interestingly, at least in hepatocytes, detectable amounts of nuclear SREBP-1c are still present after a period of 14 h without insulin (Azzout-Marniche *et al.* 2000). However, we did not detect any change in the level of the cleaved form of SREBP-1 protein in

response to insulin treatment, which confirmed, at least, the absence of variation observed in mRNA levels. However, we cannot exclude that insulin could act on SREBP-1 via post-translational mechanisms. A possible effect of insulin to induce phosphorylation of SREBP-1 protein on serine residues by mitogen-activated protein kinase was mentioned *in vitro* using transfection in a cell line (Kim *et al.* 1998, Kotzka *et al.* 1998, 2000). In cultured hepatocytes, SREBP-1c could rather be phosphorylated by downstream kinases of phosphatidylinositol-3 kinase (Foufelle & Ferré 2002); however, ongoing SREBP-1c synthesis is necessary to maintain a high level of SREBP-1c in both the membrane and nuclear compartment in rat hepatocytes (Azzout-Marniche *et al.* 2000). In conclusion, it appears unlikely that SREBP-1 transcription is involved in GH or insulin action on FAS activity in isolated porcine adipocytes. Whether both hormones could regulate SREBP-1 at a post-translational level in pig adipocytes remains to be demonstrated.

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