Transforming growth factor β_1 induces differentiation-specific gene expression in fetal rat brown adipocytes

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Received 30 March 1995

Abstract **Fetal rat brown adipocytes show a low number of transforming growth factor β_1 (TGF-β_1) binding sites of high affinity, revealing the presence of type I, II and III TGF-β_1 receptors and a minor-labeled species of approximately 140 kDa. The culture of cells in the presence of TGF-β_1 induced the expression of the tissue-specific gene uncoupling protein in a dose- and time-dependent manner. In addition, TGF-β_1 up-regulates the expression of genes involved in adipogenesis such as fatty acid synthase, glycerol 3-phosphate dehydrogenase, malic enzyme and glucose 6-phosphate dehydrogenase, as well as induces the expression of fibronectin (specific target gene for TGF-β_1). Our results suggest that TGF-β_1 is a major signal involved in initiating and/or maintaining the thermogenic and adipogenic differentiation of rat fetal brown adipocytes.**

Key words: TGF-β_1; Differentiation; Uncoupling protein; Lipogenic enzyme; Fibronectin

1. Introduction

Brown adipose tissue is a major site for lipid metabolism specialized in the non-shivering thermogenesis required to address the physiological hypothermia in the newborn mammal. The development of brown adipose tissue in the rat occurs mainly during the perinatal period and relies on two differentiation programs: the adipogenic program related to lipid synthesis and the thermogenic-program related to the induction of the tissue-specific gene uncoupling protein (UCP), in order to yield an identificable and functional tissue at birth [1]. In addition, fetal brown adipocyte primary cultures have been successfully used in differentiation studies over the last few years [2–9]. Although the nature of the circulating hormones and/or paracrine-autocrine factors involved in the fetal development of brown adipose tissue remain unknown, we have recently described, in fetal brown adipocyte primary cultures, that triiodothyronine directly induces the expression of UCP in the absence of noradrenergic stimulation [8], and also that insulin-like growth factor I induces the expression of UCP in a dose- and time-dependent manner [7,9], suggesting that both signals may be major players in the differentiation of brown adipose tissue during late fetal life. Other possible signals involved in brown adipocyte differentiation remain unexplored.

Transforming growth factor β_1 (TGF-β_1) belongs to a superfamily of multifunctional factors involved in a variety of cellular processes, including stimulation/inhibition of cell growth or differentiation, morphological transformation of the cell, and transcriptional activation of genes encoding extracellular matrix proteins [10,11]. TGF-βs are produced by many different cell types and work on a broad spectrum of target cells. Depending on cell type and culture conditions, TGF-βs either stimulate or inhibit cell differentiation. In NIH-3T3 fibroblasts and skin fibroblasts, TGF-β_1 stimulated the production of fibronectin, elastin and collagen, respectively [12,13]. However, TGF-β_1 prevented early differentiation of 3T3-L1 preadipocytes [14] and 3T3 T proadipocytes [15]. Strikingly, TGF-β_1 blocks early but not late differentiation-specific gene expression and morphological differentiation of those cells [14,15]. However, no effects of TGF-β_1 on brown adipocyte differentiation have yet been described.

TGF-β_1 is a 25-kDa homodimer [16] that binds with high affinity to three distinct integral membrane glycoproteins on the surface of target cells, identified by cross-linking to labeled TGF-β_1 and named type I (53 kDa), type II (75 kDa) and type III (280 kDa) TGF-β_1 receptors, respectively [17,18]. TGF-β_1 binds to type II receptor, a transmembrane protein with a constitutively active serine/threonine kinase domain which uses the ligand to recruit, phosphorylate and signal through receptor I [19–21]. TGF-β_1 receptors have been identified in cells derived from a variety of origins such as connective, epithelial, chondrogenic, myogenic, adipogenic and hematopoietic tissues [14,18,22]. Moreover, the occurrence of TGF-β_1 receptors in brown adipose tissue has not yet been reported.

Accordingly, the main objective of this work was to investigate the occurrence of TGF-β_1 receptors in brown adipocytes and its role in controlling the expression of differentiation-related genes upon ligand binding. Our results show that TGF-β_1, upon binding to specific receptors characterized by cross-linking analysis, induces the expression of the tissue-specific gene UCP in a dose- and time-dependent manner, monitoring a thermogenic differentiation program. Concurrently, TGF-β_1 up-regulates the expression of adipogenic-related genes and the extracellular matrix gene fibronectin.

2. Materials and methods

2.1. Cell culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day fetuses of Wistar rats and isolated by collagenase dispersion as described [2]. Cells were seeded at 1×10^6 cells/60 mm tissue culture dishes in 2.5 ml of medium Eagle's modified (MEM) supplemented with 10% FCS (Imperial Laboratories, Hampshire, UK). After 4–6 h of culture at 37°C, cells were rinsed twice with phosphate

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Abbreviations: TGF-β_1, transforming growth factor β_1; UCP, uncoupling protein; FAS, fatty acid synthase; ME, malic enzyme; G6PD, glucose 6-phosphate dehydrogenase; G3PDH, glycerol 3-phosphate dehydrogenase; PBS, phosphate buffered saline.

SSDI 0014-5793(95)00385-1

buffered saline (PBS) to remove hematopoietic cells: approximately 60% 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% (w/v) BSA (time 0 of culture), as described [7]. This time defines the starting point for TGF-β_1 stimulation. Cells were further cultured for 30 min, 2, 4, 8 and 24 h in a serum-free medium in the absence or presence of TGF-β_1 (100 pM, except in dose–response experiments) (Austral Biologicals, San Ramón, CA).

2.2. [^{125}I]TGF-β_1 binding

Cells cultured for 20 h in a serum-free medium were incubated for 3 h at 20°C with 2 pM [^{125}I]TGF-β_1 (66 μCi/μg) (Amersham, Buckinghamshire, UK) in 1 ml of binding buffer containing 25 mM HEPES-PBS and 1 mg/ml BSA, in the absence or presence of graded concentrations of unlabeled TGF-β_1. Triplicate dishes were used for each data point. At the end of the 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 solubilized in 0.1 N NaOH-1%, SDS-2%, Na_2CO_3. Radioactivity was counted in a Packard γ-counter. The radioactivity associated with cells submitted to acid wash, representing internalized [^{125}I]TGF-β_1, was negligible (<5%). Total binding in the absence of competing ligand was 45% of the radioactivity added. Non-specific binding was defined as radioactivity that remained bound in the presence of a 1000-fold excess of unlabeled TGF-β_1, and represented approximately 40% of the total binding. Maximum specific binding was approximately 60% of the total binding. Bound vs. free plot and Scatchard plot were calculated from 3 independent experiments.

2.3. [^{125}I]TGF-β_1 affinity labeling and cross-linking

The identification of receptors of TGF-β_1 was performed according to Massagué [23] with minor modifications. Cells in monolayer were incubated with 480 pM [^{125}I]TGF-β_1 as described above in the binding protocol, either in the absence or presence of a 1000-fold excess of unlabeled TGF-β_1. After incubation, the monolayer was washed 3 times with binding buffer with a final wash in the absence of BSA, and the cross-linking reaction followed in 2 ml of BSA-free binding buffer with 0.25 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) freshly prepared, at 4°C for 15 min with gentle shaking. Finally, the cells were washed and solubilized in Triton buffer (125 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, pH 7.4). After 30 min incubation at 4°C, the detergent-soluble fraction was separated by centrifugation and analysed by 6% SDS-PAGE. Subsequently, the TGF-β_1 binding proteins were identified by autoradiography.

2.4. RNA extraction and analysis

At the end of the culture time, cells were rinsed with ice-cold PBS and the monolayer was lysed directly with RNazol B (Biotecx Lab, Dallas, TX) as described [24]. For brown adipocyte primary cultures the yield was 7–10 μg of RNA per 60-mm tissue culture dishes. Total cellular RNA (10 μg) was submitted to Northern blot analysis, being electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen membranes (New England Nuclear Research Products, Bedford, MA) using a VacuGene blotting apparatus (LKB, Pharmacia, Uppsala, Sweden) and cross-linked to the membranes by UV light. Hybridization was in 0.25 mM $NaHPO_4$, pH 7.2, 0.25 M NaCl, 100 μg/ml denatured salmon sperm DNA, 7% SDS and 50% deionized formamide, containing denatured ^{32}P-labeled cDNA (10^6 cpm/ml) for 40 h at 42°C as described [25]. cDNA labeling was carried out with [α-^{32}P]dCTP to a specific activity of 10^9 cpm/μg of DNA by using a multiprimer DNA labeling system kit (Amersham, Buckinghamshire, UK). For serial hybridization with different probes, the blots were stripped and rehybridized sequentially as needed in each case. Probes utilised included β-actin (Oncor, Gaithersburg, MD) [26], UCP [27], glucose 6-phosphate dehydrogenase (G6PD) [28], fatty acid synthase (FAS) [29], glycerol 3-phosphate dehydrogenase (G3PDH) [30], malic enzyme (ME) [31] and fibronectin [32]. The resulting membranes were subjected to autoradiography for 1–3 days with Kodak X-O-MAT/AR films (Eastman Kodak Co., Rochester, NY). Relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

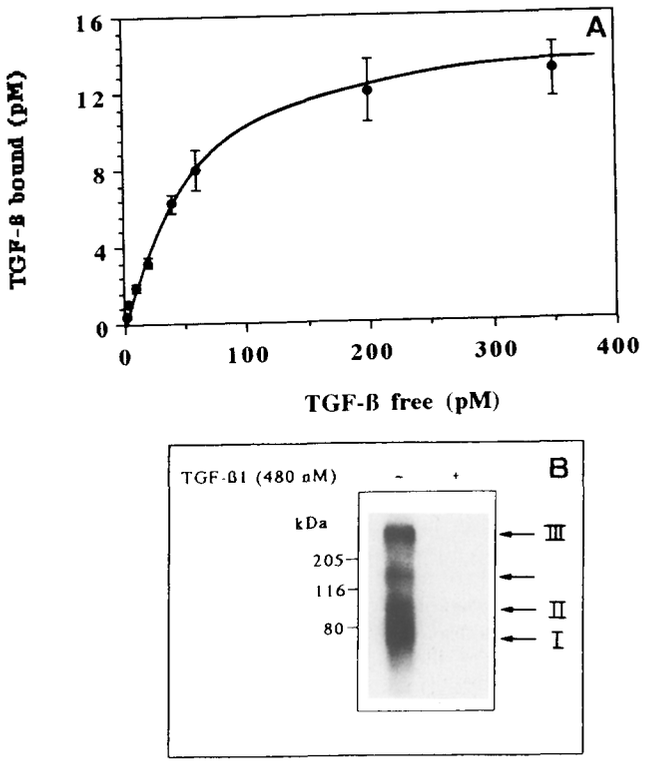

Fig. 1. TGF-β_1 specific binding (A) and analysis by affinity labeling and cross-linking of TGF-β_1 type receptors (B) for fetal brown adipocytes. (A) Brown adipocytes after culture for 20 h in the absence of serum, were incubated for 3 h at 20°C with [^{125}I]TGF-β_1 in the absence or presence of graded concentrations of unlabeled TGF-β_1 for receptor binding analysis, as described in section 2. Bound vs. free plot represents means ± S.E.M. from 3 independent experiments. (B) Affinity-labeling and cross-linking experiments were performed after culture of cells for 3 h with [^{125}I]TGF-β_1 in the absence or presence of excess unlabeled TGF-β_1, as described in section 2. A representative experiment of 3 is shown. Arrows indicate types of TGF-β_1 receptors.

3. Results and discussion

3.1. Fetal brown adipocytes bind TGF-β_1 with high affinity and expressed several TGF-β_1-type receptors

Fetal brown adipocytes, after culture for 20 h in a serum-free medium, show saturable TGF-β_1 specific binding as depicted in Fig. 1A (bound vs. free plot) with a total of 13,600 ± 1600 TGF-β_1 binding sites per cell calculated from this plot. The receptors for TGF-β_1 display a dissociation constant (K_d) of 52.6 ± 4.0 pM, calculated from the Scatchard plot (not shown). Affinity labeling studies with [^{125}I]TGF-β_1 and a chemical cross-linking agent revealed the existence of type I, II and III receptors as the major receptor species in brown adipocytes and also the presence of an additional minor labeled species of approximately 140 kDa TGF-β_1 binding protein on SDS-PAGE (Fig. 1B). Our results demonstrate that fetal rat brown adipocytes show a low number of TGF-β_1 binding sites per cell of high affinity, revealing the presence of the three canonical isoforms of TGF-β_1 receptors previously described [17], and a minor labeled-species of about 140 kDa similar to the 135 kDa isoform described in 3T3-L1 mouse fibroblasts [14].

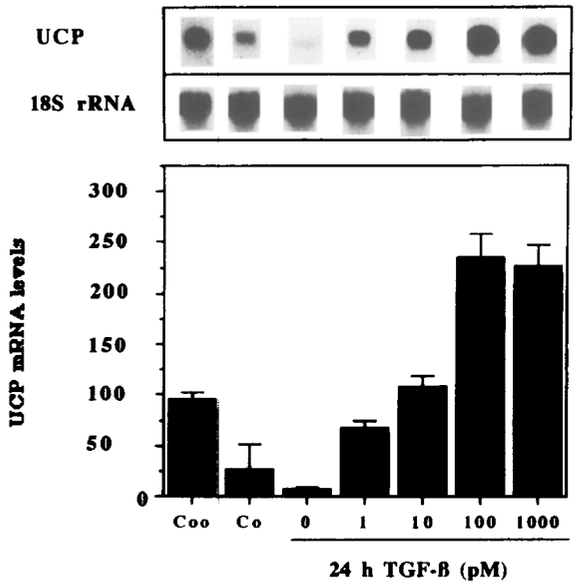

Fig. 2. Dose–response effect of TGF-β_1 on the expression of UCP mRNA in fetal brown adipocyte primary cultures. Cells at time 00 of culture (C00) were serum-starved for 20 h (C0) and further cultured for 24 h in a serum-free medium in the absence or presence of graded concentrations of TGF-β_1. Total RNA (10 μg) was submitted to Northern blot analysis and hybridized with UCP cDNA (upper panel) and 18 S rRNA cDNA (central panel). UCP mRNA levels (arbitrary densitometric units) are means ± S.E.M. from three independent experiments, and are shown in the lower panel.

3.2. *TGF-β_1 induces the expression of uncoupling protein mRNA in a dose- and time-dependent manner, in fetal brown adipocyte primary cultures*

We have studied the effect of TGF-β_1 on the expression of the tissue-specific gene UCP. Cells at time 00 of culture were serum-deprived for 20 h and further cultured for 24 h in a serum-free medium, in the presence of graded concentrations (0–1 nM) of TGF-β_1. A representative Northern blot is shown in Fig. 2 (upper panel) with the corresponding densitometric analysis represented in Fig. 2 (lower panel). The UCP mRNA was already present at time 00 of culture (C00), but after serum starvation for 20 h (C0) the expression of UCP mRNA was drastically reduced. Further culture for 24 h in the absence of serum and added external factors revealed that expression of UCP mRNA was almost undetectable. This loss of UCP expression throughout culture has been previously reported [7,9]. However, the presence of TGF-β_1 for the last 24 h of culture re-induced the UCP mRNA expression in a dose-dependent manner to levels 2.5-fold higher than those detected in brown adipocytes at time 00 of culture (Fig. 2). Since the maximal effect on UCP mRNA induction (25-fold increase) was observed at 100 pM TGF-β_1, this concentration was used in further experiments. To test the specific positive effect of TGF-β_1 on UCP mRNA expression from cells at the starting point of stimulation (20 h serum-starved cells, C0.5 in Fig. 3), a 24 h time–course of the effect of TGF-β_1 on the expression of UCP mRNA was performed (Fig. 3, upper panel). TGF-β_1 induced the UCP mRNA expression in a time-dependent manner compared to untreated cells, reaching levels 10-fold at 8 h and 24-fold at 24 h higher than their corresponding controls, as revealed by the densitometric analysis shown in Fig. 3, lower panel. Longer treatment of brown adipocytes with TGF-β_1 for 48 and 72 h did not produce any further effect on UCP mRNA expression as compared to levels reached at 24 h (data not shown). Our results demonstrate that TGF-β_1 is inducing the expression of the tissue-specific gene UCP in a dose- and time-dependent manner. Since TGF-β_1 expression has been reported during rodent fetal development regarding hematopoietic and mesenchymal tissues [33,34], we can not rule out the possibility that TGF-β_1 might play a physiological role, in an endocrine manner, as a developmental factor involved in the initiation and/or maintenance of brown adipose tissue differentiation. Although we have previously described a positive effect for insulin-like growth factor I on the expression of UCP gene in fetal brown adipocytes [7,9], this paper provides the first evidence reporting the up-regulation of UCP expression by TGF-β_1.

3.3. *TGF-β_1 induces the expression of lipogenic genes and the non-tissue specific gene fibronectin, in fetal brown adipocyte primary cultures*

In addition to the thermogenic program, we have studied (Fig. 4) the effect of TGF-β_1 on the adipogenic markers related to lipid synthesis and the non-tissue-specific gene fibronectin, a well-known target gene for TGF-β_1 [35,36]. Brown adipocytes at time 00 of culture showed a significant expression of FAS and G3PD mRNA, with the expression of ME and G6PD mRNA being much lower. After culture for 44 h in the absence of serum and added external factors, the expression of FAS, G3PDH and ME was almost undetectable, and the remaining G6PD mRNA expression essentially unmodified. However, the presence of TGF-β_1 in the culture medium for the last 24 h increased the expression of the adipogenic genes compared to

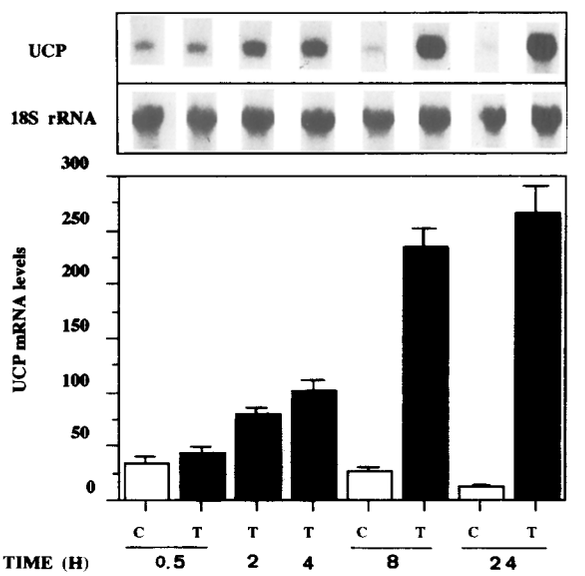

Fig. 3. Time–course of the uncoupling protein mRNA induction by TGF-β_1 in fetal brown adipocyte primary cultures. Brown adipocytes after serum deprivation for 20 h were stimulated for 30 min, 2, 4, 8 and 24 h with 100 pM TGF-β_1 (T) in a serum-free medium and compared to untreated cells at the corresponding times (C). Total RNA (10 μg) was submitted to Northern blot analysis and hybridized with UCP cDNA (upper panel) and 18 S rRNA cDNA (central panel). UCP mRNA levels (arbitrary densitometric units) are means ± S.E.M. from three independent experiments, and are shown in the lower panel.

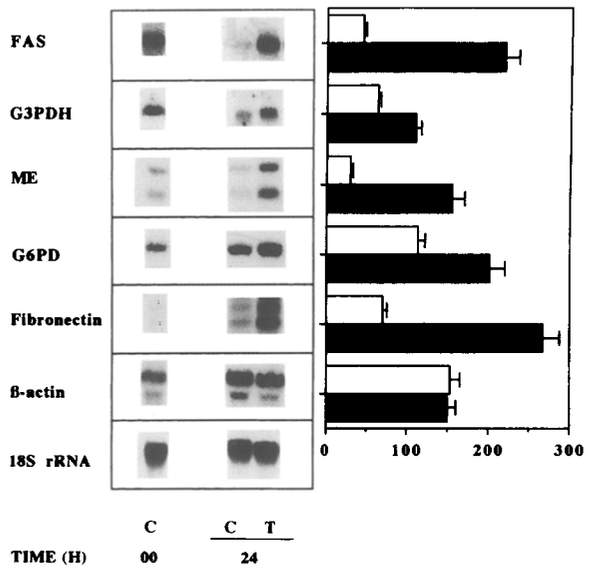

Fig. 4. TGF-β_1 up-regulates the expression of lipogenic enzymes and fibronectin in fetal brown adipocyte primary cultures. Cells at time 00 of culture (C00) were serum-deprived for 20 h and further cultured for 24 h in a serum-free medium in the absence (C24) or presence of 100 pM TGF-β_1 (T24). Total RNA (10 μg) was submitted to Northern blot analysis and hybridized with several probes as indicated in the figure (left panels). The mRNA levels at 24 h (arbitrary densitometric units) in the absence (white bars) or presence of TGF-β_1 (black bars) are means ± S.E.M. from three independent experiments, and are shown on the corresponding right panels.

untreated cells (Fig. 4). FAS and ME mRNA levels were increased by 5- and 6-fold, respectively, while the induction on G3PDH and G6PD was over 80%. Thus the expression of FAS and G3PDH in TGF-β_1-treated adipocytes reached similar levels to those found in brown adipocytes at time 00 of culture. Previous findings in the 3T3-L1 differentiation model indicated that TGF-β_1 blocked early but not late differentiation-specific gene expression (G3PDH and lipoprotein lipase) [14,15]. Fetal brown adipocytes at time 00 of culture show a medium stage of differentiation since they express various lipogenic enzymes, but not terminal differentiation markers, such as PEPCK [37] (results not shown). The culturing of brown adipocytes in the absence of serum and hormones leads to the loss of the major adipogenic markers studied. Our results suggest that TGF-β_1 could be initiating and/or maintaining the adipogenic differentiation program in fetal brown adipocytes. On the other hand, the expression of fibronectin was undetectable in cells at time 00 of culture (Fig. 4). After culture in the absence of serum for 44 h, two isoforms were barely expressed in fetal brown adipocytes primary cultures. However, TGF-β_1 present for the last 24 h of culture induced by 5-fold the expression of fibronectin mRNA, compared to untreated cells at the corresponding time of culture (Fig. 4). The expression of β-actin, a proliferation marker, slightly increased throughout the time of culture in the absence of serum, remaining essentially unmodified in the presence of TGF-β_1 (Fig. 4). Fibronectin is a target gene for TGF-β_1 [35]. The fact that fibronectin expression is induced in our model by TGF-β_1, supports the differentiation role played by TGF-β_1 in fetal brown adipocytes. However, the induction of extracellular matrix associated proteins by TGF-β_1 could be independent of other effects of TGF-β_1 on differentiation-specific gene expression, as proposed by [36].

In conclusion, TGF-β_1 up-regulates the expression of the tissue-specific gene UCP involved in the thermogenic program and also the expression of several adipogenic-related genes, suggesting that TGF-β_1 may play a major role in the initiation and/or maintenance of fetal rat brown adipocyte differentiation.

Acknowledgements: This work was supported by Grant SAF 93/0206 from Comisión Interministerial de Ciencia y Tecnologia, Spain. Teresa Teruel is recipient of a fellowship from Comunidad Autónoma de Madrid, Spain.

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