# Insulin-like growth factor I and insulin induce adipogenic-related gene expression in fetal brown adipocyte primary cultures

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Fetal rat brown adipocytes show high-affinity binding sites for both insulin-like growth factor I (IGF-I) and insulin. Cell culture for 24 h in the presence of IGF-I or insulin, independently, up-regulated the mRNA expression of adipogenic-related genes, such as fatty acid synthase (FAS), glycerol-3-phosphate dehydrogenase and insulin-regulated glucose transporter Glut4, and down-regulated the expression of phosphoenolpyruvate carboxykinase mRNA in a dose-dependent manner. Moreover, both IGF-I and insulin increased the FAS gene transcription rate at 2 h, producing a time-dependent accumulation of FAS mRNA. Furthermore IGF-I or insulin increased glucose uptake and lipid content throughout the 24 h culture period. Our results suggest that both IGF-I and insulin are major signals involved in initiating and/or maintaining the expression of adipogenic-related genes in fetal rat brown adipocytes.

## INTRODUCTION

Brown adipose tissue specializes in non-shivering thermogenesis and is also a major site for lipid metabolism. Rat brown adipocytes differentiate at the end of fetal life on the basis of two programmes: thermogenesis and adipogenesis. As regards adipogenesis, the expression of the adipogenic-related genes occurs as a multistep process: the fatty acid synthase (FAS) gene (the main lipogenic enzyme) is expressed as early as day 20 of fetal life, but its mRNA abundance significantly increases at the end of fetal development [1]. Expression of NADPH-generating enzymes (such as the malic enzyme and glucose-6-phosphate dehydrogenase) is initiated mainly on day 21 of fetal life, increasing significantly on day 22. This induction of lipogenic enzymes at the mRNA level correlates with an increase in the enzymic activities and lipogenic flux, producing an accumulation of lipids that results in the multilocular fat droplets phenotype before birth [1]. Glucose transport in adipose tissue is maintained mainly by the activity of the insulin-regulated glucose transporter (Glut4), its mRNA being detected during fetal life in brown adipose tissue and its expression increasing greatly during the last day of fetal life [1,2]. Thus fetal brown adipocytes are committed cells that accumulate lipids, but they are not terminally differentiated because they do not yet express the phosphoenolpyruvate carboxykinase (PEPCK) gene [1], a terminal differentiation marker for white adipose tissue [3], and can also be rescued for mitogenesis in response to specific mitogens in primary culture [4–6].

The adipogenesis process has been extensively investigated *in vitro* with models such as the 3T3-L1 and other preadipocyte cell lines [7], where pluripotential precursor stem cells differentiate to the mature adipocyte by converting from fibroblastic morphology to a spherical shape, with a time-course of expression of early and late mRNA markers and triacylglycerol accumulation (recently reviewed in reference [8]). Several hormonal signals, such as growth hormone, glucocorticoids, thyroid hormone and insulin-like growth factor I (IGF-I), have been shown to regulate adipocytic differentiation when tested *in vitro* [8–12].

Although the precise nature of the factors involved in the adipogenic differentiation of fetal brown adipocytes remains unknown, previous findings have demonstrated a positive role for both tri-iodothyronine and insulin in inducing lipogenesis, malic enzyme mRNA accumulation and enzymic activity in primary fetal brown adipocytes [13–15]. Furthermore fetal brown adipocytes in primary culture expressed large quantities of IGF-I receptor mRNA and cells bore a high number of high-affinity IGF-I-binding sites per cell [5]. These observations prompted us to examine the role of IGF-I and insulin in the expression of a set of adipogenic-related genes such as FAS, glycerol-3-phosphate dehydrogenase (G3PD) and Glut4, as well as in cytosolic lipid content and glucose uptake in primary cultures of fetal brown adipocytes. Our results show that IGF-I or insulin, acting independently, induced the expression of adipogenic-related genes and increased the FAS gene transcription rate, producing an accumulation of FAS mRNA in a time- and dose-dependent manner, and also increasing the cytosolic lipid content. At the same time IGF-I or insulin increased the expression of Glut4 mRNA and the uptake of glucose, inhibiting the constitutive expression of PEPCK mRNA.

## MATERIALS AND METHODS

### Materials

IGF-I was from Austral Biologicals (San Ramon, CA, U.S.A.). Insulin, actinomycin D and BSA (fraction V, essentially fatty acid free) were from Sigma (St. Louis, MO, U.S.A.). Fetal calf serum, PBS and culture media were from Imperial Laboratories (Hampshire, U.K.). Collagenase was from Boehringer (Mannheim, Germany). RNazol B was from Biotecx Lab (Dallas, TX, U.S.A.). Nylon membranes were GeneScreen® (NEN Research Products, Boston, MA, U.S.A.). Autoradiographic films were Kodak X-O-MAT/AR (Eastman Kodak Co, Rochester, NY, U.S.A.). $^{125}$I-labelled IGF-I (80 $\mu$Ci/$\mu$g), $^{125}$I-labelled insulin (80 $\mu$Ci/$\mu$g), 2-deoxy-D-[1-$^{3}$H]glucose (11.0 Ci/mmol), [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol), [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol) and the multiprimer DNA-labelling system kit were purchased from

Abbreviations used: FAS, fatty acid synthase; G3PD, glycerol-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; Glut4, insulin-regulated glucose transporter; MEM, minimal essential medium with Earle's salts; PEPCK, phosphoenolpyruvate carboxykinase.

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Amersham (Bucks., U.K.). All other reagents used were of the purest grade available. The cDNAs used as probes were FAS [16], G3PD [17], Glut4 [18], PEPCK [19] and $\beta$-actin [20].

### Cell culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day Wistar rat fetuses and isolated by collagenase dispersion as described [13]. Cells were plated at $1.5 \times 10^6$ cells per 60 mm tissue-culture dish in 2.5 ml of minimal essential medium with Earle's salts (MEM) supplemented with 10 % (v/v) fetal calf serum. After 4–6 h of culture at 37 °C, cells were rinsed twice with PBS and 70 % of the initial cells were attached to the dish forming a monolayer (time 00 of culture). Cells were maintained for 20 h in a serum-free medium supplemented with 0.2 % BSA (time 0 of culture), as described [5] This time defines the starting point for IGF-I or insulin stimulation. Cells were further cultured for 1, 2, 3, 6 and 24 h in the absence or presence of either IGF-I (1.4 or 14 nM) or insulin (1 or 10 nM). In some experiments the MEM was supplemented with glucose to a final concentration of 20 mM. In the experiments performed in the presence of actinomycin D (5 $\mu$g/ml), cells were pretreated for 14 h with the drug before stimulation with IGF-I or insulin. In all the experiments described here, the quality and purity of brown adipocyte primary cultures were tested by the detection of uncoupling protein mRNA and/or by the immunological identification of uncoupling protein, as previously described [1,5].

### Binding of $^{125}$I-labelled IGF-I and $^{125}$I-labelled insulin

Cells cultured for 20 h in a serum-free medium were incubated for 3 h at 20 °C with 0.03 nM $^{125}$I-labelled IGF-I or with 0.03 nM $^{125}$I-labelled insulin, in 1 ml of binding buffer containing 25 mM Hepes/PBS, pH 7.4, and 1 mg/ml BSA in the absence or presence of graded concentrations of unlabelled IGF-I or insulin. At the end of incubation, monolayers were rinsed either with ice-cold PBS/BSA or with ice-cold 0.3 M sodium acetate, pH 4.5 (containing 0.15 M NaCl), two more times with PBS/BSA and then dissolved in 0.1 M NaOH/1 % SDS/2 % $Na_2CO_3$, as previously described [21]. Radioactivity was counted in a Packard $\gamma$-counter. The radioactivity associated with cells subjected to an acid wash (representing internalized $^{125}$I-labelled ligand) was negligible (less than 7 %). Non-specific binding was defined as the radioactivity that remained bound in the presence of a 1000-fold excess of unlabelled ligand. Bound/free plots and Scatchard plots were calculated for triplicate dishes from three independent experiments, as described [5,21].

### Flow cytometric analysis of Nile Red fluorescence

Cytoplasmic lipid content was determined by Nile Red fluorescence emission 530 (BP 530/30 nm) in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Cells were detached from dishes by the addition of 0.05 % trypsin/0.02 % EDTA, and lipid content was determined in aliquots of $2 \times 10^5$ cells after the addition of Nile Red (0.1 $\mu$g/ml) [22]. Results represent mean intensities of fluorescence (obtained from the histograms of numbers of cells against intensity of fluorescence) and are expressed in arbitrary units.

### RNA extraction and analysis

At the end of the culture period, adipocytes were lysed directly with RNazol B following the protocol supplied by the manufacturer for total RNA isolation [23]. Total cellular RNA (10 $\mu$g) was subjected to Northern blot analysis, i.e. subjected to electrophoresis on 0.9 % agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen® membranes with a VacuGene blotting apparatus (LKB-Pharmacia, Uppsala, Sweden) and cross-linked to the membranes by UV irradiation. Hybridization was in 0.25 mM sodium phosphate, pH 7.2, containing 0.25 M NaCl, 100 $\mu$g/ml denatured salmon sperm DNA, 7 % (w/v) SDS, 50 % deionized formamide and denatured $^{32}$P-labelled cDNA ($10^6$ c.p.m./ml) for 40 h at 42 °C, as described [24]. Labelling of cDNA was performed with [$\alpha$-$^{32}$P]dCTP to a specific radioactivity of $10^9$ c.p.m. per $\mu$g of DNA by using a multiprimer DNA-labelling system kit. For serial hybridization with different probes, the blots were stripped and rehybridized sequentially as needed in each case. The resulting membranes were subjected to autoradiography for 1–3 days. Relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Each Northern blot analysis was performed on duplicate samples from three independent experiments.

### Nuclear run-on transcription assay

At the end of the culture period eight dishes were pooled for each experimental determination. Cells were washed twice and harvested in 2.5 ml of ice-cold PBS and centrifuged at 500 ***g*** for 15 min. All the subsequent procedures were performed at 4 °C. Nuclei were prepared as described [25]. The run-on transcription assay was performed with [$\alpha$-$^{32}$P]UTP. As an initial purification step, the $^{32}$P-labelled RNA was subjected to a phenol/chloroform extraction procedure by the addition of 3 vol. of solution D (4 M guanidine isothiocyanate/25 mM sodium citrate/0.5 % sarcosyl/0.72 % 2-mercaptoethanol) and 0.1 vol. of chloroform/isoamyl alcohol (49:1, by vol.). After vortexing for 1 min, the solution was incubated on ice for 30 min before phase separation by centrifugation at 500 ***g*** for 5 min. The aqueous phase was removed, and the RNA was precipitated with an equal volume of ice-cold isopropanol overnight at −20 °C. After centrifugation, the RNA pellet was resuspended in 500 $\mu$l of solution D, and then reprecipitated as described above. Finally, the RNA pellet was resuspended in 50 $\mu$l of water. Any unincorporated radiolabel was separated by centrifugation through a Sephadex G-50 spin column equilibrated in STE (0.1 M NaCl/1 mM Tris/HCl, pH 8.0). The incorporation of [$\alpha$-$^{32}$P]UTP into RNA was determined by scintillation counting, and equal radiioactive counts ($6 \times 10^6$ c.p.m./ml) were hybridized to filter-immobilized plasmids (2 $\mu$g) as described for Northern blotting. The target plasmids contained FAS cDNA and $\beta$-actin cDNA, as well as the corresponding vector pUC12. Plasmids were linearized by digestion with restriction enzymes, denatured by incubation with 0.3 M NaOH for 1 h at 65 °C, neutralized and then applied to nylon filters. After hybridization, the filters were washed twice with 2 × SSC/0.1 % SDS at room temperature for 10 min, and then twice with 0.1/SSC/0.1 % SDS at 42 °C for 30 min before autoradiography for 4 days. Quantification of the signals was performed by densitometric scanning of the slots; signals were standardized with $\beta$-actin signal. Run-on analysis was performed on triplicate samples from three independent experiments.

### Measurement of 2-deoxyglucose transport

Cells were serum-deprived for 20 h and cultured for a further 24 h in the absence or presence of both IGF-I (1.4 nM) and insulin (1 nM), together with 500 nCi/ml of 2-deoxy-D-[1-$^3$H]-

glucose. After culture, cells were washed three times with ice-cold Krebs–Ringer phosphate buffer [13 mM NaCl/5.4 mM KCl/1.4 mM $CaCl_2$/1.4 mM $MgSO_4$/10 mM sodium pyrophosphate (pH 7.4)] and solubilized in 1 ml of 1% SDS, as described [26]. The radioactivity of a 200 $\mu$l aliquot was determined in a scintillation counter. Glucose transport was determined in triplicate dishes from three independent experiments.

## RESULTS

### Fetal brown adipocytes bind IGF-I and insulin with high affinity

Fetal brown adipocytes (20 h serum-deprived) showed saturable IGF-I- and insulin-specific binding as depicted in the bound/free plots shown in Figure 1. The receptors for IGF-I displayed a dissociation constant ($K_d$) of 4.8 nM and a high number of binding sites per cell (190000), as calculated from the Scatchard plot (Figure 1). The receptors for insulin displayed a $K_d$ of 18.8 nM and 40000 binding sites per cell, as calculated from the Scatchard plot (Figure 1). These results indicated that fetal brown adipocytes in culture were potential targets for both IGF-I and insulin.

### Both IGF-I and insulin up-regulate the expression of adipogenic genes and down-regulate the expression of PEPCK in fetal brown adipocytes

Fetal brown adipocytes (after 4 h of attachment and culture for 20 h in a serum-free medium) were cultured for a further 24 h, both in the absence and the presence of IGF-I (1.4 or 14 nM) or insulin (1 or 10 nM). The expression of a set of genes involved in adipogenesis, such as FAS (the main lipogenic marker), G3PD (an esterification marker) and Glut4, together with the expression of PEPCK (a gene negatively regulated by insulin in 3T3-L1 cells and in white adipose tissue) [27,28], was studied by Northern blot, as depicted in the representative experiment shown in Figure 2; the resulting densitometric analysis is shown in Table 1. Both FAS and G3PD mRNA species were constitutively expressed in brown adipocytes before culture, but their expression decreased gradually after culture for 48 h in a serum-free medium.

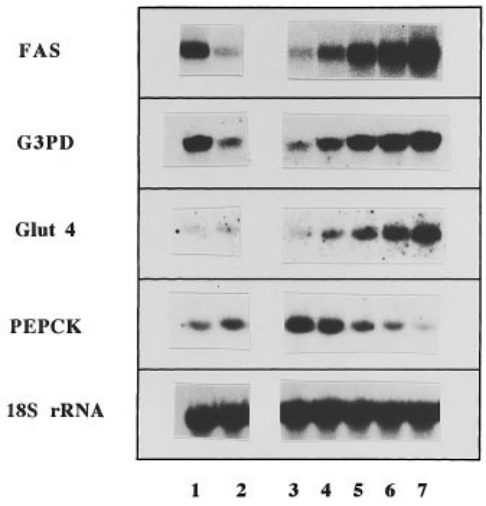


**Figure 2 IGF-I and insulin up-regulate the expression of adipogenic genes and down-regulate the expression of PEPCK in fetal brown adipocytes**

Cells at time 00 of culture (lane 1) were serum-deprived for 20 h (lane 2) and cultured for a further 24 h in a serum-free medium, in the absence (lane 3) or presence of IGF-I (1.4 or 14 nM) (lanes 4 and 5 respectively) or insulin (1 or 10 nM) (lanes 6 and 7 respectively). Total RNA (10 $\mu$g) was subjected to Northern blot analysis and hybridized with labelled FAS, G3PD, Glut4 or PEPCK cDNAs. A final hybridization with 18 S rRNA cDNA was performed for normalization. Autoradiograms are shown from a representative experiment of three.

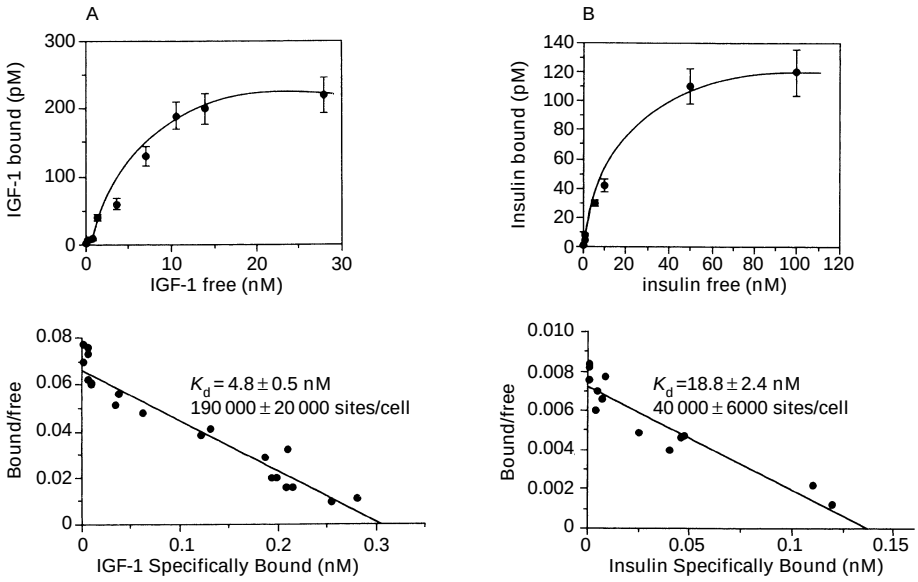


**Figure 1 Specific binding of (A) $^{125}$I-labelled IGF-I and (B) $^{125}$I-labelled insulin in primary cultures of fetal brown adipocytes**

Brown adipocytes, after being cultured for 20 h in a serum-free medium, were incubated for 3 h at 20 °C with either $^{125}$I-labelled IGF-I or $^{125}$I-labelled insulin in the absence or presence of graded concentrations of unlabelled ligand, for receptor binding analysis. Bound/free plots represent means ± S.E.M. ($n$ = 9) for three independent experiments. $K_d$ and binding sites were calculated from the corresponding Scatchard plots as described in the Materials and methods section.

**Table 1 Densitometric analysis of mRNA expression in fetal brown adipocytes**

Autoradiograms from Figure 2 were submitted to densitometric analysis and normalized with the amount of 18 S rRNA. The content of mRNA is expressed in arbitrary units (100 × density of band divided by density of 18 S rRNA), and results are means ± S.E.M. ($n = 6$) for duplicate samples of three independent experiments. The abbreviations are as in Figure 2. Data for FAS mRNA content at 24 h in C, $I_{1.4}$ and $ins_1$ columns include results from Figures 2, 3 and 4 ($n = 18$).

| Time (h) ... | 00 | 0 | 24 | | | | |
|---|---|---|---|---|---|---|---|
| Treatment ... | C | C | C | $I_{1.4}$ | $I_{14}$ | $ins_1$ | $ins_{10}$ |
| FAS | 21 ± 3 | 7 ± 1 | 6 ± 1 | 18 ± 2 | 26 ± 3 | 39 ± 3 | 49 ± 5 |
| G3PD | 20 ± 3 | 7 ± 2 | 6 ± 1 | 11 ± 2 | 15 ± 2 | 21 ± 3 | 25 ± 4 |
| Glut4 | 2 ± 0.2 | 2 ± 0.2 | 2.5 ± 0.2 | 7 ± 0.8 | 10 ± 1 | 18 ± 2 | 24 ± 3 |
| PEPCK | 5 ± 0.9 | 8 ± 1 | 18 ± 3 | 14 ± 2 | 7 ± 1 | 5 ± 1 | 2 ± 0.7 |

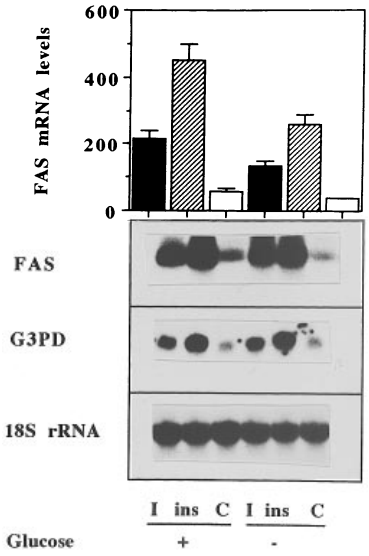


**Figure 3 Effects of glucose and IGF-I or insulin on the expression of FAS and G3PD mRNA species**

Brown adipocytes after serum deprivation for 20 h were cultured for 24 h in MEM alone (−) or supplemented with glucose to a final concentration of 2 mM (+), in the absence (lanes C) or presence of 1.4 nM IGF-I (lanes I) or 1 nM insulin (lane ins). Total RNA (10 $\mu$g) was subjected to Northern blot analysis and hybridized with labelled FAS, G3PD or 18 S rRNA cDNAs. Densitometric analysis of FAS mRNA levels after standardization with the 18 S rRNA signal is shown in the upper panel. Results (arbitrary densitometric units) are means ± S.E.M. ($n = 6$) for three independent experiments.

However, on treatment for 24 h with IGF-I (1.4 or 14 nM) and insulin (1 or 10 nM), a significant accumulation in FAS and G3PD mRNA species occurred, relative to untreated cells, this effect being higher for insulin than for IGF-I, and dose-dependent (Figure 2 and Table 1). Glut4 mRNA was almost undetectable either in cells before culture or in cells cultured for 48 h in a serum-free medium. However, the presence of IGF-I or insulin for 24 h significantly up-regulated the expression of Glut4 gene, this effect being dose-dependent for each signal. In addition, insulin was more potent than IGF-I in inducing Glut4 mRNA accumulation (Figure 2 and Table 1). Finally, the blots were also hybridized with PEPCK cDNA. PEPCK mRNA was expressed

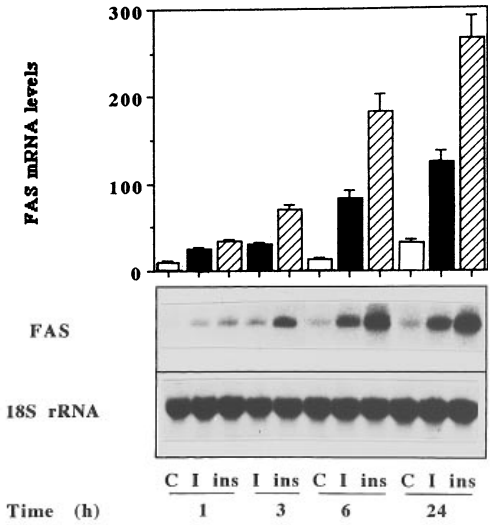


**Figure 4 Time-course of induction of FAS mRNA by IGF-I or insulin in fetal brown adipocytes**

Brown adipocytes after serum deprivation for 20 h were cultured for 1, 3, 6 and 24 h, in the absence (lanes C) or presence (lanes I) of 1.4 nM IGF-I or 1 nM insulin (lanes ins). Total RNA (10 $\mu$g) was subjected to Northern blot analysis and hybridized with labelled cDNA species for FAS and 18 S rRNA. Densitometric analysis of FAS mRNA levels after standardization with the 18 S rRNA signal is shown in the top panel. Results (arbitrary densitometric units) are means ± S.E.M. ($n = 6$) from three independent experiments.

only at low level before culture, but its expression increased significantly during culture for 48 h in a serum-free medium. However, the presence of IGF-I or insulin for the last 24 h of culture down-regulated PEPCK mRNA accumulation, in a dose-dependent manner, the inhibitory effect of insulin being higher than that of IGF-I (Figure 2 and Table 1).

It has been shown previously in cultures of white adipose tissue and isolated hepatocytes of suckling rats that glucose alone is able to induce FAS expression whereas insulin has no effect alone but potentiates the effect of glucose by enhancing its metabolism [28–30]. To test this hypothesis we cultured brown adipocytes in MEM either containing basal glucose (5 mM) or supplemented with glucose to a final concentration of 20 mM. On treatment for 24 h with 1.4 nM IGF-I, 1 nM insulin or neither hormone, under both glucose concentrations, we studied the expression of FAS and G3PD mRNAs by Northern blot (Figure 3). The presence of 20 mM glucose in the culture medium doubled the FAS mRNA content observed in cells cultured under basal glucose conditions, in the absence of hormones. However, the induction of FAS mRNA expression produced by IGF-I (3-fold) and by insulin (7-fold) compared with untreated cells was similar under both glucose concentrations. No effect was observed by 20 mM glucose alone on the expression of G3PD compared with the basal glucose conditions in the culture medium (Figure 3).

### IGF-I and insulin increase the FAS gene transcription rate

The positive effect of IGF-I or insulin on FAS mRNA accumulation observed after 24 h of hormonal treatment (Figures 2 and 3) was further investigated over a shorter period in a time-dependent Northern blot (Figure 4). Accumulation of FAS mRNA on treatment with insulin or IGF-I was observed as early as 1 h, the effect of both signals on FAS mRNA expression being

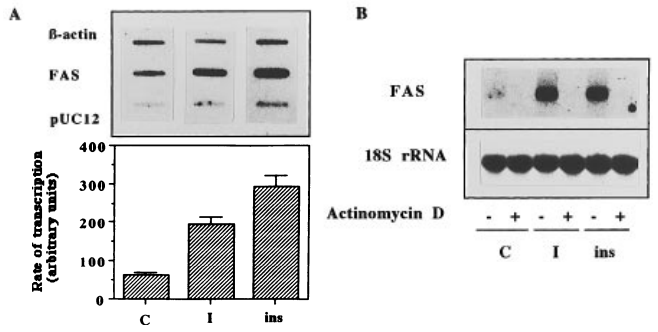


**Figure 5 IGF-I and insulin both increase FAS gene transcription rate, and actinomycin D precludes their effects on mRNA accumulation**

(**A**) After culture for 20 h in serum-free medium (lane C), cells were treated for 2 h with 1.4 nM IGF-I (lane I) or 1 nM insulin (lane ins). Nuclei were prepared and used for run-on transcription assay as described in the Materials and methods section. Representative autoradiograms of labelled transcripts representing FAS, $\beta$-actin and the vector PUC12 are shown. Densitometric analysis after standardization with the $\beta$-actin signal is shown in the lower panel. Results are expressed as arbitrary units and are means $\pm$ S.E.M. ($n = 9$) for three independent experiments. (**B**) After culture for 20 h in serum-free medium, brown adipocytes were pretreated for 14 h with actinomycin D (5 $\mu$g/ml) and further treated for 6 h in the presence (+) of actinomycin D with 1.4 nM IGF-I (lane I), 1 nM insulin (lane ins) or neither hormone (lane C), and compared with brown adipocytes under an identical protocol in the absence of actinomycin D (−). Total RNA (10 $\mu$g) was subjected to Northern blot analysis and hybridized with labelled cDNA species for FAS and 18 S rRNA. A representative autoradiogram of three independent experiments is shown.

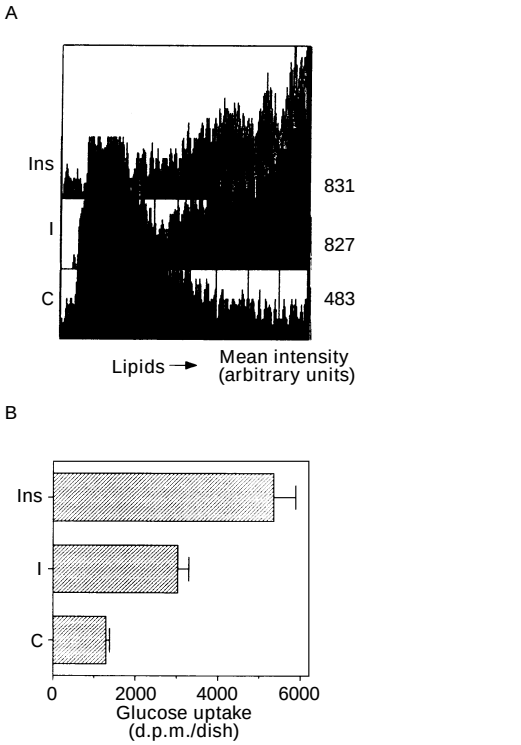


**Figure 6 IGF-I and insulin increase cytoplasmic lipid content and glucose uptake in fetal brown adipocytes**

Cells were serum-deprived for 20 h and further cultured for 24 h in the absence (lanes C) or presence of 1.4 nM IGF-I (lanes I) or 1 nM insulin (lanes Ins). (**A**) Cytoplasmic lipid content was determined by Nile Red fluorescence at the end of culturing. Mean intensities of Nile Red fluorescence from a representative experiment of three are expressed in arbitrary units. (**B**) Glucose uptake was measured throughout the last 24 h of culturing as described in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 9$) for three independent experiments and are expressed as d.p.m. per dish.

time-dependent (Figure 4). These results prompted us to determine the FAS gene transcription rate (Figure 5A). The transcription rate of the FAS gene was increased by 3- and 5-fold respectively on treatment for 2 h with IGF-I (1.4 nM) or insulin (1 nM), compared with untreated cells. Furthermore actinomycin D totally prevented the effect of IGF-I or insulin on the accumulation of FAS mRNA (Figure 5B). Likewise, IGF-I (1.4 nM) increased the FAS transcription rate at 2 h (by 3-fold) and the FAS mRNA accumulation at 24 h (by 3-fold), the effect of insulin (1 nM) on the FAS transcription rate and the FAS mRNA accumulation rate being higher (5- and 7-fold, respectively) (Figures 5 and 4).

### IGF-I and insulin increase lipid content and glucose uptake in fetal brown adipocytes

Because IGF-I and insulin up-regulated the expression of adipogenic genes, we studied Nile Red fluorescence (a sensitive detector of cytoplasmic lipid content) [31] to assess the role of those signals in the adipogenic programme. Brown adipocytes (20 h serum-deprived) were further treated for 24 h in the absence and presence of IGF-I (1.4 nM) or insulin (1 nM), and Nile Red fluorescence was analysed and quantified in a flow cytometer as shown in Figure 6A. Cells cultured in the absence of serum and signals added showed a peak of low fluorescence of Nile Red with a long tail. The mean Nile Red fluorescence shifted to a high intensity on treatment with IGF-I or insulin, indicating a doubling of the lipid content.

Because glucose is the main substrate for lipid synthesis in brown adipose tissue, and brown adipocytes in culture up-regulated the expression of Glut4 by IGF-I/insulin, we were prompted to evaluate the effect of IGF-I and insulin on glucose uptake. On treatment with IGF-I (1.4 nM) or insulin (1 nM) for 24 h, brown adipocytes increased glucose uptake by 2.5- and 4-fold respectively compared with the basal glucose uptake (Figure 6B).

## DISCUSSION

Our previous findings have suggested that IGF-I might have a role in leading brown adipose tissue to differentiate before birth [1]. In fact, 20-day fetal brown adipocytes before culture were already expressing adipogenic-related genes (FAS and G3PD), although on culture for 48 h in a serum-free medium the expression of these genes decreased markedly. The results reported here demonstrate that both IGF-I and insulin, acting independently, up-regulate the expression of adipogenic-related genes, in a dose- and time-dependent manner, in primary cultures of fetal brown adipocytes. These effects could be accounted for by the co-existence of IGF-I and insulin binding sites in fetal brown adipocytes, whereas in 3T3-L1 cells (the best characterized model for adipocyte differentiation *in vitro*) IGF-I receptors are present in pre-adipocytes (in the absence of insulin receptors); however, insulin receptors are fully expressed in terminally differentiated cells (in the absence of IGF-I receptors) [8]. In addition, IGF-I and insulin, at physiological concentrations, increased FAS gene expression at the transcriptional level. These results strongly support previous evidence of IGF-I/insulin response elements on the FAS gene promoter [11,12]. Furthermore the accumulation of FAS mRNA observed under the

basal glucose concentration (5 mM) in the culture medium is nearly doubled in the presence of a high concentration of glucose (20 mM), an effect that is still obvious in the presence of insulin or IGF-I. These results are comparable to those reported previously in cultures of white adipose tissue [29]. Accordingly, we cannot exclude the possibility that insulin and IGF-I have a potentiating effect on glucose-induced FAS mRNA expression in fetal brown adipocytes. Moreover, brown adipocytes accumulate cytoplasmic lipids in response to IGF-I and insulin. These results support the idea that both IGF-I and insulin have a role in the regulation of adipogenic-related gene expression in fetal brown adipocytes [1,32].

The expression of adipogenic-related genes induced by IGF-I or insulin in fetal brown adipocytes is accompanied by an up-regulation of Glut4 mRNA expression, resulting in increased glucose uptake. As mentioned above, Glut4 mRNA is readily detectable in brown adipose tissue just before birth (day 22 of fetal life). Consequently, 20-day fetal brown adipocytes did not express Glut4 mRNA, nor did they express it throughout culture in the absence of serum and added exogenous signals. However, Glut4 mRNA was induced at high levels on treatment with IGF-I or insulin for 24 h. Although an induction of Glut4 mRNA expression by dexamethasone has been reported [33], this paper describes the first evidence of an effect of IGF-I and insulin on Glut4 gene expression in rat brown adipocytes. In contrast, insulin suppressed the expression of Glut4 mRNA in differentiated 3T3-L1 adipocytes [34], although the expression of Glut4 mRNA is positively regulated by insulin in rat adipose tissue *in vivo* [35]. Despite the fact that the increase in glucose uptake induced by insulin in fat tissues refers to insulin-dependent movement of Glut4 protein from an intracellular membrane compartment to the cell surface, this effect occurs as an early event upon the insulin binding to its receptors. However, we found an increased glucose uptake throughout 24 h upon IGF-I and insulin treatment, probably due to the increased accumulation of Glut4 mRNA in fetal brown adipocytes.

Finally, PEPCK mRNA spontaneously accumulated during the culture of brown adipocytes in a serum-free medium, with a converse pattern when compared with lipogenic enzymes mRNAs. In addition, we have established that PEPCK gene expression is down-regulated by IGF-I and insulin in brown adipocytes, in a similar fashion to that already described for insulin in 3T3-L1 cells and in white adipose tissue [27,28], suggesting the occurrence of IGF-I and insulin response elements on the PEPCK promoter in brown adipocytes.

In conclusion, our results strongly suggest that both IGF-I and insulin are important signals involved in initiating and/or maintaining the expression of adipogenic-related genes in rat fetal brown adipocytes. However, our results also indicate that insulin is a more potent signal than IGF-I with respect to the adipogenic-related parameters studied, despite the fact that it has fewer binding sites with lower specific affinity. Thus we propose that, although the differential signalling cascades between insulin and IGF-I remain unknown, insulin might be the major factor leading to adipogenesis in fetal rat brown adipocytes.

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