Insulin increases the adrenergic stimulation of 5' deiodinase activity and mRNA expression in rat brown adipocytes; role of MAPK and PI3K

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Abstract

Type II 5' deiodinase (D2) activity produces triiodothyronine (T_3) from thyroxine (T_4) and is induced by cold and norepinephrine (NE) in brown adipose tissue. T_3 is required for and amplifies the adrenergic stimulation of D2 activity and mRNA in cultured brown adipocytes. D2 is upregulated by insulin and decrease in fasting. We now study the regulation by insulin of the adrenergically induced D2 activity and mRNA in primary cultures of rat brown adipocytes. Insulin alone does not increase D2 activity or mRNA. Insulin-depleted cells show a reduction in the adrenergically induced D2 activity, which is proportional to the length of insulin depletion and is restored after insulin addition. IGFs mimic this effect at higher doses. ERK 1/2 MAPK activity (p44/p42), stimulated by insulin, serum and NE, is an absolute requirement for the adrenergic stimulation of D2 activity and mRNA. PI3K is stimulated by insulin and serum, and NE increases the effect of insulin. The action of insulin on D2 is not due to changes in D2 half-life or in the proteasome-mediated degradation of D2, but it seems to modulate the transcriptional induction mediated by NE. D2 mRNA expression, induced by NE plus T_3 , is reduced when insulin is withdrawn at early differentiation stages. Insulin or IGF-I promotes increases in D2 mRNA. Insulin is required for the induction of D2 mRNA by T_3 . In conclusion, MAPK signaling is required for the adrenergic stimulation of D2 activity and mRNA, and insulin stimulates D2 activity via MAPK and PI3K and enhances the adrenergic pathways.

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Introduction

The deiodinases are selenoenzymes that regulate triiodothyronine (T_3) availability in peripheral tissues. Most of the T_3 present in tissues is produced from thyroxine (T_4) by 5' deiodination. Outer-ring deiodination is considered an activating pathway that produces T_3 from T_4 , while inner-ring deiodination is considered an inactivating pathway. Two isoenzymes catalyze the activating pathway: type I and type II 5' deiodinases (D1 and D2). D1 and D2 differ in their kinetic characteristics, tissular distribution and response to thyroid status. D2 prefers T_4 as substrate; its K_m is in the nM range and is insensitive to inhibition by 6-*N*-propyl-2-thiouracil (PTU). D2 is present in the brain, pituitary and brown adipose tissue (BAT) (Leonard *et al.* 1981, Silva & Larsen 1983, Visser *et al.* 1983), the pineal gland and the maternal side of the placenta (Kaplan & Shaw 1984, Rubio *et al.* 1991) and in man is also present in heart, skeletal muscle and thyroid gland (Croteau *et al.* 1996, Salvatore *et al.* 1996). D2 is upregulated in hypothyroidism (Silva & Leonard 1985, Silva & Larsen 1986a) and by a number of factors, such as adrenergic agents,

cAMP, growth factors and insulin (Silva & Larsen 1983, 1986b, Courtin *et al.* 1988).

The cDNAs coding for the three deiodinases have been isolated from rat, human and other species (Berry *et al.* 1991, Croteau *et al.* 1995, 1996). All of them contain one in-frame TGA codon that is translated as selenocysteine due to the presence of a specific structure, the selenocysteine insertion sequence (SECIS), in the 3'-untranslated region of their mRNAs (Berry *et al.* 1991, Davey *et al.* 1995).

In BAT, the best stimulation of D2 activity is obtained under cold exposure, mediated by the norepinephrine (NE) released from the sympathetic nerve endings (Silva & Larsen 1983). D2 produces most of the T_3 found in BAT (Silva & Larsen 1985), which saturates the nuclear T_3 receptors under cold exposure (Bianco & Silva 1987), and the T_3 produced is necessary for the complete thermogenic function of BAT (Carvalho *et al.* 1991), namely, the full expression of the uncoupling protein (UCP-1), a specific marker of BAT. In floating brown adipocytes, D2 is stimulated by adrenergic agents (Obregón *et al.* 1987), and synergism between the $\alpha 1$ and β adrenergic pathways has been described (Raasmaja & Larsen 1989).

In addition, BAT D2 activity is upregulated by insulin, as shown after insulin injection to diabetic rats and by low D2 activity in BAT of diabetic rats (Silva & Larsen 1986b). In floating brown adipocytes, insulin stimulates D2 activity by increasing its $V_{\max}$ (Mills *et al.* 1987).

Insulin is a key hormone for adipocytes, required for the increase in lipogenic enzymes associated with the differentiation of adipocytes. In brown adipocytes, lipogenesis and thermogenesis seem to have opposite roles, as dedifferentiation of the brown adipocytes is accompanied by increased lipid accumulation, acquisition of the white adipocyte phenotype and loss of the thermogenic marker UCP-1. On the other hand, during active thermogenesis, the lipids are the substrate used for mitochondrial combustion; therefore, lipogenesis is required for the thermogenic activity of BAT. Thus, both processes, thermogenesis and lipogenesis, are complementary, and T_3 induces the expression of both UCP-1 and lipogenic enzymes (Bianco *et al.* 1992, García-Jiménez *et al.* 1993, Guerra *et al.* 1996, Hernández & Obregón 2000).

The effect of insulin on the deiodinases has also been studied. Insulin upregulates hepatic T_3 production, as has been described in 'low T_3 syndromes'. In insulin deprivation (as in diabetes and fasting), T_3 production is low due to low hepatic D1 deiodinase activity and mRNA (O'Mara *et al.* 1993), leading to low serum T_3 levels. D2 activity in BAT is also low in these situations, reducing T_3 production, T_3 -induced thermogenesis and hence the capacity to increase energy expenditure in fasting or diabetes. BAT D2 has been proposed as a source of circulating T_3 (Fernandez *et al.* 1987), besides providing the T_3 required for UCP-1 increases.

Using primary cultures of rat brown adipocytes, we previously showed that in hypothyroid conditions NE poorly stimulates D2 activity and that T_3 is required for and amplifies the adrenergic D2 response by 10–20-fold. This effect of T_3 is observed with NE or β_3 adrenergic agents (Hernández & Obregón 1996). The stimulatory effect of T_3 on the adrenergic response of D2 requires *de novo* protein synthesis and is inhibited by actinomycin. Recently, we have shown that T_3 is an absolute requirement for the adrenergic stimulation of D2 mRNA and that T_3 itself increases D2 mRNA (Martínez-deMena *et al.* 2002).

In the present paper, we studied the regulation of the adrenergic stimulation of D2 by insulin in primary cultures of rat brown preadipocytes. We show that insulin plays a fundamental role, upregulating the adrenergic stimulation of D2 activity and mRNA expression in brown adipocytes. The signaling pathways and mechanisms involved in the insulin action, ERK 1/2 and PI3K, are also examined. We conclude that insulin and the stimulation of the ERK 1/2 pathway are essential for the D2-mediated production of T_3 in brown adipocytes.

Materials and methods

Materials

The source of most of the reagents used has been previously described (Hernández & Obregón 1996). We purchased newborn calf serum (NCS) from Flow (Paisley, UK) or from Gibco Life Technologies (Uxbridge, UK); collagenase, bovine insulin, ascorbic acid, dithiothreitol (DTT), 6-*n*-propyl-2-thiouracil (PTU), NE and T_3 from Sigma; insulin-like growth factor (IGF)-I and IGF-II from Boehringer Mannheim (Mannheim, Germany); wortmannin, PD-98059 (PD), LY-294002 (LY) and the proteasome inhibitor MG132 from Calbiochem (Schwalbach, Germany); oligo(dT) cellulose from New England Biolabs (Beverly, MA, USA); p-ERK (MAP kinases, p44 and p42) antibodies and the Western blotting luminol reagent kit from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and Phospho-AKT (PKB) rabbit antibodies from PharMingen (Becton Dickinson).

Primary cultures of brown adipocytes

Precursor cells were obtained from the interscapular BAT of 20-day-old rats (Sprague-Dawley), isolated by the method described by Néchad *et al.* (1983), by collagenase digestion (0.2%) and filtration through 250 μ m silk filters. Mature cells were allowed to float, and the infranatant was put through 25 μ m silk filters and centrifuged. Precursor cells were seeded at a density of 1500–2000 cells/cm² on day 1, and grown in DMEM supplemented with 10% NCS, 3 nM insulin, 10 mM HEPES, 50 IU penicillin and 50 μ g streptomycin/ml, and 15 μ M ascorbic acid. Culture medium was changed on day 1 and every second day thereafter. Precursor cells proliferate actively under these conditions, reach confluence at day 4 or 5 after seeding (40 000–60 000 cells/cm²) and differentiate into mature brown adipocytes. All studies and treatments were done in fully differentiated brown adipocytes (at days 8–9 after seeding).

Both NCS and hypothyroid serum were used for culture. Hypothyroid serum, obtained as previously described (Samuels *et al.* 1979), contained 10% or less of the original amount of thyroid hormones, as assessed by RIA (Morreale de Escobar *et al.* 1985). Total and free T_4 and T_3 in NCS and hypothyroid serum have been recently described (Martínez-deMena *et al.* 2002).

Determination of D2 activity

Cells were scraped, collected in buffer (0.32 M sucrose, 10 mM Hepes and 10 mM DTT, pH 7.0) and homogenized. D2 activities were determined in homogenates by measuring the release of iodide, by the

method previously described (Leonard *et al.* 1983) with modifications (Obregón *et al.* 1989), using as final concentrations 2 nM T_4 (50 000 c.p.m. $^{125}\text{I}-T_4$), 1 μM T_3 , 50 mM DTT, 1 mM PTU and 80–100 μg protein/100 μl total volume, pH 7.0, for 1 h at 37 °C. Cell homogenates were tested in triplicates, using 2–3 culture flasks per treatment. Results were expressed in fmols/h per mg protein.

High specific activity $^{125}\text{I}-T_4$ (>3000 $\mu\text{Ci}/\mu\text{g}$) was obtained in our laboratory with chloramine T and T_3 as substrate (Obregón *et al.* 1989, Hernández & Obregón 1996). Before each assay, $^{125}\text{I}-T_4$ was purified by paper electrophoresis to separate the contaminating iodide. Assay blanks were always less than 1% of the total radioactivity. The validation of the assay has been published (Hernández & Obregón 1996).

RNA preparation and Northern blot analysis

For isolation of Poly(A)⁺ RNA, cells were harvested, and mRNA was isolated using oligo(dT) cellulose, as previously described (Vennström & Bisop 1982). For Northern analysis, Poly(A)⁺ (5 μg) was denatured and electrophoresed on a 2.2 M formaldehyde/1% agarose gel in 1 MOPS buffer and transferred to nylon membranes, as previously described (García-Jiménez *et al.* 1993, Hernández *et al.* 1998). A 1.988 kb fragment of a rat D2 cDNA clone (Croteau *et al.* 1996), including the entire coding region, was used as a probe by labeling with [α - ^{32}P]dCTP, using random primers. Filters were hybridized with D2 cDNA and cyclophilin as a control to correct for differences between lanes in the amount of Poly(A)⁺ mRNA (Danielson *et al.* 1988). Autoradiograms were obtained from the filters and quantified by computer-assisted densitometry (Molecular Dynamics, Sunnyvale, CA, USA). All the experiments were repeated at least two times. The more complete or representative experiments are shown.

Western blotting

Total protein extracts (50 $\mu\text{g}/\text{well}$) were resolved by SDS-PAGE and transferred to PVDF transfer membranes (Biotechnology Systems, NEN Research Products, Boston, MA, USA), in a buffer containing 25 mM Tris, 200 mM glycine and 20% methanol. After blocking of the membrane with 5% low-fat dried milk in Tris-buffered saline containing 0.05% Tween-20, the levels of phosphorylated ERK (p44 and p42) were detected with a 1:5000 dilution of the specific antibody (pERK), followed by a 1:2000 dilution of goat antimouse IgG-HRP (Santa Cruz). Phospho-AKT was detected with a 1:1000 dilution of a specific rabbit antibody (anti-pAKT) from Pharmingen (Becton Dickinson), followed by a 1:5000 dilution of goat antirabbit immunoglobulin (Ig) G (Santa Cruz). Immunoreactive

bands were visualized by enhanced chemiluminescence (Western blotting luminol reagent, Santa Cruz).

Statistical analysis

Mean values $\pm$ s.e. are given. When required, one-way analysis of variance was done, after homogeneity of variance was ensured by square root or logarithmic transformations, if not found with the raw data. The significance of differences between groups was assessed by the protected least-significant-difference test. All the calculations were done as described by Snedecor and Cochran (1980).

Results

Insulin increases the adrenergic stimulation of D2 activity: effect of IGFs

Brown adipocytes grown in the presence of insulin had many lipid droplets and round shapes, while no lipid accumulation was observed in the insulin-depleted cells.

Insulin upregulates D2 activity in floating rat brown adipocytes (Mills *et al.* 1987). However, in cultured rat brown adipocytes, we did not observe increases in D2 activity after addition of human or bovine insulin (from 3 to 3000 nM for 2–6 h) when it was added alone (without NE and T_3) (not shown). But, as insulin improves the adrenergic stimulation of D2, all the experiments were done in the presence of NE plus T_3 , in conditions previously tested (Hernández & Obregón 1996, Martínez-deMena *et al.* 2002).

Figure 1 shows that the adrenergic stimulation of D2 activity improved in the presence of insulin. Figure 1A shows the time-course response of D2 to NE in the presence or absence of insulin. D2 reached a peak 8 h after addition of NE, and the adrenergic response was much lower in the absence of insulin. The effect of insulin depletion for a short and a long time was also examined. Insulin depletion for 1 day (Fig. 1B) decreased D2 activity by 35%, and D2 was fully recovered after a brief exposure to insulin (6 h). Prolonged insulin depletion (3 days; Fig. 1C) caused a 50% reduction in D2 activity. Addition of insulin during the last 6 h had no effect, but D2 activity was partially restored 24 h after addition of insulin. Therefore, the adrenergic stimulation of D2 is upregulated in the presence of insulin, and the recovery of D2 activity is dependent on the insulin-deprivation status of the adipocytes. The maximal inhibition of D2 was observed after 2–3 days of insulin depletion (30–50% of control values), and the inhibition was maintained thereafter; insulin replacement had a clear effect at 24 h.

We previously reported that prolonged exposure to T_3 for 3 days increases the adrenergic stimulation of D2 activity (Martínez-deMena *et al.* 2002). However, the

Figure 1 Effect of insulin depletion on the adrenergic induction of D2 activity. Eight-day-old cultures of brown adipocytes were used. Cells were cultured in medium supplemented with 10% hypothyroid serum and 10 nM T_3 during the last 24 h. NE (5 μ M) was added to all the flasks during the last 6 h. (A): Time response to NE in the presence (closed circles) or absence of insulin (open circles). Cells were cultured in the presence or absence of insulin during the last 3 days and collected at 0, 2, 4, 6, 8, 10, 14, 16 and 20 h after adrenergic stimulation with NE plus T_3 (see above). Data are means $\pm$ s.e. ($n=2$). (B and C): Effect of short and long insulin depletion and insulin replacement on D2 activity. Cells were cultured in insulin-depleted medium during the last 1 or 3 days (B or C), and 3 nM insulin (Ins) was added to some flasks during the last 6 or 24 h, as specified. Data are means $\pm$ s.e. ($n=14$ –22 flasks/point) from nine cultures, expressed as percentages for panels B and C. * $P < 0.05$ vs control cells. # $P < 0.05$ vs insulin-depleted cells. (D): Effect of short and long T_3 exposure on D2 activity. Cells were cultured in insulin-depleted medium during the last 24 h, and 3 nM insulin was added to some flasks for the last 6 h. Cells were treated with T_3 (10 nM) for 1 or 3 days, and NE was added as described above. * $P < 0.05$ vs 1-day treatment with T_3 .

higher D2 activities obtained with prolonged T_3 exposure (Fig. 1D) did not overcome the decreases in D2 activity observed after insulin deprivation.

To test whether the insulin effect was mediated through the IGF receptors, we investigated the effect of increasing doses of insulin, IGF-I and IGF-II on the adrenergic stimulation of D2 activity. Figure 2 shows that insulin increases the adrenergically stimulated D2 activity at very low doses (twofold at 0.03 nM), in a dose-dependent way, and this effect is reproduced only at much higher doses of IGF-I or IGF-II. Maximal stimulation of D2 activity is already obtained with physiologic doses of insulin (3 nM). Insulin is more potent than IGFs, suggesting that its effects are not mediated through the IGFs receptors.

The adrenergic stimulation of D2 activity requires ERK1/2 MAPK and PI3K activities

The actions of insulin are mediated through the binding to its specific membrane receptors that, by activating the insulin receptor tyrosine kinase, leads to the phosphorylation of several substrates, mainly IRS-1, which acts as the anchor protein activating several pathways as PI-3 kinase (PI3K), and also results in the sequential activation of the Ras, Raf and MAPK cascade.

We first tested whether the ERK 1/2 signaling pathway is implicated in the regulation of D2 by insulin. Confluent preadipocytes were depleted from insulin for 4 days. Insulin, added during the last 24 h (Fig. 3), partially restored D2 activity ($P < 0.05$). Addition of PD

Figure 2 Dose–response curves for insulin, IGF-I and IGF-II in the adrenergic stimulation of D2 activity. Eight-day-old cultures of brown adipocytes were maintained in insulin-depleted medium from day 4 (for 4 days). Cells were exposed to different doses of insulin (0.03–300 nM; closed circles), IGF-I or IGF-II (1–100 nM; open circles) for the last 24 h, in medium supplemented with 10% hypothyroid serum and 10 nM T_3 . NE (5 μ M) was added to all the flasks during the last 6 h. Data are means $\pm$ s.e. ($n=4$ –6 flasks/point) from three different cultures. In the ordinates, D2 values for cells grown in standard conditions. prot, protein.

Figure 3 Effect of the inhibitors of MAPK and PI-3 kinase on D2 activity. Eight-day-old cultures of brown adipocytes were maintained in insulin-depleted medium from day 4 (4 days of insulin depletion). Some of the cells received 3 nM insulin (ins) during the last 24 h in the presence or absence of PD 98059 (PD, 50 μ M) or LY-294002 (LY, 10 μ M), added 20 min before the addition of insulin for 24 h. Cells were cultured in the presence of 10 nM T_3 for the last 24 h, using medium supplemented with 10% hypothyroid serum. NE (5 μ M) was added to all the flasks during the last 6 h. Data are means $\pm$ S.E. ($n=8-19$) from nine experiments, expressed as percentage of control. * $P<0.05$ vs control cells. # $P<0.05$ vs insulin-depleted cells. & $P<0.05$ vs insulin-depleted cells plus insulin (24 h).

resulted in inhibition of the insulin effect, indicating that ERK 1/2 is involved in the regulation of the adrenergic stimulation of D2 activity by insulin. We also tested whether PI3K is involved in the signaling pathway by adding two inhibitors of PI-3 kinase, wortmannin (W) and LY-294002 (LY), the latter reported to be more specific and stable. Addition of 20 nM W did not suppress the insulin effect (even after repeated additions, possibly due to its instability in aqueous medium (results not shown), while LY inhibited D2 with an effect similar to PD, indicating that both pathways (ERK 1/2 and PI3K) are involved in the activation of D2 by insulin.

As the growth factors present in the serum also act through the same signaling pathway, we tested the effect of the inhibitors PD and LY with or without serum and insulin. Brown adipocytes were cultured during the last 24 h in the presence or absence of insulin, and in the

presence (Fig. 4A) or absence of serum (Fig. 4B). Cells were always stimulated with NE and T_3 . In the presence of 10% serum (Fig. 4A), insulin depletion decreased D2 activity by 25%, and addition of PD or LY decreased D2 activity, in both the presence and the absence of insulin, indicating that both pathways are possibly stimulated by serum in insulin-depleted cells. Using serum-free medium (Fig. 4B), the adrenergic stimulation of D2 was observed only when insulin was present, but was very low in the absence of insulin and serum, indicating that ERK 1/2 and PI3K are required for the adrenergic stimulation of D2 activity. The addition of LY plus PD did not induce a stronger inhibition (not shown). We conclude that the adrenergic stimulation of D2 by NE and T_3 is not enough by itself to increase D2 activity and requires the presence of an ERK 1/2 or PI3K activity induced by insulin and/or serum. Interestingly, the basal levels in the presence of T_3 increased in the absence of insulin plus serum, suggesting that both agents inhibit the basal D2 activity in T_3 -treated cells.

Activation of ERK 1/2 and PI3K activities

We further checked the activation of ERK 1/2 and PI3K by Western blots under the three stimuli used: insulin, serum and NE. Figure 5A shows that all three stimulate ERK 1/2 (p42 and p44), and serum was more potent than insulin and NE. All three stimuli were inhibited by PD, although the inhibition of serum was not complete. The effect of NE is dose and time dependent (maximum at 10 min and at 0.5–2 μ M NE) (results not shown). Figure 5B shows that T_3 alone has no effect on ERK 1/2, while the addition of insulin to NE plus T_3 increases ERK 1/2 activity, showing an additive effect between the signaling pathways of NE and insulin.

PI3K activation was explored by probing the phosphorylation of AKT (PKB) (downstream of the PI3K cascade). Figure 5C shows that insulin increases pAKT, which is inhibited by LY, but not by PD. The addition of LY plus PD resulted in complete inhibition of pAKT. The addition of serum also induced pAKT, which was slightly inhibited by LY.

We also tested the effect of NE on pAKT. NE by itself did not induce pAKT (Fig. 5D), but enhanced the effect of insulin (lane 5) or insulin plus serum (lane 7), suggesting an interaction between NE and insulin.

Effect of insulin on the adrenergic stimulation of D2 mRNA expression

We tested whether the effects of insulin observed on D2 activity were also observed at the mRNA level. Different results were obtained when cells were depleted of insulin for a long time, around cellular confluence (from

Figure 4 Effect of insulin depletion, in the presence (A) and absence of serum (B), on the adrenergic induction of D2 activity. Effect of the inhibitors PD and LY. Eight-day-old cultures of brown adipocytes were maintained in the presence (3 nM) or absence of insulin for the last 24 h, using medium supplemented with 10% hypothyroid serum (A) or serum-free medium (B) supplemented with 10 nM T_3 . NE (5 μ M) was added to all the flasks during the last 6 h. Some of the flasks received PD (50 μ M) and others LY (10 μ M) during the last 24 h. Data are means $\pm$ S.E. ($n=6$ flasks/point) from three cultures. * $P<0.05$ vs control cells, all in the presence of insulin. # $P<0.05$, insulin-treated cells vs insulin-depleted cells. * $P<0.05$, insulin-depleted cells vs insulin-depleted cells treated with the inhibitor.

day 4, for 5 days) or for a shorter time, during the differentiation period (from day 6, for 3 days). When insulin was suppressed very early, D2 mRNA expression decreased by 50% (Fig. 6A) in agreement with the findings for D2 activity. The addition of physiologic

doses of insulin or IGF-I for 24 h increased D2 mRNA expression by 4.5- and 2.7-fold respectively above control values. However, when insulin was withdrawn for a shorter time and during the differentiation period (at day 6, Fig. 6B), we found small variations and the

Figure 5 (A) Stimulation of p42/p44 MAPK (ERK 1/2) activity by serum, insulin and NE, and inhibition by PD. Nine-day-old cultures of brown adipocytes were cultured in the absence of insulin and serum during the last 24 h. Insulin (Ins) (3 nM), 10% NCS and NE (3 μ M) were added, and cells were collected 10 min later. PD (50 μ M) was added 30 min before the addition of serum, insulin or NE. (B) Additive effect of NE and insulin on MAPK activity. Cells were cultured as described above. T_3 was added as specified. NE (3 μ M) or insulin (3 nM) was added for 10 min. Western blots were prepared with cellular extracts and probed with specific antibodies against p42/44-MAPK. (C) Stimulation of PI3K (pAKT) activity by insulin or 10% NCS in cells cultured as described above. The inhibitors LY, PD or LY+PD were added 30 min before insulin or serum. (D) Stimulation of PI3K (pAKT) activity by insulin or 10% NCS in cells cultured as described above. The inhibitors LY, PD or LY+PD were added 30 min before insulin or serum. (E) Stimulation of PI3K (pAKT) activity by insulin or 10% NCS in cells cultured as described above. The inhibitors LY, PD or LY+PD were added 30 min before insulin or serum.

Figure 6 Effect of insulin depletion on the adrenergic induction of D2 mRNA at day 4 or 6 after seeding. Nine-day-old cultures of brown adipocytes were maintained during the last 72 h (from day 6) in medium supplemented with 5% hypothyroid serum. Insulin was withdrawn from the medium on day 4 (A) or day 6 (B) after seeding. Insulin (Ins) or IGF-I (3 nM) were added during the last 24 h. T_3 (10 nM) and NE (5 μ M) were added during the last 24 h and 6 h respectively. An amount of 5 μ g Poly(A)⁺ RNA was loaded per lane. Hybridization with cyclophilin (Cy) cDNA was used to correct for differences between lanes, and the D2/Cy ratio is shown, taking the control values as 100%. The graph shows the mean of five experiments (day 4, A) and 10 experiments (day 6, B) (three experiments for insulin and IGF-I addition each) (means $\pm$ S.E.).

addition of insulin or IGF-I led to small increases (n.s.) in D2 mRNA expression. This experiment was repeated several times, and the mean values are represented in Fig. 6A and B. Therefore, D2 mRNA decreases only after prolonged insulin depletion, in contrast to the changes observed in D2 activity.

The small increases in D2 mRNA observed in the absence of insulin (Fig. 6B) are not caused by stabilization of the mRNA half-life, because we tested the disappearance of D2 mRNA during 5 h in the presence of actino-

mycin D, and no difference was observed between the presence or absence of insulin (results not shown).

PD decreased D2 mRNA stimulated by insulin or serum (results not shown), confirming the ERK 1/2 dependency of the adrenergic stimulation of D2 also at the transcriptional level. We did not observe the inhibitory effect when using LY.

The action of actinomycin D and cycloheximide (CHX) previously studied in control cells (Martínez-deMena *et al.* 2002) was tested in cells depleted of insulin

Figure 7 (A) Effect of actinomycin D (Act) and cycloheximide (CHX) on the adrenergic stimulation of D2 mRNA, in the presence or absence of insulin (Ins). Nine-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothyroid serum. Insulin was withdrawn from the medium on day 4 or 6 after seeding. Cells were cultured in the presence of 10 nM T_3 during the last 24 h, and 5 μ M NE were added during the last 7 h. Act (5 μ g/ml) or CHX (25 μ M) were added 15 min before NE. An amount of 5 μ g Poly(A)⁺ RNA was loaded per lane. Hybridization with cyclophilin (Cy) cDNA was used to correct for differences between lanes (two experiments). (B) With T_3 alone, we used the same protocol as above, except that T_3 was added for 72 h (without NE), and Act or CHX was added during the last 14 h (two experiments).

at days 4 and 6, and we observed similar results in all three situations. Actinomycin D decreases D2 mRNA levels to undetectable levels, and CHX leads to accumulation of D2 mRNA in all three cases (Fig. 7A). These results suggest that insulin acts as a modulator of the transcriptional increases due to adrenergic stimulation of D2. When T_3 was used alone (Fig. 7B) (T_3 for 72 h without adrenergic stimulation), as previously reported (Martinez-deMena *et al.* 2002), insulin depletion led to complete loss of the D2 mRNA signal, and actinomycin and CHX had the same effect as described with NE plus T_3 . These data suggest that insulin is required for stimulation by T_3 of D2 mRNA.

To determine whether the low D2 mRNA found after insulin depletion is due to a delayed differentiation state of the brown adipocytes, we analyzed D2 mRNA expression at days 5 and 9 (Fig. 8). Higher D2 expression was found near cellular confluence. In fact, D2 mRNA decreases during differentiation (results not shown), showing that a delayed differentiation state could not cause the D2 mRNA decreases in insulin-depleted cells. The capacity of the cells to respond to adrenergic stimulation of D2 mRNA decreases along with differentiation.

Mechanisms of degradation of D2 activity and insulin

As we did not observe a direct action of insulin by itself when it was added to the cells, we investigated whether

the depletion of insulin could decrease D2 activity half-life and modify its degradation pathways. We analyzed D2 activity half-life in the presence or absence of insulin; in the latter, we did not find a higher turnover of D2 activity (Fig. 9). In some experiments, the absence of insulin induced a slight stabilization of D2 activity. Therefore, the decreases observed in the absence of insulin are not due to a higher turnover rate of D2 activity.

We also tested whether insulin influences the degradation pathways of D2. Recently, it has been reported that D2 is degraded via the proteasome (Steinsapir *et al.* 1998, 2000), and also that insulin inhibits proteasome activity (Bennett *et al.* 2000). If so, the absence of insulin would result in higher proteasome activity and higher D2 degradation rates. We tried to inhibit proteasome activity with several proteasome inhibitors (such as PSI and MG132). Unfortunately, most inhibitors of proteasome (PSI, MG132 and lactacystin) strongly induce c-Jun kinase (JNK-1) (Meriin *et al.* 1998), a pathway that inhibits adrenergic stimulation by interaction with the cAMP response element (CRE) element (Yubero *et al.* 1998). We did time-course and dose-response experiments with both MG132 and PSI, and the results were similar with both (not shown). Simultaneous addition of MG132 and NE for 7 h strongly inhibited the adrenergic stimulation of D2 (by 86% at 3 and 10 μ M MG132) (not shown). After

Figure 8 Effect of the differentiation state of the cells and insulin depletion on the adrenergic stimulation of D2 mRNA. Nine-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothroid serum. In some plates, insulin was withdrawn from the medium on day 4. T_3 (10 nM) and NE (5 μ M) were added during the last 24 and 6 h respectively. Some plates were collected on day 5 after treatment with NE plus T_3 . An amount of 5 μ g Poly(A)⁺ RNA was loaded per lane. Hybridization with cyclophilin (Cy) cDNA was used to correct for differences between lanes, and the D2/Cy ratio is shown (means $\pm$ S.E. from two experiments, as 100% of control values on day 9).

several experimental conditions had been tested, the proteasome inhibitor (MG132, 1 and 10 μ M) was added during the last 1–2 h after a long stimulation with NE (18 h), to avoid interference with the initial CRE response (Fig. 10, left panel). D2 increases (180%) were observed with 10 μ M MG132 for 1–2 h. As previously reported (Steinsapir *et al.* 1998), MG132 is also able to reverse the inhibition of D2 activity by CHX (Fig. 10, right panel). Finally, we compared the effect of MG132, added during the last hour of adrenergic stimulation, with or without insulin (Table 1). The use of MG132 led to increases in D2 activities (166%). In the absence of insulin, a smaller increase was observed (138%). This excludes a higher D2 turnover in the absence of insulin, as less D2 is degraded in this situation, in terms of both percentage and absolute units. It also indicates that insulin does not inhibit proteasome activity, because we would have found higher degradation rates in the absence of insulin.

Discussion

Insulin plays a fundamental role by increasing the lipogenic pathways by adipocytes. Besides its action

Figure 9 Half-life of D2 in the presence and absence of insulin. Eight-day-old cultures of brown adipocytes were maintained in the presence of insulin (INS) or in insulin-depleted medium for 3 days. T_3 (10 nM) was added during the last 24 h, using medium supplemented with 10% hypothroid serum. NE (5 μ M) was added to all the flasks during the last 6 h. Then, CHX (25 μ M) was added, and cells were harvested at 0, 20, 30, 40, 60, 75 and 90 min, using controls without CHX at 60, 75 and 90 min. Data are means $\pm$ S.E. from seven experiments ($n=3$ –15/point).

Figure 10 Effect of proteasome inhibitor MG132 on D2 activity. Dose- and time-response experiments. *Left panel.* Eight-day-old cultures of brown adipocytes were maintained in the presence of T₃ (10 nM) during the last 24 h in medium supplemented with 10% hypothyroid serum. NE (5 μM) was added during the last 18 h. MG132 (1 and 10 μM) was added during the last 1 or 2 h of adrenergic stimulation. Data are means±s.e. (n=2). *Right panel.* Cells were cultured as described above, and NE was added during the last 7 h. CHX was added during the last 75 min and MG132 10 min before CHX. Data are means±s.e. (n=4; two different experiments). *P<0.05 vs control cells. #P<0.05 vs CHX-treated cells.

on lipogenic enzymes, insulin seems to control the production of T₃ by regulating both enzymes involved in T₃ production, D1 and D2. Hepatic D1, the major source of circulating T₃, is also regulated by insulin. This seems to have a physiologic meaning, keeping low T₃ levels in conditions with low metabolic requirements, such as in fasting or diabetes. The local production of T₃ in BAT is also decreased in diabetes, providing reduced lipids for thermogenic responses.

Previous reports found large increases of D2 activity after insulin injections to rats (Silva & Larsen 1986b) or a

progressive increase in V_{max} when insulin was added to floating brown adipocytes (Mills *et al.* 1987). In the present study, we found a clearly positive effect of insulin on D2 activity and mRNA, once adrenergically stimulated, but we did not find an effect of insulin by itself, as described in rats, implying that the *in vivo* effect could be mediated through other pathways (Silva & Larsen 1986b). Insulin stimulates the V_{max} of D2 activity in floating brown adipocytes, as shown with higher insulin dosages (3000 μU/ml=25 nM). Our study used lower insulin concentrations (3 nM), but we cannot exclude that floating adipocytes are more mature and respond better to insulin than cultured adipocytes. It has been suggested that the changes in V_{max} are due to an increase in D2 protein synthesis (Mills *et al.* 1987), but the decrease of D2 degradation or the stabilization of D2 half-life that could also lead to accumulation of D2 was not examined.

We observed that adrenergically stimulated D2 activity decreases in the absence of insulin and is induced at very low concentrations (0.05 nM), maximal increases being obtained at physiologic doses (3 nM). Insulin acts through its own receptors, not through IGF receptors, and the recovery of the D2 response depends on the length of insulin depletion. Our results also indicate that the adrenergic stimulation, though lower, is maintained even in the absolute absence of insulin, but in this case the presence of serum is required.

Table 1 Effect of proteasome inhibitor MG132 on D2 activity. Eight-day-old cultures of brown adipocytes were cultured in insulin-depleted medium for 1–2 days. Cells were cultured in the presence of 10 nM T₃ during the last 24 h, using medium supplemented with 10% hypothyroid serum. NE (5 μM) was added to all the flasks during the last 14 h. Half of the cells received 10 μM MG132 during the last hour

	–MG132	+MG132
Treatment		
Control	100% ±3 (n=12)	166% ±7 (n=12) ^a
–Insulin	100% ±8 (n=6)	138% ±7 (n=6) ^{b,c}

Data are means±s.e. (as percentage of its respective control or insulin depleted). Control: six experiments; insulin-depleted cells: three experiments.
^aP<0.05 vs control cells. ^bP<0.05 vs insulin-depleted cells.
^cP<0.05 vs control plus MG132.

The experiments done to delineate the signaling pathway of insulin on D2 activity indicate that insulin acts through both signaling pathways, ERK 1/2 and PI-3 kinase. Most interesting is the fact that the activation of ERK 1/2 by insulin and/or serum is required for the adrenergic stimulation of D2 activity and mRNA. This new finding points to the interrelationship between different signaling pathways and agrees with the report that NE activates ERK 1/2 MAPK (Shimizu *et al.* 1997). We further show an increase in the phosphorylation of ERK 1/2 MAPK when insulin and NE are added together (Fig. 5B). It has also been reported that β 3 adrenergic stimulation activates p38 MAPK in murine brown adipocytes, and this pathway seems to be required for this stimulation (Cao *et al.* 2001). In rat pinealocytes, NE stimulates the phosphorylation of p42/44 MAPK, which in turn is modulated by the activation state of p38 MAPK (Man *et al.* 2004). The link between these two signaling pathways (MAPKs and CRE) becomes more important and involves routes different from the simple stimulation of the CRE. The fact that D2 is stimulated by fibroblast growth factors in astroglial cells (Courtin *et al.* 1990) also supports the role of ERK 1/2 signaling pathways in the stimulation of D2. D2 increases in situations in which recruitment of BAT is found, together with an increased proliferation rate (cold exposure and hypothyroidism), indicating a possible role for the relationship between D2 activities and MAPK signaling pathways. The other pathway explored, PI3K, stimulated by insulin and serum, but not by NE, is also enhanced by the combined presence of NE, insulin and serum.

The mechanisms of the insulin action on D2 are not clear. Insulin could act at different levels, as by increasing the transcriptional effect of NE. Insulin has been reported to stimulate both CREB phosphorylation and transcriptional activation in HepG2 and 3T3-L1 cells, through serine 133, the PKA site, and this process is blocked by PD98059 (Klemm *et al.* 1998). Our results confirm the positive effect of insulin on the transcriptional activation of D2 by NE and disclose a synergy between the PKA and ERK 1/2 pathways, a novel aspect of the adrenergic regulation of D2 in brown adipocytes. The CRE is present in the human and rodent proximal D2 promoter, and seems to play an important role in the full D2 expression. The functional interaction of insulin with CREB remains to be proven for D2. A putative serum response element (SRE) is found in the rat D2 promoter.

Insulin could also act at the post-transcriptional level, modulating D2 half-life or the activity of proteasome (degradation pathways); finally, it could act by promoting specific differentiation aspects necessary for D2 expression. We have analyzed several of these aspects. Our results exclude a shorter D2 half-life or a higher turnover rate of D2 activity as the cause of the

low D2 values found in the absence of insulin. The inactivation of D2 activity has been reported to occur through the proteasome-dependent pathway (Steinsapir *et al.* 1998). Our study of the proteasome activity confirms the role of this pathway in D2 degradation and excludes that the absence of insulin results in a higher degradation of D2. The role of insulin as an inhibitor of proteasomal activity (Bennett *et al.* 2000) is not confirmed in our cells, pointing to pretranslational events.

Our studies at the mRNA level indicate that the adrenergically increased D2 mRNA is rather stable and decreases only after prolonged insulin depletion or when it starts at very early stages (before confluence). These decreases are not observed in shorter insulin depletion (3 days), and small increases (n.s.) in D2 mRNA are even observed (Figs 6 and 7). These increases are not due to accumulation of D2 mRNA, as D2 mRNA half-life does not change in the absence of insulin. Therefore, the decreases in D2 activity due to the absence of insulin are found at the mRNA level, only after prolonged insulin depletion. Another possible explanation is that the early depletion of insulin delays the switch to the differentiation program of the adipocytes, which occurs around cellular confluence (Bronnikov *et al.* 1999). But this is not the case, as higher D2 mRNA expression is found around confluence. The response to insulin is larger in this situation (Fig. 6A), a fact not observed once the adipocytes are already differentiated (Fig. 6B).

The experiments performed with inhibitors of transcription or protein synthesis gave similar results to those reported in control cells under adrenergic stimulation (Fig. 7), suggesting that insulin modulates the transcriptional increases due to adrenergic stimulation.

From Fig. 4, it is obvious that T_3 itself has a very small effect in increasing basal D2 activity in brown adipocytes and its action is inhibited by insulin and serum, in contrast with the T_3 effects on D2 mRNA, where insulin is required for induction of D2 mRNA after prolonged exposure to T_3 .

D2 is an enzyme highly regulated at both the transcriptional and post-transcriptional levels. The present report studies most of the modulators of D2 activity in brown adipocytes: adrenergic stimulation (NE), thyroid hormones (T_3), insulin and growth factors (serum) and the interaction of their signaling pathways. This emphasizes the tight regulation of T_3 production in brown adipocytes and its importance for thermogenesis. D2 has been recently found to be very low in C/EBP α -null mice, together with low BAT T_3 and impairment of many genes involved in thermogenesis (UCP-1 and transcription factors) (Carmona *et al.* 2002). This implies that BAT D2 is linked to the differentiation program of brown adipocytes, which is highly dependent on C/EBP α , and D2 is possibly required for the full achievement of the thermogenic capacity. It also indicates that D2 seems to be a target of C/EBP α and

that its functionality depends on the full activity of these transcription factors.

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