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Growth factor regulation of uncoupling protein-1 mRNA expression in brown adipocytes

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García, Bibian, and Maria-Jesús Obregón. Growth factor regulation of uncoupling protein-1 mRNA expression in brown adipocytes. *Am J Physiol Cell Physiol* 282: C105–C112, 2002. First published October 3, 2001; 10.1152/ajpcell.01396.2000.—To study the effect of the mitogens epidermal growth factor (EGF), acidic and basic fibroblast growth factors (aFGF and bFGF), and vasopressin on brown adipocyte differentiation, we analyzed the expression of uncoupling protein-1 (UCP-1) mRNA. Quiescent brown preadipocytes express high levels of UCP-1 mRNA in response to triiodothyronine (T3) and norepinephrine (NE). The addition of serum or the mitogenic condition aFGF + vasopressin + NE or EGF + vasopressin + NE decreases UCP-1 mRNA. A second addition of mitogens further decreases UCP-1 mRNA. Treatment with aFGF or bFGF alone increases UCP-1 mRNA, whereas the addition of EGF or vasopressin dramatically reduces UCP-1 mRNA levels. The continuous presence of T3 increases UCP-1 mRNA levels in cells treated with EGF, aFGF, or bFGF. The effect of T3 on the stimulation of DNA synthesis also was tested. T3 inhibits the mitogenic activity of aFGF and bFGF. In conclusion, mitogens like aFGF or bFGF allow brown adipocyte differentiation, whereas EGF and vasopressin inhibit the differentiation process. T3 behaves as an important hormone that regulates both brown adipocyte proliferation and differentiation.

proliferation; differentiation; thermogenesis

THE MAIN FUNCTION of brown adipose tissue (BAT) is to produce extra heat by nonshivering thermogenesis, especially in demanding situations such as cold exposure. This function is accomplished by the mitochondrial protein, thermogenin, also called uncoupling protein-1 (UCP-1), a BAT-specific protein present only in the inner mitochondrial membrane of brown adipocytes (27). UCP-1 provides heat by uncoupling the oxidative phosphorylation, dissipating as heat the energy that otherwise would be stored as ATP (21).

BAT development occurs in mammals during the perinatal period, and in the rat, it reaches its maximal activity and thermogenic capacity during the first week of postnatal life, due to the norepinephrine (NE) released from the sympathetic fibers in response to the cold experienced by the newborn after delivery (20, 22). With increasing age and body size, BAT degenerates, loosing its thermogenic capacity, but can be reactivated in situations such as cold exposure or overeating. The reactivation implies increases in cell proliferation of brown adipocyte precursor cells present in the tissue and the differentiation of those cells to thermogenic brown adipocytes (8, 11, 19, 25). Several studies in vivo have shown that the NE released from the sympathetic nerve endings is the main mediator of the proliferative stimulus in BAT activation (8, 11, 25) and also stimulates the proliferation of brown fat cells in primary cultures in the presence of serum (7). In a previous study, our group showed that NE has a low mitogenic effect per se but increases the stimulation of DNA synthesis exerted by serum, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or vasopressin in quiescent brown preadipocytes (10). NE also regulates the differentiation process, mainly by inducing UCP-1 expression at the transcriptional level (5, 27). The thyroid hormone triiodothyronine (T3) has been reported to be required for the optimal UCP-1 gene expression in response to the noradrenergic stimulus in vivo (2, 31) and in dispersed and cultured brown adipocytes (1, 13). T3 per se is able to induce the transcription of UCP-1 gene and stabilizes its mRNA in fetal rat brown adipocytes in primary culture (12). The same group has reported that IGF-I stimulated UCP-1 expression in brown adipocytes from rat fetuses (17). Besides these effects of NE, T3, and IGF-I, little is known about other hormones or growth factors involved in the acquisition of the differentiated state. In our previous study (10), we showed that brown adipocytes cultured in the presence of different mitogenic conditions, including serum, express UCP-1 mRNA when stimulated adrenergically in the presence of T3, but the expression level was variable, depending on the mitogenic combinations used. In the present work we analyze *1*) the effect of the growth factors EGF, acidic fibroblast growth factor (aFGF), and bFGF and the hormone vasopressin on the expression level of UCP-1 gene in brown adipocytes and *2*) the role of T3 in modulating the effects of these mitogens in UCP-1 expression as well as on DNA synthesis stimulation.

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MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO (Uxbridge, UK). Newborn calf serum (NCS) was from Flow (Paisley, UK). Antibiotics were from a local pharmacy. Bovine serum albumin (BSA; in solution at 22%, pH 7.2) was from Ortho Diagnostic Systems, Johnson & Johnson (Raritan, NJ). Collagenase, bovine insulin, ascorbic acid, guanidinium-HCl, 3-(*N*-morpholino)propanesulfonic acid (MOPS), dithiothreitol (DTT), NE, and vasopressin were from Sigma (St. Louis, MO). aFGF, bFGF, and EGF were from Boehringer Mannheim (Mannheim, Germany). aFGF was used in the presence of 50 μg/ml heparin (final concentration), as recommended for proper biological activity. PD-98059 (PD), bisindolylmaleimide I (Bis), and LY-294002 (LY) were from Calbiochem (Darmstadt, Germany). [α-^{32}P]dCTP (3,000 Ci/mmol) and [^{3}H]thymidine were from Amersham International (Amersham, UK). Formamide was from Fluka or Merck, and the oligolabeling system was from Pharmacia (Uppsala, Sweden). Nytran membranes for RNA blotting were purchased from Schleicher & Schuell (Dassel, Germany), and the glass fiber filter mats for thymidine incorporation were from Wallac Oy (Turku, Finland). All other chemicals were reagent grade or molecular biology grade.

Cell isolation and culture. Brown fat precursor cells were isolated from the interscapular brown adipose tissue of 20-day-old rats as described by Néchad et al. (18), with the difference that we did not perform the hypoosmotic shock. The process involved collagenase digestion, separation of mature adipocytes by flotation, and subsequent filtration through 25-μm silk filters, obtaining precursor cells by centrifugation. The precursor cells obtained from each animal were divided into two culture flasks (Nunclon, 25 cm^2; Nunc, Roskilde, Denmark), each containing 5 ml of culture medium consisting of DMEM supplemented with 3.5 nM insulin, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, 25 μM sodium ascorbate (culture medium), and 10% NCS. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air with 95% humidity. Cells were washed on *day 1* (2,000 cells/cm^2), and culture medium was changed every other day.

For proliferation assays, preconfluent cells (*day 2–3*) were harvested using 0.2% collagenase in DMEM, washed, and seeded in 24-multiwell tissue culture plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) at a density of 8,000 cells/cm^2 with 1 ml of culture medium supplemented with 10% NCS. After 6 h, the cells were washed twice with medium and maintained for 48 h in culture medium supplemented with 0.2% BSA (*time 0*). This was the starting point for mitogenic stimulation.

For RNA analysis, preconfluent cells (*day 3*) were used. After being washed and maintained for 48 h in culture medium supplemented with 0.2% NCS, quiescent cells were stimulated by the addition of the different growth factors and hormones. After 2 days in culture, 5 nM T3 was added and, 16 h later, RNA isolation was performed, after a 4-h pretreatment with 10 μM NE.

Proliferation assays. Growth factors, hormones, or serum was added to the cells at *time 0* (10), at the concentrations indicated in each experiment. For [^{3}H]thymidine incorporation assays, quiescent cells were stimulated with the appropriate mitogens and hormones at *time 0* in the presence of [^{3}H]thymidine (1 μCi/ml). After 40 h of exposure, the medium was discarded, and the cells were removed from the plate with the use of a trypsin-EDTA solution. Thereafter, the contents of each well were harvested onto glass fiber filters with a cell harvester from Inotech (Dottikon, Switzerland), and the radioactivity incorporated into DNA was determined with a beta-scintillation counter (MicroBeta) from Wallac Oy.

RNA analysis. At the end of the experiments, the cells were dissolved in 1 ml of hot guanidine-HCl extraction buffer, and RNA was isolated after ethanol precipitation as described previously (14). RNA concentration was determined by measuring the optical density (OD) at 260 nm, and the ratio of OD at 260 nm to that at 280 nm (260/280) was always around 2.0. Samples of total RNA (20 μg) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. Methylene blue staining revealed the presence of equal amounts of RNA in each lane. RNA was blotted into nylon filters (Nytran, NY13). Hybridization and washing were carried out as previously described (10) by using a rat UCP-1 cDNA (6) and cyclophilin cDNA probes labeled by random oligopriming. The quantitation of the signal was performed by using an Instant Imager (Packard).

Statistics. Mean values given were obtained from at least three different culture flasks or wells. All experiments presented were repeated at least three times, and representative Northern blots are shown. The SE values given in figure legends are the mean SEs calculated by ANOVA. Data were submitted to one-way ANOVA after having been tested for homogeneity of variance by using Bartlett's procedure for groups of unequal size. Square root or logarithmic transformations were used to ensure homogeneity of variance when not achieved with the raw data. Significant differences among groups were assessed by using the protected least significant difference (LSD) test. All statistical calculations were performed by following the method described by Snedecor and Cochran (32).

RESULTS

Stimulation of proliferation by serum and growth factors. In a previous study, we described several growth factors that stimulate DNA synthesis in quiescent brown preadipocytes, such as EGF, aFGF, and bFGF (10). The addition of vasopressin plus NE induced a substantial increment in DNA synthesis stimulation elicited by each growth factor, reaching levels comparable to those found in response to 10% serum.

When quiescent cultures are stimulated with these mitogenic combinations for 72 h, a substantial stimulation of cell proliferation is observed. Figure 1 shows microphotographs of cultures untreated or treated with serum or EGF (5 ng/ml), aFGF (1 ng/ml), or bFGF (5 ng/ml) plus vasopressin (20 nM) and NE (1 μM) for 72 h. There is evidence that the mitogenic combinations and serum stimulate cellular proliferation and that cultures reach confluence, as compared with quiescent unstimulated cells.

UCP-1 mRNA level in quiescent and serum-treated cultures. We recently reported that quiescent brown adipocytes cultured in the presence of serum or the different mitogenic combinations mentioned above were able to express the differentiation marker UCP-1 only when stimulated adrenergically in the presence of T3 (10). In the present study, first of all, we wanted to measure UCP-1 mRNA level in quiescent cultures to study the state of differentiation of quiescent brown adipocytes. For this purpose, cultures of brown preadipocytes were maintained during 48 h in low-serum conditions, as described in MATERIALS AND METHODS. Af-

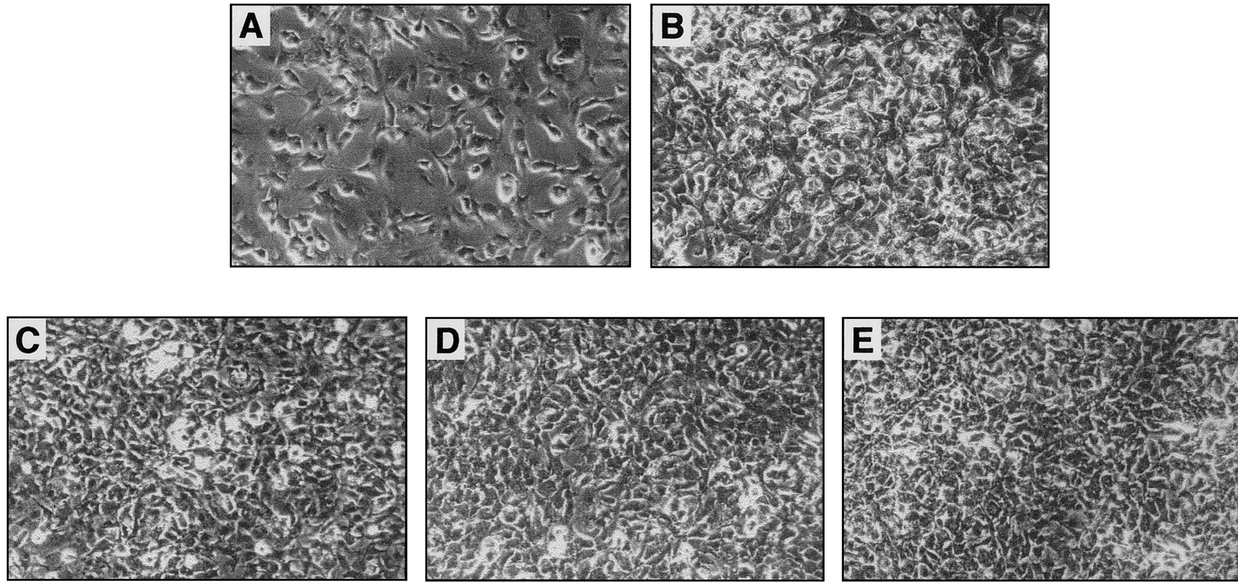

Fig. 1. Brown adipocyte proliferation in response to different mitogenic conditions. Quiescent preadipocytes (maintained for 48 h in low-serum conditions) were cultured for 72 h in absence (*A*) or presence (*B*) of newborn calf serum (NCS; 10%) or the following mitogenic combinations: vasopressin (20 nM) plus norepinephrine (NE; 1 μM) plus epidermal growth factor (EGF; 5 ng/ml) (*C*), acidic fibroblast growth factor (aFGF; 1 ng/ml) (*D*), or basic fibroblast growth factor (bFGF; 5 ng/ml) (*E*).

terward, RNA isolation was performed in half of the flasks containing quiescent cells, whereas the remaining flasks were treated with 10% serum, and RNA was analyzed 72 h later. All flasks received 5 nM T3 and 1 μM NE 16 and 4 h, respectively, before RNA isolation. Figure 2 shows that quiescent cells express UCP-1 mRNA when stimulated adrenergically in the presence of T3; however, when those cells are stimulated with serum, UCP-1 gene expression substantially decreased. Cy mRNA is shown as control.

Effect of mitogenic stimulation on UCP-1 mRNA level. Given that serum treatment stimulates proliferation and inhibits the ability of cells to express the

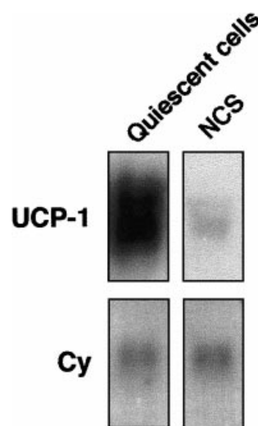

Fig. 2. Uncoupling protein (UCP)-1 gene expression in quiescent preadipocytes. Brown preadipocytes were maintained 48 h in low-serum conditions to induce a quiescent state. At that time, RNA isolation was performed in half of the flasks while the other half were treated with 10% NCS for a further 72 h, and RNA isolation was then performed. All flasks received triiodothyronine (T3; 5 nM) and NE (10 μM) 16 and 4 h, respectively, before RNA isolation. A representative Northern blot is shown. Cy, cyclophilin; shown as control.

differentiation marker UCP-1, we tested the effect of successive additions of the mitogenic combinations described above on UCP-1 mRNA expression. For this purpose, quiescent cells were cultured in the absence or presence of aFGF (1 ng/ml) plus vasopressin (20 nM) plus NE (1 μM) or EGF (5 ng/ml) plus vasopressin plus NE or 10% NCS for 72 h. Half of the flasks received a second addition of mitogens 24 h after the first addition. All flasks received 5 nM T3 and 10 μM NE 16 and 4 h, respectively, before RNA isolation. Results are shown in Fig. 3. The UCP-1 mRNA level in quiescent cells was much higher than that in stimulated cells ($P < 0.05$). The second addition of mitogens resulted, in all cases, in a further reduction of UCP-1 mRNA level, and the transcript was almost undetectable in cultures that received two additions of the mitogenic combination of EGF plus vasopressin plus NE.

UCP-1 mRNA level in cells cultured in the presence of EGF, aFGF, and bFGF: effect of chronic T3 treatment. Although the mitogenic stimulation results in an important reduction of UCP-1 gene expression compared with the level found in quiescent cells, the different mitogenic combinations produced quite different effects. Thus we studied the effect of different growth factors, added alone, on the expression of UCP-1 mRNA. In the same assay, the effect of the continuous presence of T3 was analyzed. Quiescent cultures were left untreated or were treated with aFGF (1 ng/ml), EGF (5 ng/ml), or bFGF (5 ng/ml) in the absence or presence of 5 nM T3, and UCP-1 mRNA was analyzed after 72 h of exposure. Before RNA isolation, T3 (5 nM) and NE (1 μM) were added, as described above. Figure 4 shows that cells treated with aFGF but without vasopressin and NE expressed the differentiation marker UCP-1, and the mRNA level observed is higher

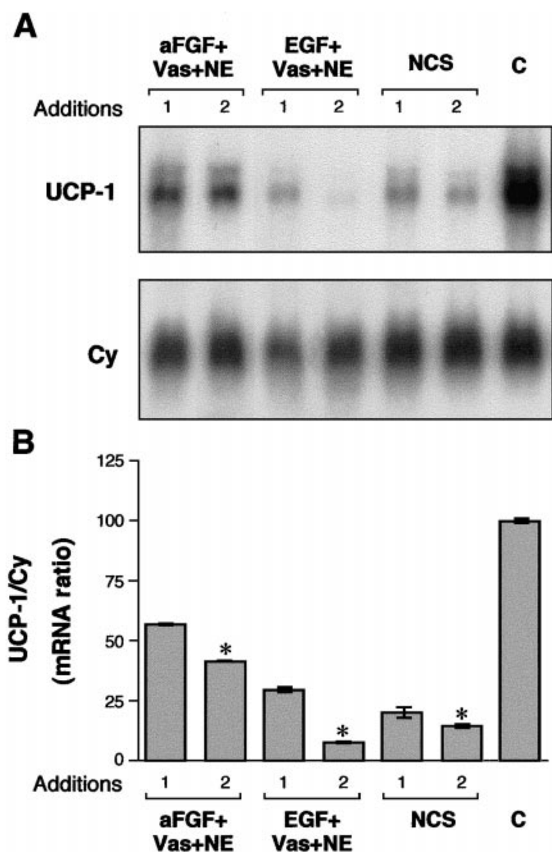

Fig. 3. Effect of different mitogenic conditions on UCP-1 mRNA level. Quiescent brown preadipocytes were untreated (C) or treated with NCS (10%) or the mitogenic combinations vasopressin (20 nM) plus NE (1 μM) plus either aFGF (1 ng/ml) or EGF (5 ng/ml) for 72 h. Twenty-four hours after the first addition, half of the flasks received a second addition of serum or mitogens. All flasks received T3 (5 nM) and NE (10 μM) 16 and 4 h, respectively, before RNA isolation. *A*: representative Northern blot. *B*: relative UCP-1 mRNA levels, expressed in arbitrary units, obtained by quantitation of UCP-1 mRNA signal of 3 independent experiments and normalized by using Cy values. The mean SE = 1. $^*P < 0.05$, double vs. single addition of mitogens.

than that detected in quiescent unstimulated cells. Treatment with bFGF exhibited the same effect as aFGF. However, the presence of EGF resulted in a substantial reduction in the UCP-1 mRNA level compared with that in quiescent unstimulated cells (Fig. 4).

The continuous presence of T3 resulted in an increase of UCP-1 mRNA expression in quiescent cells as well as in cells treated with the growth factors EGF, aFGF, or bFGF.

Vasopressin and NE effect on UCP-1 expression. As with the growth factors aFGF, bFGF, and EGF, we wanted to study the role of vasopressin and NE in the regulation of UCP-1 gene expression. Figure 5 shows that the incubation of quiescent cultures during 72 h with 20 nM vasopressin prevents the induction of UCP-1 gene expression by T3 and NE ($P < 0.05$) compared with the level reached by quiescent cells. Furthermore, the presence of vasopressin reduced UCP-1 mRNA levels in cells treated with the growth factors EGF (5 ng/ml), aFGF (1 ng/ml), and bFGF (5 ng/ml) and in cells treated with NE (1 μM). The addition of NE produced a further decrease in UCP-1 expression in cells treated with the different growth factors plus vasopressin.

Effect of the protein kinase C inhibitor Bis. In an attempt to understand the differential effect of EGF and FGFs on the noradrenergic stimulation of UCP-1 mRNA expression, we tested the effect of several inhibitors of different signaling pathways. First, Bis, a known inhibitor of protein kinase C (PKC; isoforms α, β, γ, δ, and ε) was used. Figure 6 shows that Bis increases the expression of UCP-1 in cells treated with EGF and aFGF and completely reverts the inhibition exerted by EGF on UCP-1 mRNA to the level observed in quiescent cells. These results suggest the presence of a PKC with inhibitory effect on the stimulated UCP-1 mRNA expression.

In other experiments we also used PD, an inhibitor of mitogen-activated protein kinase kinase (MAPKK) activity (MEK), as well as LY, a specific inhibitor of phosphatidylinositol (PI) 3-kinase. Both PD and LY inhibited UCP-1 mRNA expression in the presence of EGF, aFGF, or bFGF (results not shown), suggesting that both MAPKK (MEK) and PI 3-kinase signaling pathways are cooperative signals for UCP-1 mRNA expression using NE plus T3.

T3 effect on DNA synthesis stimulation. Although T3 is necessary for BAT development and thermogenesis

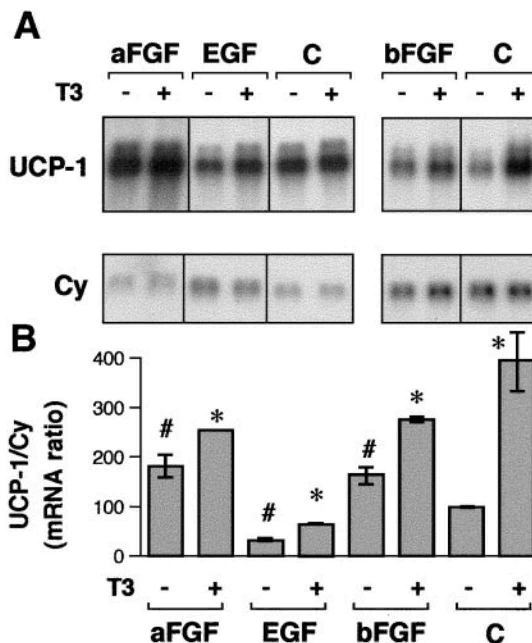

Fig. 4. Effect of EGF, aFGF, bFGF, and T3 on UCP-1 gene expression. Quiescent brown preadipocytes were treated with aFGF (1 ng/ml) or EGF (5 ng/ml) (*A, left*) or with bFGF (5 ng/ml) (*A, right*) in the absence (−) or presence (+) of T3 (5 nM) for 72 h, and RNA isolation was then performed. All flasks received T3 (5 nM) and NE (10 μM) 16 and 4 h, respectively, before RNA isolation. *A*: 2 representative Northern blots. *B*: relative UCP-1 mRNA levels, expressed in arbitrary units, obtained by quantitation of UCP-1 mRNA signal of 3 independent experiments and normalized by using Cy values. The mean SE = 21.5. $^*P < 0.05$ vs. corresponding cells in the absence of T3. $\#P < 0.05$ vs. quiescent cells (C) in the absence of T3.

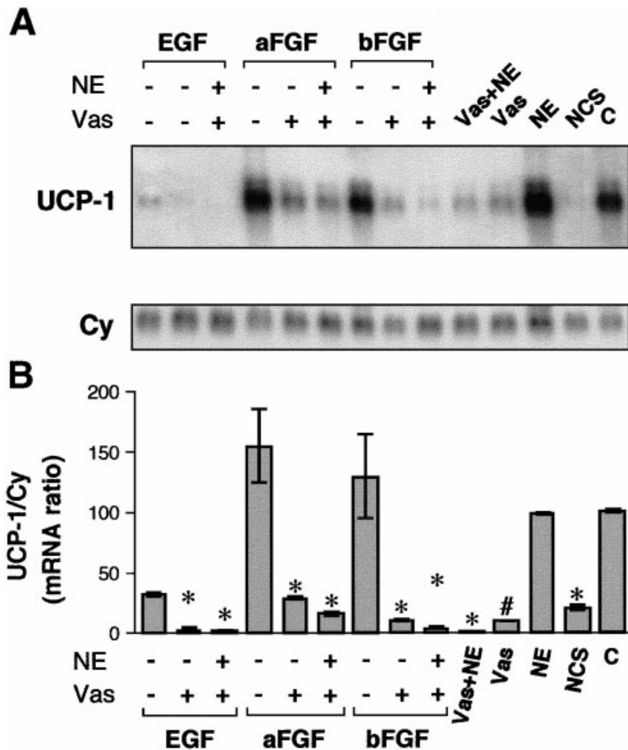

Fig. 5. Effect of vasopressin and NE on expression of the UCP-1 gene. Quiescent brown preadipocytes were treated with NCS (10%), EGF (5 ng/ml), aFGF (1 ng/ml), or bFGF (5 ng/ml) in the absence or presence of vasopressin (20 nM) or vasopressin plus NE (1 μM) for 72 h. Other flasks were treated with vasopressin or NE, or with both hormones. All flasks received T3 (5 nM) and NE (10 μM) 16 and 4 h, respectively, before RNA isolation. Vas, vasopressin. *A*: representative Northern blot. *B*: relative UCP-1 mRNA levels, expressed in arbitrary units, obtained by quantitation of UCP-1 mRNA signal of 3 independent experiments and normalized by using Cy values. The mean SE = 0.84. *$P < 0.05$ vs. each treatment (EGF, aFGF, bFGF, or NE) in the absence of vasopressin. #$P < 0.05$ vs. quiescent cells (C).

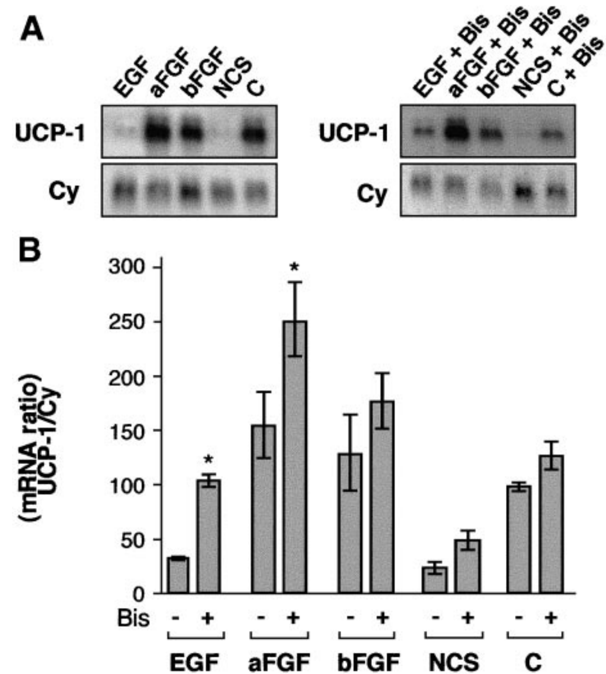

Fig. 6. Effect of bisindolylmaleimide I (Bis) on UCP-1 gene expression. Quiescent brown preadipocytes were untreated (C) or treated with NCS (10%), EGF (5 ng/ml), aFGF (1 ng/ml), or bFGF (5 ng/ml) for 72 h. Half of the flasks received Bis (1 μM) 1 h before growth factors were added. All flasks received T3 (5 nM) and NE (10 μM) 16 and 4 h, respectively, before RNA isolation. *A*: representative Northern blot. *B*: relative UCP-1 mRNA levels, expressed in arbitrary units, obtained by quantitation of UCP-1 mRNA signal of 3 independent experiments and normalized by using Cy values. The mean SE = 16. *$P < 0.05$ vs. corresponding cells in the absence of Bis.

(3, 23), its role on brown adipocyte proliferation has not been studied yet. In this report we analyze the effect of T3 addition on DNA synthesis stimulation elicited by the growth factors EGF, aFGF, and bFGF and by vasopressin. The results shown in Fig. 7 reveal that the presence of T3 did not alter the [^{3}H]thymidine incorporation level reached in response to vasopressin and decreased slightly the mitogenic activity of EGF. However, T3 considerably reduced the stimulation elicited by aFGF and inhibited the effect of bFGF on DNA synthesis stimulation. T3 when added alone had no effect on [^{3}H]thymidine incorporation compared with untreated quiescent cells (data not shown).

DISCUSSION

During BAT recruitment in response to cold exposure, there must be a variety of growth factors and hormones regulating the proliferation of brown preadipocytes, as well as the differentiation of those cells into mature thermogenic adipocytes. In our previous study, we identified several growth factors and hormones (EGF, platelet-derived growth factor, aFGF, bFGF, and vasopressin) that stimulated DNA synthesis in brown preadipocytes, and we showed that NE was able to potentiate the effect of some of those growth factors. We established mitogenic conditions that stimulated DNA synthesis to a level comparable with that reached in response to 10% NCS. Those cells growing in these mitogenic conditions (growth factor +

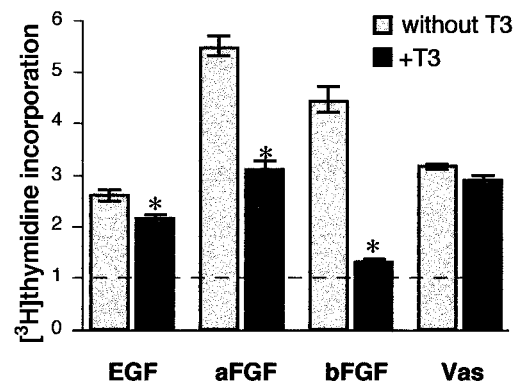

Fig. 7. Effect of T3 on the mitogenic activity of growth factors. Quiescent brown preadipocytes were exposed for 40 h to EGF (5 ng/ml), aFGF (1 ng/ml), bFGF (5 ng/ml), or vasopressin (20 nM) in the absence or presence of T3 (5 nM) and in the continuous presence of 1 μCi/ml [^{3}H]thymidine. T3 added alone had no effect on [^{3}H]thymidine incorporation. Results are expressed as fold increases relative to values in untreated quiescent cells. The mean SE = 14.5. *$P < 0.05$ vs. corresponding cells in the absence of T3.

vasopressin + NE) were able to express UCP-1 gene when stimulated by T3 and NE. In this work we were interested in studying the effect of each mitogen on UCP-1 mRNA levels and the role of T3 in regulating UCP-1 mRNA content in response to these mitogens. For this purpose, we first analyzed the differentiation state of quiescent brown preadipocytes before their stimulation with the mitogens. We have shown that nonconfluent cultures of quiescent brown preadipocytes (maintained 48 h in low-serum conditions) are able to express UCP-1 gene when stimulated by T3 and NE. The addition of 10% serum to these cultures produces a substantial reduction in the UCP-1 mRNA level. The observed reduction in UCP-1 mRNA level in response to serum could be due to *1*) the mitogenic stimulation that makes cells enter the cell cycle and acquire a proliferative state and *2*) certain growth factors and hormones present in serum that could interfere with the stimulatory action of T3 and/or NE on the UCP-1 gene expression.

We report evidence that both situations might take place. First, we have shown that the treatment of quiescent brown preadipocytes under the mitogenic conditions we described in a previous study (10), growth factor plus vasopressin + NE, stimulates cellular proliferation and results in a reduction of UCP-1 mRNA level compared with quiescent unstimulated cells. The UCP-1 mRNA level declines even more when cultures are treated with a second addition of mitogens or serum. These results confirm that the quiescent state favors cellular differentiation even in cultures that have not yet reached confluence and that the mitogenic stimulation reduces the ability of brown adipocytes to express the differentiation marker UCP-1.

Second, quiescent brown preadipocytes cultured in the presence of aFGF or bFGF keep the ability to express UCP-1 mRNA, but cells treated with EGF or vasopressin fail to express UCP-1 mRNA in response to T3 and NE. Thus growth factors like aFGF and bFGF, besides being potent mitogens for brown adipocytes, allow differentiation. One of those growth factors, bFGF, has been shown to be involved in BAT enlargement during cold acclimation (34), and its expression is greatly enhanced in response to NE (35). These findings, in addition to our results, confirm that bFGF is an important growth factor for brown fat cell proliferation and differentiation.

Conversely, EGF or vasopressin regulates the expression of the UCP-1 gene negatively, interfering with the stimulatory action of NE and/or T3 on UCP-1 mRNA expression. Many studies in vitro, as well as in vivo, have established EGF as an inhibitor of white adipocyte differentiation. EGF inhibits white preadipocyte differentiation in primary cultures (29, 33) and, when administered to neonatal rats, increases the number of white adipocyte precursors, reducing the ability of these cells to undergo differentiation (30), and elicits a significant reduction in midscapular skin temperature as an indicator of brown adipose tissue thermogenesis (9). EGF is also a potent mitogen that prevents the adipogenic differentiation of 3T3-L1 cells (4). The same behavior has been observed in thyroid cells, where EGF stimulates cellular proliferation and prevents the acquisition of the differentiated state (28). In this study we report, for the first time, evidence of EGF action on the differentiation of brown precursor cells. Our results confirm EGF as an important growth factor for adipose cells and suggest that EGF, in addition to vasopressin, could play an important role interfering in the process of brown adipocyte differentiation. In this respect, high levels of c-Jun, present in nondifferentiated brown adipocytes as compared with differentiated cells, have been reported to repress the basal and cAMP-mediated expression of UCP-1 gene (36). These authors postulate that c-Jun levels could determine the cellular state, e.g., proliferative or differentiated in brown adipocytes. c-Jun is a transcription factor that stimulates progression of the cell cycle (26) and mediates the proliferative response to growth factors in multiple cell types (15). It remains to be elucidated whether c-Jun is involved in the dedifferentiative effect of EGF and vasopressin.

To understand the molecular mechanisms underlying the differential effects of these growth factors, we analyzed the signaling pathways involved in the effect of these growth factors on UCP-1 mRNA expression. It is well accepted that after the binding of the growth factors to their tyrosine kinase receptors, several signaling pathways are initiated, leading to the activation of a cascade of events that involve the activation of MAPK, PI 3-kinase, and PKC (16). The results obtained with the PI 3-kinase and MEK inhibitors did not allow us to discriminate between the effect of the different growth factors, since both inhibited UCP-1 mRNA expression regardless of which mitogen was tested. Our results with the PKC inhibitor Bis suggest the involvement of an unknown PKC isoform in the inhibition of UCP-1 mRNA expression. Bis potentiated the effect of aFGF and completely reverted the inhibitory effect of EGF on UCP-1 expression, suggesting that this PKC isoform could be involved in the dedifferentiative effect of EGF. Further work needs to be done to identify the specific PKC involved in the modulation of UCP-1 expression.

In the present paper we also have analyzed the role of T3 in the regulation of UCP-1 expression and of DNA synthesis stimulation in response to these mitogens. We have shown that the continuous presence of T3 increases UCP-1 mRNA content in quiescent cells as well as in cells treated with the growth factors EGF, aFGF, or bFGF. We also have reported that T3 reduces the mitogenic effect of aFGF and inhibits the stimulation of DNA synthesis elicited by bFGF, indicating that T3 interferes with the mitogenic signaling pathways activated in response to these growth factors. Although an interaction of thyroid hormone receptors and the transcription factor AP1, which is induced in response to many growth factors, has been reported (37), the precise mechanism underlying the role of T3 in brown adipocyte proliferation remains to be studied.

Our findings are in agreement with the inhibitory effects of T3 on proliferation in N2a cell lines (neuro-

blastoma), where T3 leads to decreases in the levels of the cyclin D1, a key step in the progression of the cell cycle, and to increases in cyclin kinase inhibitor $p27^{kip1}$, which are associated with increases in the cyclin-dependent kinase 2 (Cdk2) (24).

In conclusion, our results describe for the first time a permissive role of aFGF and bFGF and a negative role for EGF and vasopressin on the differentiation of brown adipocytes, measured as UCP-1 mRNA level. T3 behaves as an important hormone that may control both brown adipocyte proliferation and differentiation.

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