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Thyroxine 5′-Deiodination Mediates Norepinephrine-Induced Lipogenesis in Dispersed Brown Adipocytes*

ANTONIO C. BIANCO, SUZY D. CARVALHO, CELSO R. F. CARVALHO, ROGÉRIO RABELO, AND ANSELMO S. MORISCOT

Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

ABSTRACT

In euthyroid rats, maximal sympathetic nervous system stimulation (*e.g.* during cold exposure) results in a 3- to 4-fold increase in brown adipose tissue lipogenesis, a response that is blunted in hypothyroid rats. To further investigate this phenomenon, the role of local type II 5′-deiodinase (5′-DII) was studied in freshly isolated brown adipocytes. In a typical experiment, 1.5×10^6 cells were incubated for up to 48 h in a water-saturated 5% CO_2-95% O_2 atmosphere. After incubation with medium alone or with different concentrations of T_4, T_3, and/or norepinephrine (NE), lipogenesis was studied by measuring 1) the rate of fatty acid synthesis as reflected by ^3H_2O incorporation into lipids and 2) the activity of key rate-limiting enzymes, *i.e.* acetyl coenzyme A carboxylase and malic enzyme, and the results are reported in terms of DNA content per tube. Lipogenesis decreased progressively over time (~40%) when no additions were made to the incubation medium. T_4 or T_3 partially prevented that inhibition at physiological concentrations (65×10^{-9} and 0.77×10^{-9} M, respectively), whereas a receptor-saturating concentration of T_3 (154×10^{-9} M) doubled the lipogenesis rate. The addition of 10^{-6} M NE inhibited lipogenesis acutely (~50% by 12 h) and was followed by a progressive stimulation that reached ~2-fold by 48 h, but only in the presence of T_4. Furthermore, NE did not attenuate T_3 (154×10^{-9} M)-induced lipogenesis. Both the inhibition and the stimulation of lipogenesis caused by NE showed a strong dose-response relationship within the range of 10^{-11}-10^{-5} M. The role of local 5′-DII was further tested by incubating brown adipocytes with 10^{-6} M NE and T_4 (65×10^{-9} M) in the presence of 100 μM iopanoic acid, a potent inhibitor of 5′-DII. Although iopanoic acid did not affect the T_3 stimulation of lipogenesis, it did block the ~2-fold stimulation of lipogenesis triggered by NE in the presence of T_4, confirming the mediation of 5′-DII in this process. In conclusion, lipogenesis in brown adipose tissue is under complex hormonal control, with key roles played by NE, thyroid hormones, and local 5′-DII. As in other tissues, NE-generated signals acutely (12 h) inhibited lipogenesis. However, the presence of the 5′-DII generated enough T_3 to stimulate lipogenesis and gradually reverse the short-lived NE-induced inhibition, leading to the 2- to 3-fold response observed at later time points. (*Endocrinology* **139:** 571–578, 1998)

BROWN adipose tissue (BAT) is an important site of facultative thermogenesis in small mammals, including human newborns. The local biochemical mechanisms involved in heat liberation are partially understood and include a major inflow and subsequent oxidation of glucose and fatty acids, energy substrates for this tissue (1). Although glucose is plasma borne, fatty acids can either be derived from the hydrolysis of circulating triglyceride-rich lipoproteins or be synthesized *de novo*. In this respect, lipogenesis is a very active pathway in BAT. Indeed, it is estimated that BAT lipogenesis accounts for about 10–20% of rat lipogenesis and possibly more than 50% during cold acclimation (2). BAT lipogenesis is particularly important because it generates fuel (fatty acids) for the abundant BAT mitochondria (1) and also represents a potential thermogenic mechanism *per se*, as in other lipogenic tissues (3).

Norepinephrine (NE) released by the profuse sympathetic nervous system (SNS) innervation triggers most of the BAT biochemical pathways, eventually leading to heat liberation. In addition to modulating several processes that are primarily dependent on the cAMP/protein kinase A (PKA) pathway, NE causes a several-fold stimulation of type II 5′-deiodinase (5′-DII) (4), an enzyme that produces T_3 from T_4 within the brown adipocytes. Shortly after cold exposure is initiated, the occupancy of BAT nuclear T_3 receptors is enhanced from its baseline of ~70% up to virtual saturation (5). As a result, processes that are primarily dependent on thyroid hormone are also activated. Therefore, the physiological changes that take place during cold/NE stimulation of BAT are actually a composite of interactions between NE- and thyroid hormone-generated signals that eventually lead to heat liberation. In fact, the role of local 5′-DII appears to be so important for BAT physiology that, by being increased several-fold during hypothyroidism, the local generation of T_3 reduces the effects of hypothyroxinemia on BAT nuclear T_3 receptor occupancy (6).

We have shown that locally produced T_3 interacts with NE-dependent signals to stimulate uncoupling protein (UCP) gene expression, which is the limiting mitochondrial protein for BAT thermogenesis (7–9). Furthermore, data obtained *in vivo* (10–12) indicate that the SNS stimulation of BAT during cold exposure increases the activity and/or expression of key lipogenic enzymes, *i.e.* malic enzyme (ME), glucose-6-phosphate dehydrogenase, acetyl coenzyme A (CoA) carboxylase (ACC), as well as tissue lipogenesis measured by ^3H_2O incorporation (13, 14). In addition, hemidenervation of the interscapular BAT of rats maintained at thermoneutrality resulted in a rapid ~30–40% drop in ME activity (12).

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Address all correspondence and requests for reprints to: Antonio C. Bianco, M.D., Ph.D., Departamento de Fisiologia e Biofísica, ICB-USP, Avenida Prof. Lineu Prestes 1524, 05508–900 Sao Paulo, Sao Paulo, Brazil. E-mail: acbianco@usp.br.
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It is intriguing, however, that the SNS induces lipogenesis in BAT, the opposite of what NE causes in liver and lactating mammary gland, the other two important lipogenic tissues (15–17). In studies involving differentiating brown adipocytes (18, 19), where the role of local 5′-DII was not accounted for, NE and other inducers of the cAMP pathway, such as glucagon and forskolin, all caused moderate to complete inhibition of S_{14} gene expression, a nuclear protein closely linked to tissue lipogenesis (20). This would, in turn, suggest an indirect effect(s) in SNS-induced BAT lipogenesis. In previous publications (10, 12), we tentatively explained the apparent discrepancy of NE modulation of BAT lipogenesis by the local generation of T_3. Our *in vivo* data suggest that the T_3-induced stimulation of lipogenesis may overcome the inhibition directly caused by NE. Nevertheless, in addition to providing only a tentative explanation, the *in vivo* model has limitations in terms of distinguishing direct from indirect effects, which in the case of lipogenic enzymes are particularly important given their multifactorial regulation.

The aim of the present study was to investigate the mechanisms involved in the hormonal regulation of 1) *de novo* lipogenesis as estimated by ^3H_2O incorporation and 2) the activities of ME and ACC in freshly dispersed brown adipocytes, particularly the individual roles of NE and thyroid hormones. ME is an NADPH-generating enzyme whose activity positively correlates with the rate of *de novo* fatty acid biosynthesis and that of ACC, which is, in turn, the rate-limiting enzyme for this pathway (10, 21).

Materials and Methods

Animals and isolation and culture of brown adipocytes

Male Wistar rats (200–250 g) were obtained from our breeding colony and maintained under controlled conditions of light (12-h dark, 12-h light cycle) and temperature (21–22 C). All animals were kept in plastic cages, five or six to a cage, and had free access to food (rat chow) and water. This study was performed in accordance with the highest standards of animal care. To reduce the sympathetic activity to BAT, all animals were acclimated at 28–30 C for 5–7 days before being killed by decapitation, without anesthesia, in the early morning of the day of each experiment. The isolation of brown adipocytes was based on the method originally reported by Fain *et al.* (22) and subsequently modified by Sundin *et al.* (23) and Obregon *et al.* (24). Previous work from our group (25) and others (26–28) has shown that this isolation protocol yields brown adipocytes that retain their functional regulation of 5′-DII and the ability to respond *in vitro* to NE and thyroid hormones by increasing the expression of the UCP gene. Additional modifications were introduced for the culture of these cells as described below, including extension of the incubation time up to 48 h, with minimal repercussions on cell viability.

Unless otherwise specified, all drugs and reagents were purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO). After the animals were killed, the interscapular and cervical BAT pads were rapidly dissected and placed in basic medium kept at room temperature. This was DMEM buffered with 44 mM sodium bicarbonate and 20 mM HEPES, pH 7.4. On the morning of each experiment the medium was supplemented with 4% fatty acid-free BSA (final concentration). After being freed from visible white fat and minced with scissors, the tissue was suspended in 1 ml medium/rat containing 2 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ). Digestion was carried out at 37 C in a Nalgene (Nalge Company, Rochester, NY) bottle gassed with 5% CO_2-95% O_2 and incubated in a Dubnoff shaker set at 140 cycles/min. After 10 min, the cells were harvested on a chiffon nylon membrane, transferred to a clean Nalgene bottle, and returned to the incubator for an additional 20–25 min. The end of the digestion time was determined by the appearance of homogeneous turbidity in the medium and the disappearance of most visible fragments. The digestion was stopped by the addition of 1 vol collagenase-free medium. The cells were then filtered into plastic graduated centrifuge tubes and left to float. The undigested fragments were discarded. The infranatant was carefully aspirated with a glass micropipette, and the cells were resuspended with new collagenase-free medium. The procedure was repeated two or three times. Finally, the cells were transferred to a clean Nalgene bottle and counted under the microscope. The yield was usually 5–6 $\times$ 10^6 cells/rat.

In any given experiment, cells obtained from BAT pads of 20–25 rats were pooled and then separated into different 2.0-ml Eppendorf tubes containing culture medium down to a final volume of 1.5 ml at a concentration of 1 $\times$ 10^6/ml. The caps of all tubes were perforated with a 30-gauge disposable needle to allow ventilation during incubation. Depending on the experiment, the incubations lasted 2–48 h and were carried out in a cell culture incubator with a water-saturated atmosphere containing 5% CO_2 and 95% O_2.

At the end of the incubation period, cell viability was regularly estimated by trypan blue exclusion and was always greater than 95%. Another indication that the cells remained alive during the many hours of incubation was that in some experiments when the amount of DNA of paired tubes was measured at the beginning and the end of the incubation period, the differences were never greater than 10% of the initial amount. During pilot experiments, 5′-DII was measured in isolated adipocytes as reported previously (24), and typically the addition of 10^{-6} M NE to the incubation medium induced a 3- to 4-fold increase in enzyme activity. The effect of NE was maximal at ~8 h and remained so throughout the incubation period (data not shown).

In all experiments, each time point represents the average of the results obtained in experiments involving sets of 3–4 tubes, carried out in triplicate, for a total of 9–12 tubes per time point. Each of these triplicate tubes was handled identically until the end of incubation. Then, one tube was used to measure ^3H_2O incorporation into lipids, a second tube was used for measurement of ME activity, and the third tube was used for measurement of ACC activity. At the end of the incubation period, a 500-μl aliquot of cell suspension was collected from all tubes for DNA measurement as described by Giles (26). Values ranged from 8–14 μg DNA/tube and were used to calculate the lipogenesis rate and both enzyme activities. The medium from each tube was then aspirated, and the cells were processed for lipid extraction or enzymatic activities.

Hormones and drug additions

For drug addition, the substances were dissolved in volumes of medium ranging from 10–100 μl. NE was added every 24 h along with ascorbic acid (1 mM final) to prevent oxidation. Appropriate control tubes containing no NE also received ascorbic acid. T_3 and T_4 in the free acid form were dissolved in 40 mM NaOH and diluted in medium to the desired concentration. Our previous work indicated that in the presence of 4% BSA, the dialyzable fractions of T_4 and T_3 were only 1.3- to 1.4-fold the dialyzable fractions of T_4 and T_3 in rat plasma (25). Iopanoic acid was dissolved in 40% propylene glycol and then diluted in incubation medium to a final concentration of 100 μM. Appropriate controls had the same final concentration of propylene glycol in the tube (~0.7%).

Measurement of fatty acids synthesis by ^3H_2O incorporation

Rates of *in vitro* lipogenesis were measured in isolated brown adipocytes as the incorporation of ^3H_2O into lipid by a combination of the methods described by Saggerson and Greenbaum (30), Saggerson *et al.* (31) and Folch *et al.* (32). Briefly, ^3H_2O (1.4 Ci/mol; New England Nuclear, Boston, MA) was diluted to 50 μl with incubation medium and added to each tube to a final specific activity of 5 μCi/ml. One hour later, cells were extracted with chloroform-methanol (2:1; 1 ml cell suspension-10 ml of the mixture) and mixed thoroughly with 0.2 vol of a salt solution containing 4.8 mM $CaCl_2$, 3.6 mM $MgCl_2$, and 10 mM NaCl. The lower phase containing the lipids was then transferred to a clean tube, evaporated to dryness at 50 C, and saponified with 2 ml 5% ethanolic KOH at 70–80 C for 2 h. After 2 ml water were added to each tube, and the ethanol was evaporated completely at 45 C, the contents of each tube were washed with 7 ml petroleum ether followed by titration with 0.04% green bromocresol and acidification with 6 N H_2SO_4 during vortexing. The lipids were then extracted three times with petroleum ether. The

combined fractions were evaporated to dryness, redissolved in scintillation liquid (Atomlight), and counted for ^3H. The rates of lipogenesis are expressed as microgram-atoms of ^3H incorporated into lipid per h/100 μg brown adipocyte DNA.

Pilot experiments were carried out to determine the time course of ^3H_2O incorporation into lipids with freshly dispersed cells and with cells that had been incubated for as long as 48 h. Under both conditions the incorporation of ^3H_2O into lipids was fairly linear (two or three experiments per time point), indicating that the whole process was reliable, and the cells were viable up to 48 h from the dispersion time point. The basal rate of lipogenesis in cells incubated with medium alone ranged from 0.6–1.0 μg-atoms/h·100 μg DNA.

Measurement of enzyme activities

At the end of the incubations and after the medium had been carefully aspirated, cells were resuspended in 1 ml ice-cold homogenization buffer according to the enzyme to be assayed. For ME, we used 10 mM Tris-HCl buffer, pH 7.4, containing 0.32 M sucrose, 2 mM EDTA, and 5 mM 2-mercaptoethanol. For ACC, we used 50 mM Tris-HCl buffer, pH 7.5, containing 20 mM sodium citrate, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol. From this step on, all procedures were carried out at 4 C. The cells were homogenized in a motor-driven Teflon-glass homogenizer and centrifuged for 1 h at 100,000 $\times$ *g* to obtain the cytosolic fraction. All samples were stored at −70 C for further processing.

ME was assayed by the method of Ochoa, adapted by Hsu and Lardy (33), in 0.4 M triethanolamine buffer, pH 7.4, containing 0.03 M malate, 0.12 M $MnCl_2$, 3.4 mM $NADP^+$, and about 100 μg diluted cytosolic protein, enough to cause an absorbance change of 0.05–0.1 OD/min. The formation of the NADPH was followed for up to 5 min at 340 nm and then plotted against time. The basal activity of ME in cells incubated with medium alone ranged between 1.0–1.5 ΔOD/min·100 μg DNA.

ACC was assayed by the method of Inove and Lowenstein (34). To separate the enzyme-enriched fraction from internal cellular inhibitors, the whole citosolic fraction was size-fractionated through a 2.5 $\times$ 30-cm Sephadex G-25 column equilibrated with 1 mM dithiothreitol (DTT) dissolved in 20 mM Tris-HCl, pH 7.5. The fraction containing the enzyme was collected and diluted to 1 ml with 50 mM Tris-HCl, pH 7.5, containing 20 mM sodium citrate, 20 mM $MgCl_2$, 1 mM DTT, and 0.5 mg/ml BSA. Subsequently, the enzyme was activated at 37 C for 30 min and immediately assayed for activity. The assay was carried out in 400 μl of a 100 mM Tris-HCl, pH 7.5, buffered solution containing 1 mM DTT, 0.2 mM acetyl-CoA, 20 mM $NaH^{14}CO_3$ (New England Nuclear; 42.6 Ci/mol), 5 mM ATP, 20 mM sodium citrate, 20 mM $MgCl_2$, 0.5 mg/ml BSA, and usually 100 μl activated enzyme at 37 C for 5–10 min. The reaction was interrupted by the addition of 100 μl 4 N HCl, dried under a N_2 stream, redissolved in 1 ml H_2O, and then mixed with 10 ml Atomlight before counting. The results are expressed as units per 100 μg DNA; 1 U ACC activity is equal to 1 μmol [^{14}C]malonyl CoA formed in 1 min at 37 C. The basal activity of ACC in cells incubated with medium alone ranged from 500–800 mU/100 μg DNA.

Statistical analysis

Data were analyzed by one-way ANOVA followed by multiple comparisons using the Newman-Keuls test. Results are expressed as the mean ± SD. For simplicity, statistical significance is only mentioned when it is relevant to an argument.

Results

Time course of lipogenesis and activity of lipogenic enzymes in isolated brown adipocytes

Isolated brown adipocytes were incubated in the absence or presence of T_3 or T_4 at respective concentrations of 0.77 $\times$ 10^{-9} and 65 $\times$ 10^{-9} M to study separately the effects of near-physiological concentrations of these hormones or in the presence of 154 $\times$ 10^{-9} M T_3 to study the effects of a receptor-saturating T_3 concentration. The results are shown in Fig. 1. When no thyroid hormones were present, the lipogenesis rate decreased progressively over time. At 12 h, it was already ~20% lower ($P < 0.05$), and by the end of the incubation period, the drop had reached ~45% (Fig. 1A). ME activity also showed a tendency to decrease over time, although the fall did not reach statistical significance (Fig. 1B). Nevertheless, the ACC activity did decrease substantially

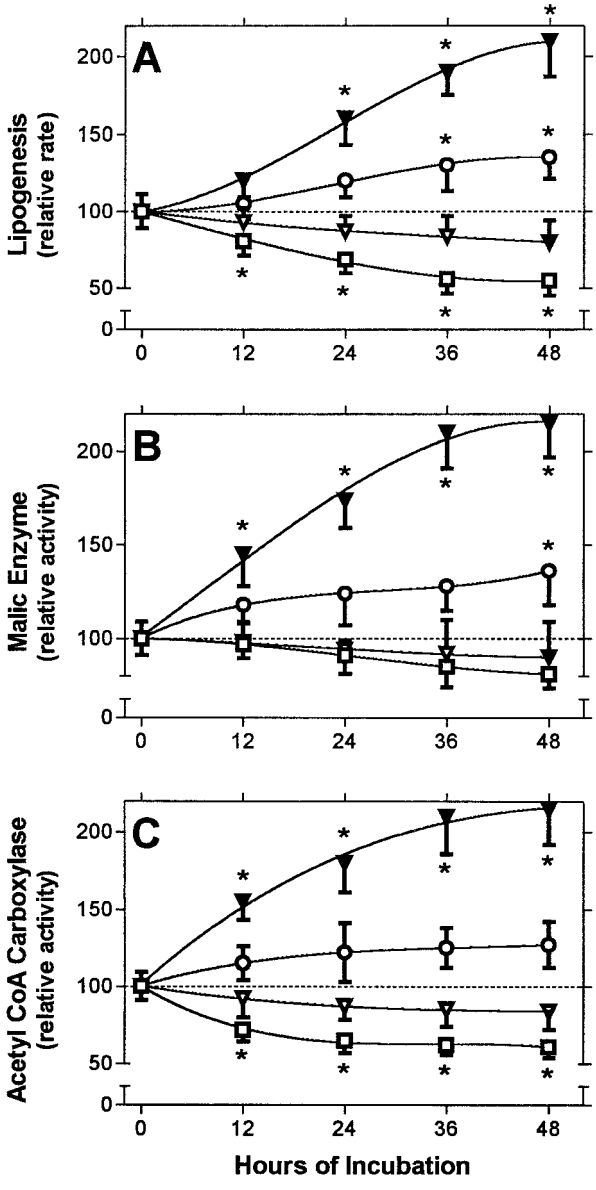

FIG. 1. Effects of thyroid hormones on the time course of lipogenesis and on ME and ACC activities of isolated brown adipocytes. All cells were isolated from intact rats, pooled, and then incubated up to 48 h with medium containing no thyroid hormone (*open squares*), 65 $\times$ 10^{-9} M T_4 (*open circles*), or T_3 at two different concentrations, *i.e.* 0.77 $\times$ 10^{-9} M (*open triangles*) or 154 $\times$ 10^{-9} M (*closed triangles*). At the end of each period, cells were harvested and immediately processed as described in *Materials and Methods*. Each time point represents the average of the results obtained from experiments involving sets of three or four tubes carried out in triplicate. One tube was used to measure ^3H_2O incorporation into lipids, a second tube was used for the measurement of ME activity, and the third tube was used for the measurement of ACC activity. All entries are relative to the values found at time zero. A, ^3H_2O incorporation into fatty acids; B, activity of ME; C, activity of ACC. Each entry is the average of three or four tubes ± SD. *, $P < 0.05$ *vs.* time 0.

under unstimulated conditions; it had fallen by ~30% at 12 h ($P < 0.05$) of incubation and by ~40% at the end of the incubation period (Fig. 1C). In the same experiment, when cells were incubated with a near-physiological T_3 concentration (0.77×10^{-9} M), the lipogenesis rate and the activity of both enzymes (ME and ACC) continued to decrease over the incubation period, although to a much lesser extent and not reaching statistical significance (Fig. 1, A–C). On the other hand, when T_3 was added to the medium at a receptor-saturating concentration, the lipogenesis rate and the activity of both lipogenic enzymes increased markedly (Fig. 1, A–C). By 12 h of incubation, the increment in lipogenic activity had already reached levels 20–50% above basal values ($P < 0.05$ for both ME and ACC). At the end of the incubation, the ~2-fold stimulation mark had been exceeded (Fig. 1, A–C), indicating that even during a 48-h incubation, cells retained the capacity to respond to thyroid hormones. Interestingly enough, cells incubated in the presence of T_4 showed a shallow, but progressive, increase in the lipogenesis rate ($P < 0.05$ by 36 h) and in the activity of the two lipogenic enzymes, which tended to plateau when values were 20–35% above basal. This is an indication that in the presence of T_4, the impact of thyroid hormones on these cells is greater than when they are incubated with a near-physiological T_3 concentration. It is therefore likely that even under these unstimulated conditions, the basal 5′-DII activity generates enough T_3 to sustain a higher lipogenesis rate in brown adipocytes, provided enough T_4 is present. It is worth noting that here and also in the following experiments, DNA concentration did not decrease significantly during incubation (data not shown), indicating that these findings represent a real change in lipogenesis-related activity.

Time and dose dependence of NE-stimulated lipogenesis and the activity of lipogenic enzymes in isolated brown adipocytes

Figure 2 shows the time course of the lipogenesis rate and the activity of lipogenic enzymes when NE was added to the incubation medium at a fairly high concentration (10^{-6} M). The lipogenesis rate decreased significantly by 12 h of incubation, down to ~50% of the basal values ($P < 0.05$), regardless of the presence of thyroid hormones in the medium (Fig. 2A). With regard to ME activity (Fig. 2B), the addition of NE alone resulted in only a mild decrease in enzymatic activity (~10%) by 12 h; however, the inhibition progressed and reached 35% ($P < 0.05$) by 48 h of incubation (Fig. 2B). ACC activity decreased markedly at the early time point; it was reduced to ~40% by 12 h ($P < 0.05$) and remained at that level throughout the incubation period (Fig. 2C). The addition of T_3 at a near-physiological concentration (0.77×10^{-9} M) did not change the NE-mediated down-regulation of lipogenic activity during the entire 48-h incubation period. However, as shown in Fig. 2, A–C, when T_3 was added at a receptor-saturating concentration (154×10^{-9} M), the NE-mediated inhibition of lipogenic activity was reversed, reaching levels significantly higher than basal at 12 h for ME activity (up by 25%; $P < 0.05$) and at 36 h for lipogenesis rate (up by 24%; $P < 0.05$) and ACC activity (up by 69%; $P < 0.05$). In addition, when T_4 and NE were added to the incubation medium, the stimulatory phase of lipogenic

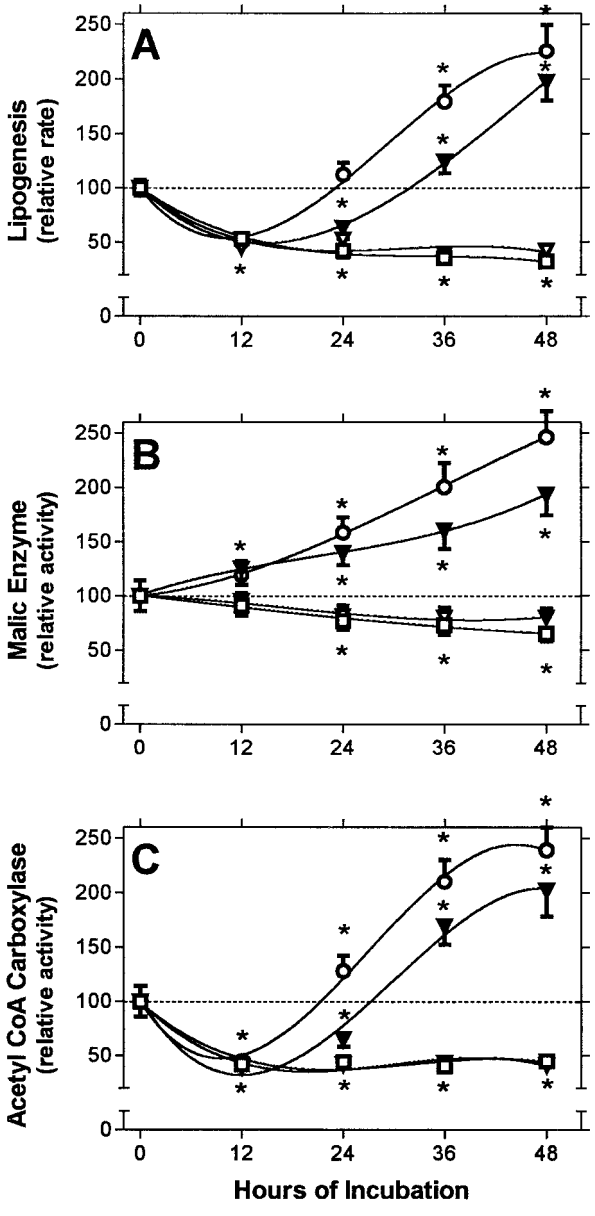

FIG. 2. Effect of thyroid hormones on the time course of lipogenesis and on ME and ACC activities of isolated brown adipocytes incubated in the presence of NE (10^{-6} M). Cells were isolated and processed as described in Fig. 1. Medium contained 5 mM ascorbic acid to minimize NE oxidation. A, Results of ^3H_2O incorporation into fatty acids; B, ME activity; C, ACC activity. Each entry is the average of three or four tubes ± SD. *, $P < 0.05$ *vs.* time 0.

activity was more pronounced than that observed with receptor-saturating T_3 concentrations and caused a 2.2- to 2.5-fold stimulation over basal values (Fig. 2, A–C).

As shown in Fig. 3, we next investigated the dose-response curve for NE inhibition (12-h incubation) or stimulation (48-h incubation) of the lipogenesis rate and for the activity of both lipogenic enzymes. Brown adipocytes were incubated with different NE concentrations (10^{-11}–10^{-5} M) with or without T_3 or T_4. Figure 3A shows that when brown adipocytes were incubated with a wide range of NE concentrations for only 12 h, the lipogenesis rate decreased progressively with in-

creasing NE concentrations in the medium. Statistical significance was reached with 10^{-9} M NE when the lipogenesis rate was decreased by 35%. The presence of a near-physiological T_3 concentration did not change this pattern, whereas T_4 or high T_3 concentrations slightly shifted the curve to the right, but were still unable to prevent the NE-mediated inhibition of lipogenesis. Interestingly, as shown in Fig. 3B, ME activity decreased only mildly (by about −15% at 10^{-5} M NE; $P < 0.05$) when no thyroid hormone was added or when T_3 was added at a near-physiological concentration. Again, when either T_4 or a receptor-saturating concentration of T_3 was added, the dose-response curve was slightly shifted to the right (Fig. 3B), minimizing the inhibitory effect of NE on ME activity. The behavior of ACC activity was similar to that observed for the lipogenesis rate (Fig. 3C). Regardless of the presence of any given thyroid hormone, ACC activity decreased markedly and progressively as the NE concentration increased. Statistical significance was reached at 10^{-9} M NE, and at 10^{-5} M NE, ACC activity was reduced to only ~30% of the basal values.

Next, brown adipocytes were incubated with different NE concentrations (10^{-11}-10^{-5} M) with or without T_3 or T_4 for 48 h to investigate the role of thyroid hormones in NE-stimulated lipogenesis at later time points. Figure 4 shows that, as expected, in the absence of thyroid hormones or in the presence of T_3 at a near-physiological concentration, in-

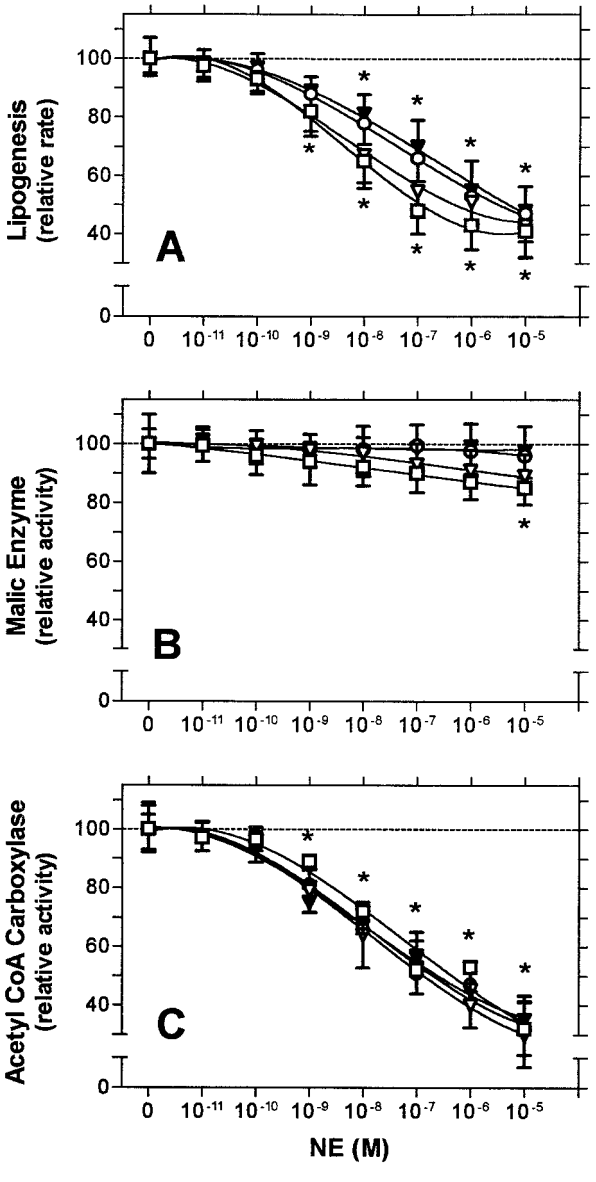

FIG. 3. NE dose-response curve of lipogenesis and ME and ACC activities of isolated brown adipocytes incubated for 12 h: effects of thyroid hormones. Cells were isolated and processed as described in Figs. 1 and 2. However, incubation of all cells lasted only 12 h. A, Results of ^3H_2O incorporation into fatty acids; B, ME activity; C, ACC activity. Each entry is the average of three or four tubes ± SD. *, $P < 0.05$ *vs.* basal.

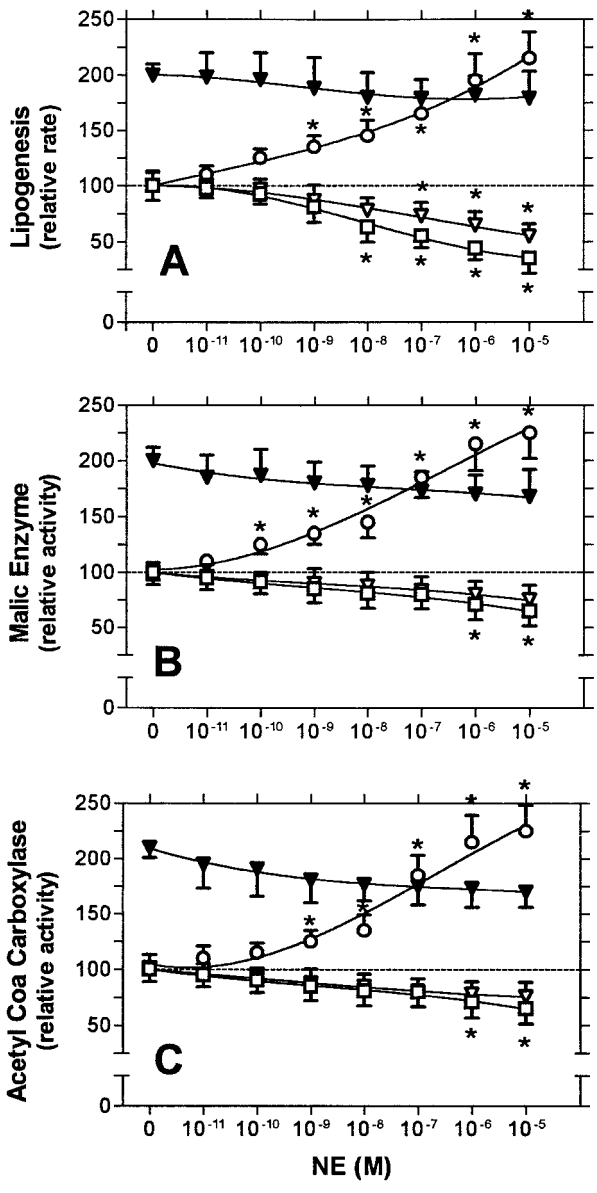

FIG. 4. NE dose-response curve of lipogenesis and ME and ACC activities of isolated brown adipocytes incubated for 48 h: effects of thyroid hormones. Cells were isolated and processed as described in igs. 1 and 2. However, incubation of all cells lasted 48 h. A, Results of ^3H_2O incorporation into fatty acids; B, ME activity; C, ACC activity. Each entry is the average of three or four tubes ± SD. *, $P < 0.05$ *vs.* basal.

creasing NE concentrations resulted in decreased lipogenesis and a 40–50% reduction of ME and ACC activities. On the other hand, when brown adipocytes were incubated with a receptor-saturating T_3 concentration for 48 h, lipogenesis and both enzyme activities were stimulated up to ~210%. Increasing NE concentrations tended to slightly reduce all three lipogenic parameters, particularly ACC activity (Fig. 4C). Nevertheless, at any given NE concentration, lipogenesis and ME and ACC activities were well above basal values, at least by a factor of ~1.7 (Fig. 4). When cells were incubated with T_4, the profiles obtained for all three parameters were startling. NE stimulated lipogenesis and the activity of both lipogenic enzymes in a dose-dependent fashion, exceeding ~2.3-fold stimulation at the maximum NE concentration (10^{-5} M).

Effects of IOP on NE-stimulated lipogenesis and the activity of lipogenic enzymes in isolated brown adipocytes

The potency of T_4 compared with that of a near-physiological T_3 concentration in allowing NE to stimulate lipogenic activities suggests, as in previous *in vivo* experiments (12), that the NE-induced activation of the local 5′-DII plays an important role. To test this hypothesis, we added IOP (100 μM) to the incubation medium and treated the brown adipocytes with either T_4 (65×10^{-9} M) or receptor-saturating concentration of T_3 in the presence of NE (10^{-6} M). All incubations were carried out for 48 h, and the results are presented in Fig. 5. As before, in the absence of T_4, NE failed to stimulate either the lipogenesis rate or enzyme activities. On the other hand, as anticipated from the experiments described above, the addition of T_4 to the incubation medium plus NE resulted in stimulation of lipogenesis and both enzyme activities, and the mechanism involved was fully blocked by the addition of IOP to the incubation medium (Fig. 5A). A similar extent of stimulation was detected when the cells were incubated with NE and a receptor-saturating T_3 concentration, except that in this case IOP treatment did not blunt the lipogenic response to T_3, nor did it affect the cells when added in combination with NE.

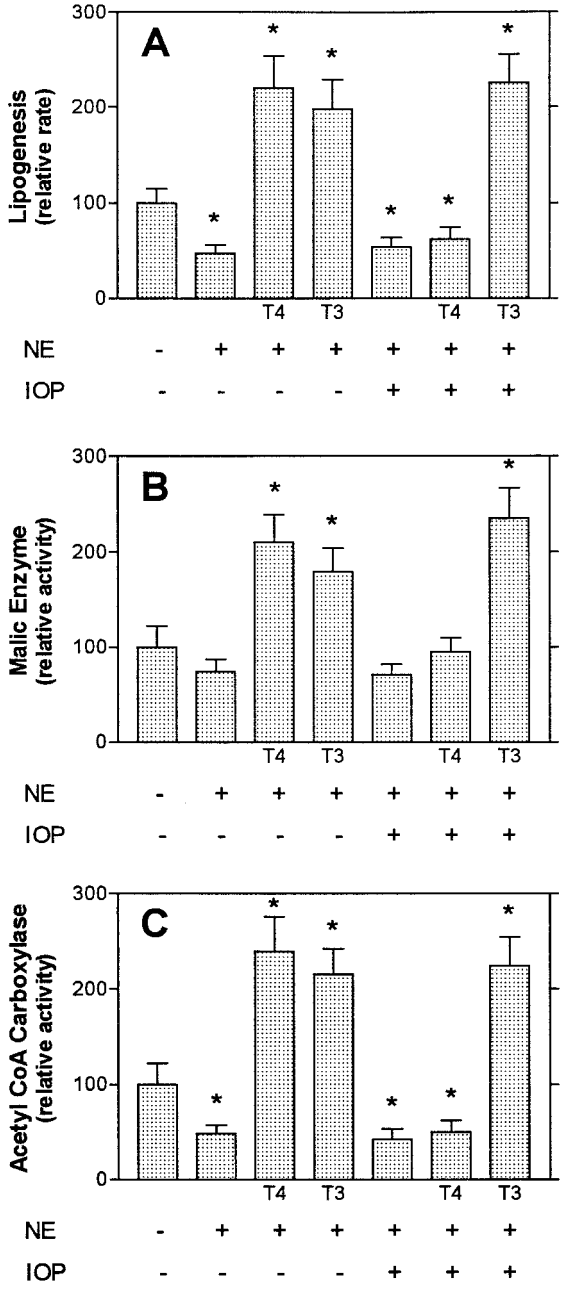

FIG. 5. Stimulation of lipogenesis and ME and ACC activities by NE and thyroid hormones: influence of IOP. Cells were isolated and processed as described in Figs. 1 and 2. Concentrations of T_4 and T_3 were 65×10^{-9} and 154×10^{-9} M, respectively. IOP was used at 100 μM. Incubation lasted for 48 h. Each entry is the average of three or four tubes ± SD. *, $P < 0.05$ *vs.* basal.

Discussion

The synthesis of long chain fatty acids is an energy expensive process. As discussed in detail previously (3) the equivalent of 27 mol ATP is required for each mole of palmitate synthesized. When esterification, export and storage are considered, the estimated molar cost is 40 ATP/palmitate synthesized. Because key lipogenic enzymes are stimulated by thyroid hormones, it has been suggested that ~3–4% of the thermogenic effects of these hormones on obligatory thermogenesis could be accounted for by their stimulatory role in lipogenesis (3, 35).

However, less is known about the contribution of lipogenesis to facultative thermogenesis. BAT lipogenesis accounts for a substantial fraction of the whole rat lipogenesis (2), raising the possibility that in this tissue, thyroid hormone-induced lipogenesis may play a more important thermogenic role than previously recognized. Based on published values for BAT lipogenesis (2) and oxygen consumption of animals acclimated at room temperature (9), it has been estimated that the caloric cost of BAT lipogenesis is ~10% of the total BAT oxygen consumption, a figure that is expected to rise during cold stimulation.

In previous publications, we (10, 12) and others (11) have investigated *in vivo* the stimulation of BAT lipogenesis by the SNS and/or thyroid hormones. The results indicated, however, that these are not independent stimuli; BAT T_3 production, via local T_4 5′deiodination, is stimulated severalfold by the SNS (4, 36), increasing the BAT T_3 concentration

and its nuclear T_3 receptor occupancy (5). Therefore, it was difficult to quantitate the individual role and contribution of each hormonal input because thyroid- and SNS-dependent signals are concurrently generated whenever BAT is physiologically stimulated. Another complicating factor is that thyroid hormones increase BAT cAMP production in response to NE by altering the expression of adrenergic receptors, adenylyl cyclase subtype, and other proteins involved in cAMP production (37), therefore modulating BAT sensitivity to the SNS.

The first aspect that is noteworthy about lipogenesis in isolated brown adipocytes is that in the absence of hormonal additions to the incubation medium, the rate of lipogenesis and the activity of ME and ACC tended to decrease substantially over time, down to ~60% by 48 h. Because cells remained viable throughout the incubation period, these results indicate that the high rates of lipogenesis sustained *in vivo* are directly dependent on neural/endocrine stimulation. Indeed, the addition of either T_4 or T_3 to the incubation medium at physiological concentrations did minimize the time-dependent down-regulation of lipogenesis. Furthermore, lipogenesis was stimulated up to ~2-fold when a receptor-saturating concentration of T_3 was added. This confirms our *in vivo* data showing that thyroid hormones positively regulate lipogenesis in the BAT (10, 12) and indicates that T_3 vigorously stimulates lipogenesis in isolated brown adipocytes *in vitro*, independently of NE.

The results obtained in the present investigation indicate that NE modulates lipogenesis in brown adipocytes in a complex manner, and the final biological effect depends on 1) whether T_4 is present and 2) the incubation span. Alone, NE rapidly inhibited lipogenesis in a dose-dependent fashion, whereas in the presence of a physiological T_4 concentration and longer incubation times (48 h), NE stimulated lipogenesis ~2-fold, also in a dose-dependent manner, to a level similar to that observed when cells were incubated with a receptor-saturating concentration of T_3. These results resemble previous findings showing that fatty acid synthesis (38) and ACC messenger RNA levels studied by primer extension analysis (39) decrease sharply during the first 4–6 h of cold exposure, only to increase by a factor of 2–3 after 24–48 h of continued cold stimulation.

On the one hand, the present results confirm the capacity of NE to acutely inhibit lipogenesis, in consonance with the inhibitory effects of NE and other inducers of the cAMP pathway, such as glucagon and forskolin, on lipogenesis and/or the activity/expression of lipogenic enzymes/proteins in the liver, lactating mammary gland (15–17), BAT (40), and brown adipocytes (18, 19). On the other hand, the results obtained also indicate the capacity of NE to induce lipogenesis in isolated brown adipocytes, provided T_4 is present and the incubation is allowed to proceed for 24–48 h, in accordance with the 3- to 4-fold stimulation of lipogenesis detected *in vivo* during cold exposure, via SNS (2).

There is extensive *in vivo* (10, 12) and now *in vitro* evidence that the mechanism responsible for reversing the inhibitory role of NE on BAT lipogenesis includes the activation of 5′-DII by NE. This would, in turn, intensify the impact of T_3 in this tissue by increasing the BAT nuclear T_3 receptor occupancy. As a consequence, even in the presence of NE, lipogenesis was vigorously stimulated, as when cells were incubated with receptor saturating concentrations of T_3. Nevertheless, the operation of such a mechanism requires the T_3 induction of lipogenesis to be more potent than the NE inhibitory effect. Indeed, our data strongly support this assumption. Even at the highest concentration, NE could not inhibit or prevent the ~2-fold lipogenesis stimulation caused by receptor-saturating concentrations of T_3.

ME and ACC gene expressions are known to be up-regulated by T_3 and down-regulated by cAMP/PKA-dependent nuclear proteins (15–17, 41). Fatty acid synthase gene expression is also specifically inhibited by cAMP/PKA (42). Based on the time course of our results, it is likely that an early NE-dominant phase (negative) would be followed by a later T_3-dominant phase (positive), up-regulating the transcription of these genes. In fact, we did report that ACC messenger RNA levels in BAT are ~2-fold increased after 48–96 h of cold stimulation (39), confirming the T_3-induced lipogenic phase. However, in addition to these nuclear effects, ACC is known to be very sensitive to phosphorylation by PKA. NE and other inducers of the cAMP/PKA pathway inhibit lipogenesis, allowing PKA to inactivate the enzyme by phosphorylation (42). This might be the reason we found a greater inhibition of ACC activity when cells were incubated with NE (~60%) compared with the mild 10–20% inhibition of ME activity, which is known not to be regulated by allosteric effectors or covalent modifications. At this time we do not understand the mechanism by which T_3 would overcome ACC allosteric inactivation by PKA.

Nonetheless, the present findings in isolated brown adipocytes confirm the idea put forward in previous publications from several groups (43, 44) and our own (5–7) that the intracellular T_3 pool at any given moment represents a composite of the relative contributions of T_3 derived as such from the circulation and T_3 generated locally via 5′-D. This is particularly true in the BAT, given the expression of 5′-DII and the magnitude of physiological regulation it undergoes. Because 5′-DII is stimulated several-fold by NE, the local impact of thyroid hormones increases substantially, triggering the local thermogenic effects of thyroid hormones without affecting other tissues. We have shown that this mechanism is limiting for the full expression of the UCP gene (7) and the BAT thermal response during NE infusion (45).

In the present investigation we expanded this concept to include BAT lipogenesis. In this system, 5′-DII is so critical that the stimulatory effects of NE on local lipogenesis are necessarily mediated by the activation of local T_4 to T_3 conversion. In addition, one more piece of evidence favoring the critical role of 5′-DII in this process was obtained when isolated brown adipocytes were incubated in the presence of IOP, a specific inhibitor of T_4 deiodination. The blockade of 5′-DII with IOP did not interfere with the down-regulation of lipogenesis caused by NE. However, it did blunt the NE-induced lipogenesis in these cells, emphasizing the key role of locally generated T_3 in allowing stimulation of lipogenesis by NE in BAT.

Apart from the effects discussed above, the actions of NE on BAT elicit a powerful activation of the hormone-sensitive lipase within minutes of SNS stimulation. Lipolysis is so intense that it greatly exceeds BAT's capacity to oxidize fatty

acids, and BAT becomes an exporter of fatty acids (46), a fact that might contribute to fuel shivering thermogenesis. In the present *in vitro* investigation we found that during a similar time frame, the first few hours of NE stimulation, NE decreases lipogenesis by half, indicating that the initial sympathetic surge to BAT shifts the balance between lipolysis and lipogenesis, stimulating the former and inhibiting the latter. After ~24 h, however, as the biological effects of the rapidly rising local T_3 production start to take place, the inhibitory effects of NE on lipogenesis are minimized by a greater T_3 impact on the tissue, and a new steady state is reached, where the rate of lipogenesis approaches and eventually equals that of lipolysis. This assumption is based on previous data (47) indicating that the size of the intracellular brown adipocyte fat depot, as estimated by the diameter of the lipid vacuoles and cell area, decreases by half during the first hours of cold exposure, only to be restored by 24 h of continued cold stimulation. T_4 and the 5′-DII are critical in this process. By stimulating several-fold the local transformation of T_4 to T_3, NE induces lipogenesis and as a consequence sustains the high rates of electron transfer in BAT mitochondria and the capacity to export fatty acids.

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