Mice with Targeted Disruption of the Dio2 Gene Have Cold-Induced Overexpression of the Uncoupling Protein 1 Gene but Fail to Increase Brown Adipose Tissue Lipogenesis and Adaptive Thermogenesis

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The Dio2 gene encodes the type 2 deiodinase (D2) that activates thyroxine (T4) to 3,3′,5-triiodothyronine (T3), the disruption of which (Dio2$^{-/-}$) results in brown adipose tissue (BAT)-specific hypothyroidism in an otherwise euthyroid animal. In the present studies, cold exposure increased Dio2$^{-/-}$ BAT sympathetic stimulation ~10-fold (normal ~4-fold); as a result, lipolysis, as well as the mRNA levels of uncoupling protein 1, guanosine monophosphate reductase, and peroxisome proliferator–activated receptor γ coactivator 1, increased well above the levels detected in the cold-exposed wild-type animals. The sustained Dio2$^{-/-}$ BAT adrenergic hyperresponse suppressed the three- to fourfold stimulation of BAT lipogenesis normally seen after 24–48 h in the cold. Pharmacological suppression of lipogenesis with ββ′-methyl-substituted α-ω-dicarboxylic acids of C14–C18 in wild-type animals also impaired adaptive thermogenesis in the BAT. These data constitute the first evidence that reduced adrenergic responsiveness does not limit cold-induced adaptive thermogenesis. Instead, the resulting compensatory hyperadrenergic stimulation prevents the otherwise normal stimulation in BAT lipogenesis during cold exposure, rapidly exhausting the availability of fatty acids. The latter is the preponderant determinant of the impaired adaptive thermogenesis and hypothermia in cold-exposed Dio2$^{-/-}$ mice. *Diabetes* 53:577–584, 2004

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ACC, acetyl CoA carboxylase; α-MT, α-methyl parathyrosine; BAT, brown adipose tissue; D2, type 2 iodothyronine deiodinase; GMPr, guanine monophosphate reductase; HSL, hormone-sensitive lipase; IBAT, interscapular BAT; NE, norepinephrine; PGC-1α, peroxisome proliferator–activated receptor γ coactivator 1-α; SNS, sympathetic nervous system; T3, 3,3′,5-triiodothyronine; T4, thyroxine; TG, triglyceride; TR, thyroid hormone receptor; UCP-1, uncoupling protein 1; WT, wild-type.

Adequate quantities of thyroid hormone are required for the maintenance of basal energy expenditure (1,2) and are also critical for adjustments in energy homeostasis during acute exposure to cold, without which survival is not possible (3). These adjustments in nonshivering adaptive thermogenesis are initiated by an increase in the activity of the sympathetic nervous system (SNS). In human newborns and other small mammals, brown adipose tissue (BAT) is the main site of the sympathetic-mediated adaptive thermogenesis. During cold exposure, there is an acute ~50-fold increase in type 2 iodothyronine deiodinase (D2) activity in BAT that accelerates thyroxine (T4) to 3,3′,5-triiodothyronine (T3) conversion (4). This increases thyroid hormone receptor (TR) saturation and leads to intracellular thyrotoxicosis specifically in this tissue (5), which in turn increases adrenergic responsiveness (6–8) in a feed-forward mechanism that allows BAT to produce heat in a sustainable manner.

The current paradigm of thyroid-adrenergic synergism is based on the principle that hypothyroidism causes a generalized decrease in adrenergic responsiveness and, therefore, frustrates the homeostatic role of the SNS, including the stimulation of BAT (9,10). However, these studies are largely based on the hypothyroid animal as a model, which has serious limitations for this purpose. The reduced obligatory energy expenditure caused by systemic hypothyroidism leads to a generalized and gradual increase in sympathetic activity that, in the BAT, activates adaptive energy expenditure to sustain normal core temperature, even at room temperature (11). However, chronic norepinephrine (NE) stimulation typically sets off a series of desensitization mechanisms designed to limit adrenergic responsiveness, decreasing the capacity for further increase in the adrenergic signal transduction as required during an acute exposure to cold. Thus, it is difficult to differentiate the primary effects of hypothyroidism in BAT from those caused by the compensatory increase in sympathetic activity.

Conversely, the recently created mouse with targeted disruption of the D2 gene (Dio2$^{-/-}$) constitutes an improved system to study thyroid-adrenergic interactions.

These animals are systemically euthyroid, as their serum T3 is normal and serum T4 is only slightly elevated. Thus, they do not develop homeostatic adaptations at room temperature (12,13). However, the absence of D2 impairs BAT thermogenesis by precluding the adaptive increase in T4 to T3 conversion. As a result, cold-exposed Dio2$^{-/-}$ mice activate shivering, a less efficient thermogenic pathway that ensures survival but does not prevent mild hypothermia (13).

It is not entirely clear why the D2-mediated high T3 receptor saturation is critical for BAT thermogenesis. On the basis of studies performed in hypothyroid rat and mouse models, the Dio2$^{-/-}$ mouse would have been expected to have impaired uncoupling protein 1 (UCP-1) expression (14–16) and decreased adrenergic responsiveness (7,8). However, our previous studies indicate that the nonstimulated Dio2$^{-/-}$ BAT has normal amounts of mitochondria and normal UCP-1 concentration (13). At the same time, Dio2$^{-/-}$ brown adipocytes do have decreased cAMP generation capacity in response to different adrenergic stimulants (13), indicating that the latter is the mechanism of impaired thermogenesis. Unexpectedly, the present studies reveal that cold-exposed Dio2$^{-/-}$ mice have a compensatory approximately ninefold increase in BAT SNS stimulation that bypasses the relative adrenergic insensitivity. At the same time, we now report that the compensatory supernormal sympathetic stimulation causes intense lipolysis and suppresses the otherwise normal lipogenic surge observed during cold exposure, thus rapidly depleting the brown adipocytes of its source of fatty acids, resulting in impaired adaptive thermogenesis.

RESEARCH DESIGN AND METHODS

Chemicals and drugs. Unless otherwise specified, all drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The ββ′-methyl-substituted α-ω-dicarboxylic acids of C14–C18 chain length (MEDICA-16), a dead-end inhibitor of acetyl CoA carboxylase (ACC) and a competitive inhibitor of citrate lyase (17), was a gift from Dr. Jacob Bar-Tana (Department of Nutrition and Metabolism, Hebrew University Medical School, Jerusalem, Israel).

Animals and treatments. All studies were performed under a protocol approved by the Standing Committee on Animal Research. Some experiments were performed on male Wistar rats that weighed 200–260 g, obtained from our breeding colony as described (18). Surgical thyroidectomy was performed under light ether anesthesia and was followed by administration of 0.05% methimazole in the drinking water. Other studies were performed in C57BL/6J or B6129SF2/J mice that weighed 20–30 g and were either purchased from The Jackson Laboratories (Bar Harbor, MA) or bred in our laboratory as described (13). Dio2 genotyping was by PCR using a wild-type (WT) sense primer (5′-GTTTAGTCATGGAAGCAGCACTATG-3′), a Dio2$^{-/-}$ sense primer (5′-CGTGGGATCATTGTTTTTCTCTTG-3′), and a common antisense primer (5′-CATGGCGTTAGCCAAAACTCATC-3′), which generates an ~400-bp and an ~450-bp fragment corresponding to WT and Dio2$^{-/-}$, respectively.

Interscapular BAT pad thermal response and NE turnover. The interscapular (IBAT) thermal response to NE was performed as described (16,19) in mice anesthetized with urethane (1.2 g/kg i.p.). Raw data were plotted over time and expressed in terms of maximum ΔIBAT temperature (°C). NE turnover was measured in mice acclimated at room temperature or during cold exposure by blocking NE synthesis with 300 mg/kg α-methyl parathyrosine (α-MT) as previously described (20). Mice were killed at 0, 1, 2, 3, or 4 h after the α-MT injection, and the IBAT was processed for NE measurement by radioimmunoassay (Alpco Diagnostics, Windham, NH).

IBAT processing for histologic studies. Animals were killed by perfusion with ice-cold 0.9% NaCl over a period of 15 min, under chloral hydrate anesthesia (33%; 0.1 ml/100 g body wt i.p.), followed by perfusion with 4% paraformaldehyde in 0.05 mol/l phosphate buffer (pH 7.4). The IBAT was rapidly removed and processed for light microscopy or electron microscopy as described (21). Sections were analyzed under a Nikon Eclipse E600 light microscope or at 80 KV with JEOL 100CXII or JEOL 1010 transmission electron microscopes.

Biochemical determinations and IBAT enzymatic activities. The IBAT was processed by homogenization and isolation of the cytosolic fraction as described (18). Malic enzyme and glucose 6-P-dehydrogenase were assayed using 50–100 μg of cytosolic protein (18,22,23). ACC was assayed as described (24,25), and the results are expressed as units per minute per milligram of protein; 1 unit of ACC activity is equal to 1 mmol of [^{14}C]malonyl CoA formed in 1 min at 37°C. Hormone-sensitive lipase (HSL) activity was measured as described (26,27), and the results are expressed as nanomoles of free fatty acid released per minute per milligram of protein. For measuring in vivo BAT lipogenesis, $^{3}H_2O$ (5 mCi; New England Nuclear, Boston, MA) dissolved in 0.5 ml of saline was injected intravenously (jugular vein) into fed mice, which were killed 1 h later. The IBAT was weighed and processed for lipid extraction as described (25). Rates of lipid synthesis were calculated assuming that the specific activity of intracellular water was identical to that of plasma water; each glycerol and each fatty acid incorporated into triglycerides (TG) contained 3.3 and 13.3 atoms of tritium, respectively. TG content was measured by enzyme-linked immunosorbent assay (L-Type TG H; Wako Chemicals USA, Richmond, VA) after extraction with chloroform/methanol (2:1).

IBAT mRNA analysis. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (28) or using TRIzol (Invitrogen, Carlsbad, CA). Northern blots were performed using current standard techniques (29) and specific mouse UCP-1 or peroxisome proliferator–activated receptor γ coactivator 1-α (PGC-1α) cDNA probes provided by Dr. Brad Lowell (Division of Endocrinology, Beth Israel Deaconess Medical Center) or rat guanine monophosphate reductase (GMPr) (30) cDNA probe. RT-PCR was performed as previously described (31,32). PCR cDNA was synthesized using 2.5 μg of total RNA, the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), and the Robocycler thermocycler (Stratagene, La Jolla, CA). The cDNA product was used in an RT-PCR reaction using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). The cycle conditions were 15 min at 94°C (Hot Start), 30–50 s at 94°C, 30–50 s at 55–60°C, and 45–60 s at 72°C for 40 cycles. A final extension at 72°C for 5 min was performed as well as the melting curve protocol to verify the specificity of the amplicon generation. Standard curves consisting of five points of serial dilution (factor of 5) of mixed experimental and control groups cDNA were performed in each assay and used as calibrators. β-actin was used to correct for the loaded amount of cDNA. r^2 was >0.99 for all standard curves, and the amplification efficiency varied between 80 and 100%.

Statistical analysis. One-way ANOVA was used to compare more than two groups, followed by the Student-Newman-Keuls test to detect differences between groups. The Student's t test was used to compare the differences between two groups. $P < 0.05$ was used to reject the null hypothesis.

RESULTS

Exaggerated sympathetic responsiveness in cold-exposed hypothyroid and Dio2$^{-/-}$ animals. Brown adipocytes are typically multilocular, with the bulk of the cell being occupied by numerous round-shaped TG inclusions of various sizes permeated by a profusion of mitochondria separated by a cytoplasmic matrix (Fig. 1) (33,34). Acute cold exposure causes a reduction in the size and number of lipid inclusions (Fig. 1). By 6 h, the round inclusions have developed indentations, and by 12 h, virtually all lipid inclusions have disappeared and the mitochondria have become larger and less dense. By 24 h of cold exposure, however, the cytoplasm is once more filled with small lipid inclusions that permeate the space between the mitochondria (Fig. 1). As these lipid inclusions fuse (48 h), their sizes increase, and by 7 days of cold exposure, they almost have returned to that observed at thermoneutrality.

The typical multilocular aspect is preserved in the hypothyroid brown adipocytes (Fig. 1). Remarkably, after only 6 h of cold exposure, the size and number of the lipid inclusions are decreased, and the nuclei can now be seen at the center of the brown adipocytes (Fig. 1). It is notable that this lipolytic response is greater than in control cells. However, hypothyroid animals die of hypothermia if exposed for >6 h to cold, precluding longer studies of

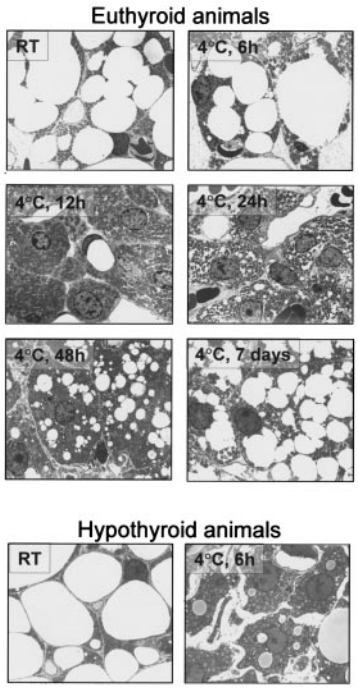

FIG. 1. Cold-induced ultrastructural changes in IBAT of euthyroid and hypothyroid rats. Electron microscopy of brown adipocytes during 0–7 days of cold exposure. Ambient temperature and times in the cold are indicated in the upper left corner of each slide. Pictures are representative of 12–20 images obtained per animal in groups of three to four animals. This experiment was performed two to three times. Magnification ×2,000.

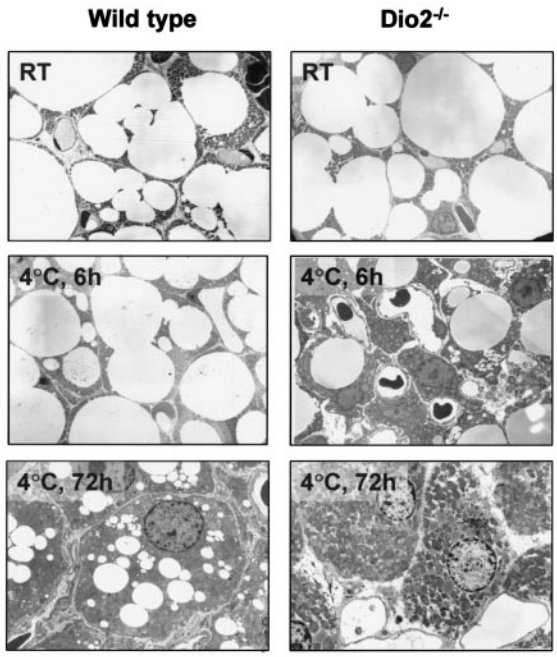

FIG. 2. Cold-induced ultrastructural changes in IBAT of WT and Dio2$^{-/-}$ mice. Electron microscopy of brown adipocytes during 0–72 h of cold exposure. Ambient temperature and times in the cold are indicated in the upper left corner of each slide. Mouse strains are indicated. Pictures are representative of 18–32 images obtained per animal in groups of four animals. This experiment was performed two times. Magnification ×2,000.

the role of thyroid hormone in sustaining BAT adaptive thermogenesis.

To bypass this limitation, we turned to the Dio2$^{-/-}$ mouse model (12,13). Whereas the BAT of WT mice undergoes a similar pattern of ultrastructural changes during cold exposure, the BAT of Dio2$^{-/-}$ mice presents a much more intense lipolytic phase in the first 6–12 h of cold exposure, similar to the hypothyroid BAT (Fig. 2, Table 1). This hyperresponse is characterized by higher HSL activity, lower TG content (Table 1), and marked reduction in the number and size of the lipid inclusions (Fig. 2). Remarkably, even after 72 h of cold exposure, there was no reorganization of the lipid inclusions in the Dio2$^{-/-}$ brown adipocytes (Fig. 2).

Because sympathetic responsiveness in isolated Dio2$^{-/-}$ brown adipocytes is decreased two- to threefold (13), we hypothesized that this super-normal lipolysis might be due to a compensatory increase in the SNS stimulation. In WT animals, basal NE disappearance was ~5%/h (Fig. 3*A*). During cold exposure, NE turnover rate peaked at 12 h (~25%/h) and decreased progressively to ~18% by 48 h. Although the BAT of Dio2$^{-/-}$ mice had a similar basal NE turnover, measurements performed during cold exposure revealed that NE turnover increased to 45–50%/h by 6–8 h and remained at approximately these high levels throughout the time at 4°C (Fig. 3*A*). Hypothyroid mice not only had an increased basal BAT NE turnover but also developed a hyperresponse when exposed to cold (Fig. 3*A*).

Next, we looked for other well-known cAMP-responsive markers in brown adipocytes as additional surrogates of adrenergic signal transduction, namely the gene expression of UCP-1 (35), GMPr (30), and PGC-1α (36). In WT animals, the UCP-1 mRNA levels increased 2- to 2.5-fold by 6 h of cold exposure and remained at these levels until 24 h. Although basal UCP-1 mRNA levels were not different in the Dio2$^{-/-}$ mice (Fig. 3*B*, Table 2), these animals had a super UCP-1 response to cold exposure. UCP-1 mRNA levels were up to ~4-fold higher already at 6 h of cold exposure and kept increasing to ~5.2-fold by 24 h (Fig. 3*B*, Table 2). This translated into significantly more mitochondrial UCP-1 after the 24 h of cold exposure (up 1.4 ± 0.23-fold in WT vs. 2.2 ± 0.37-fold in Dio2$^{-/-}$ mice; $P < 0.05$). Likewise, basal levels of GMPr mRNA were not

TABLE 1
HSL activity and TG content in BAT of cold-exposed mice

Mouse	Hours at 4°C	HSL (nmol FFA · min^{-1} · mg protein^{-1})	TG (mg/IBAT × 10^{-1})
WT			
	0	5.0 ± 0.9	2.5 ± 0.5
	6	9.4 ± 1.2	3.0 ± 0.3
	24	7.8 ± 1.1	1.0 ± 0.2*
Dio2$^{-/-}$			
	0	4.8 ± 0.6	3.5 ± 1.0
	6	13.9 ± 2.0*	1.3 ± 0.4*†
	24	15.2 ± 2.6*	0.5 ± 0.2*†
Hypothyroid			
	0	6.3 ± 1.3	2.0 ± 0.8
	6	12.4 ± 1.9†	0.3 ± 0.3*†

Data are means ± SD of four to five animals. *$P < 0.001$ vs. the same time in WT animals; †$P < 0.01$ vs. the same time in WT animals by ANOVA. Hypothyroid mice are WT. This experiment was performed twice.

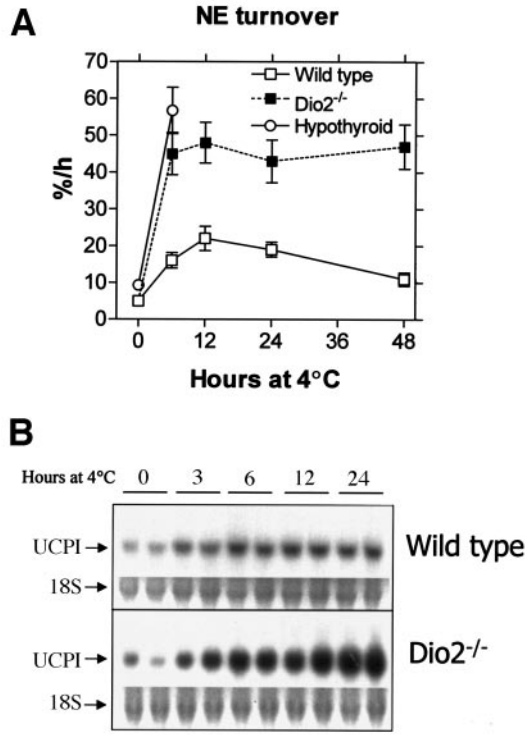

FIG. 3. IBAT NE turnover mRNA levels of cAMP-responsive genes of WT and Dio2$^{-/-}$ mice during cold exposure. *A*: Animals received α-MT and were killed at the indicated times. Values are the mean ± SD of four to five animals. *B*: Northern blot analysis of UCP-1 mRNA levels. The signal was quantified using a phosphoimager (Molecular Dynamics). The average of the relative intensity of each pair is, in the WT animals, 1 (room temperature), 2.2 (3 h at 4°C), 2.5 (6 h at 4°C), 2.6 (12 h at 4°C), and 2.3 (24 h at 4°C), and in the Dio2$^{-/-}$ animals, 1.1 (room temperature), 2.3 (3 h at 4°C), 4.0 (6 h at 4°C), 4.0 (12 h at 4°C), and 5.2 (24 h at 4°C). The ethidium bromide–stained image of the 18S RNA is shown for each gel. Mouse strains and times in the cold are indicated. The experiments were performed two to three times.

different between WT and Dio2$^{-/-}$ mice (Table 2). However, the cold stimulation of GMPr mRNA levels was also significantly higher in Dio2$^{-/-}$ mice (Table 2). When analyzed at 6 and 24 h in the cold, WT animals presented an ~13-fold and ~16-fold increase in GMPr mRNA, respectively, whereas the stimulation in Dio2$^{-/-}$ mice reached ~18-fold by 6 h and remained at this level for up to 24 h of cold exposure. Similar results were obtained in the analysis of the PGC-1α mRNA levels (36) (Table 2), confirming the increased NE stimulation of the BAT of cold-exposed Dio2$^{-/-}$ mice.

BAT lipogenesis during cold exposure. The reorganization of the lipid inclusions in the normal brown adipocytes that follows the initial 12 h of cold exposure indicates a shift from a predominant lipolytic phase to a situation in which lipogenesis and esterification of fatty acids progressively increase and predominate. It is interesting that this takes place despite continued increased β-oxidation and energy expenditure (37). Because cold exposure and T3 stimulate BAT lipogenesis (18,38), we tested whether changes in BAT lipogenesis paralleled the ultrastructural modifications in brown adipocytes. BAT lipogenesis in WT and Dio2$^{-/-}$ mice, measured as the rate of incorporation of ^3H_2O into lipids, decreased during the first 12 h of acute cold exposure, reaching values as low as ~50% of controls after 6–12 h at 4°C (Fig. 4*A*). This was accompanied by a reduction in the activity of the two key NADPH-generating enzymes, malic enzyme (Fig. 4*B*) and glucose 6-P-dehydrogenase (data not shown), and activity and mRNA levels of ACC, the rate-limiting enzyme of the lipogenic pathway (Fig. 4*C* and *D*). It is interesting that the inhibition in lipogenesis was transient. The incorporation of ^3H_2O into lipids and the activity and mRNA of those key enzymes increased from a nadir at 6–12 h to values three- to fourfold higher than in controls after 72 h of continued cold exposure (Fig. 4*A*–*C*).

Remarkably, Dio2$^{-/-}$ BAT lipogenesis, as studied by all four parameters discussed above, was inhibited in the first hours of cold exposure and remained low throughout the 72 h of cold exposure (Fig. 4). A similar decrease in BAT ACC mRNA levels was also observed in the hypothyroid mice after 6 h of cold exposure (Table 2).

To assess the roles that T3 and NE play in regulating ACC and Spot-14 mRNA levels, we treated WT mice with T3 and/or NE as follows: a single T3 injection (15 μg i.p.) at −24 h; five NE injections (3 μg/10 g body wt i.p.) at −7 h, −6 h, −5 h, −3 h, and −1 h; control animals received an injection of saline; all animals were killed at 0 h. T3 treatment doubled ACC and Spot-14 mRNA levels, whereas NE significantly decreased this by ~30% (Table 3). Most important, when both treatments were combined, NE antagonized the effects of T3, blunting the T3-induced increase in ACC and Spot-14 mRNA levels (Table 3). Note that D2 mRNA levels, a gene that is positively regulated by NE, changes in the opposite direction (Table 3). Thus,

TABLE 2
mRNA levels of positively or negatively adrenergic-regulated genes in BAT of cold-exposed mice

Mouse	Hours at 4°C	ACC	UCP-1	GMPr	PGC-1α
WT	0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.1
	6	0.5 ± 0.2	2.4 ± 0.2	13.2 ± 2.2	1.2 ± 0.1
	24	1.1 ± 0.2	2.4 ± 0.1	16.5 ± 1.5	3.9 ± 0.6
Dio2$^{-/-}$	0	1.0 ± 0.3	0.8 ± 0.1	1.2 ± 0.3	0.9 ± 0.2
	6	0.3 ± 0.2	3.8 ± 0.4*	17.9 ± 1.8*	3.4 ± 0.5*
	24	0.5 ± 0.2*	5.2 ± 0.6*	18.2 ± 2.9	2.9 ± 0.3
Hypothyroid	0	1.0 ± 0.3	0.6 ± 0.2*	ND	ND
	6	0.3 ± 0.1*	1.2 ± 0.3*	ND	ND

Data are means ± SD of four animals. ACC mRNA levels were determined by RT-PCR. UCP-1, GMPr, and PGC-1α mRNA levels were determined by Northern blot, and the bands of interest were cut from the RNA-containing filter and counted in a liquid scintillation counter. The signal in each lane was corrected for RNA loading by the intensity of the 28S ribosomal RNA band. *$P < 0.05$ vs. the same time in WT animals by ANOVA. Hypothyroid mice are WT. This experiment was performed twice.

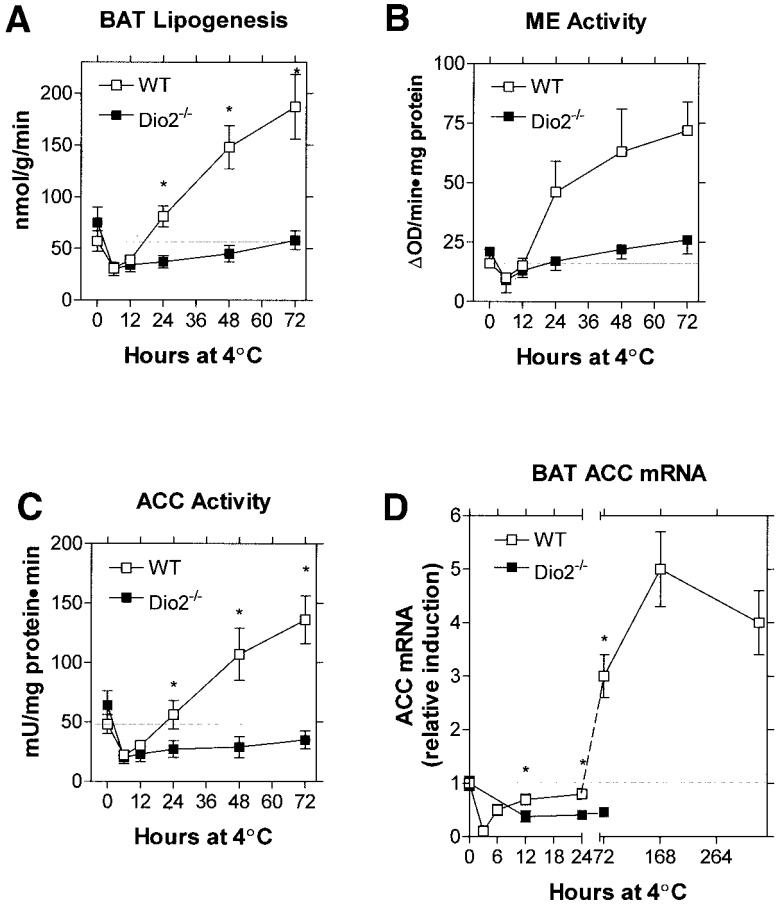

FIG. 4. Lipogenesis and lipogenic enzymes in the BAT of cold-exposed WT and Dio2$^{-/-}$ mice. *A*: Rates of BAT lipogenesis, as measured by incorporation of ^3H_2O into lipids. This experiment was performed twice. *B*: Activity of malic enzyme. *C*: Activity of ACC. *D*: ACC mRNA was measured by real-time PCR and expressed as changes in the ratio ACC/β-actin; values are the mean ± SD of three to four animals assayed twice in triplicate; *$P < 0.05$ vs. WT animals at the same time point by ANOVA. The experiments were performed three to four times.

sustained NE stimulation prevents the otherwise potent T3 induction of ACC and Spot-14 mRNA in BAT.

De novo fatty acid synthesis is critical for thermal homeostasis. To test whether cold-induced lipogenesis is vital for sustained adaptive thermogenesis, WT mice were fed 0.25% (wt/wt) MEDICA-16 (or standard diet) for 5 days and then exposed to 4°C for 48 h. This regimen was previously shown to inhibit lipogenesis in liver and adipose tissue (17) and blocked >90% incorporation of ^3H_2O into IBAT lipids in our animals. Whereas standard diet–fed animals sustained their core temperature well throughout the experimental period in the cold, MEDICA-16–fed mice did so only during the first 24 h (Fig. 5*A*). By 48 h, a time at which lipogenesis is highly stimulated in cold-exposed animals (Fig. 4), MEDICA-16–fed mice presented hypothermia, with a core temperature ~1°C lower than WT animals (Fig. 5*A*). The perirenal white adipose tissue depot was dissected and weighed, and no differences were found between standard diet– and MEDICA-16–fed groups (data not shown), indicating that the length of the treatment was not sufficient to significantly decrease adiposity. Also, calorie intake was not affected by MEDICA-16 treatment (data not shown).

For testing the role of lipogenesis in BAT thermogenesis, this was assessed directly in MEDICA-16–fed normal mice by measuring changes in the IBAT temperature during infusion of NE in anesthetized mice, as described (19). Because the unstimulated BAT contains a substantial TG depot, all animals were first exposed to cold during 12 h to deplete the brown adipocytes of multilocular fat depots (Fig. 1). They were then moved to room temperature for 24 h to allow restoration of intracellular fat by de novo synthesis of fatty acids, a mechanism that is >90% suppressed in MEDICA-16–fed mice. The IBAT temperature of mice that were fed standard diet increased ~5°C, indicating a normal BAT thermal response (Fig. 5*B*). However, despite normal mitochondrial UCP-1 levels, the IBAT thermal response of mice that were fed MEDICA-16 was lower at every time point, remaining ~50% below the response observed in the control animals (Fig. 5*B*).

TABLE 3
ACC, Spot-14, and D2 mRNA levels in BAT of WT mice treated with T3 and/or NE

Enzyme mRNA	Saline	T3	NE	T3+NE
ACC	1.0 ± 0.3	1.8 ± 0.3*	0.7 ± 0.1*	1.2 ± 0.3
Spot-14	1.0 ± 0.4	2.3 ± 0.3*	0.7 ± 0.1*	1.3 ± 0.1
D2	1.0 ± 0.3	0.1 ± 0.1*	3.3 ± 0.4*	1.0 ± 0.4

Data are means ± SD of four animals. mRNA levels were determined by RT-PCR and corrected by β-actin mRNA. *$P < 0.05$ vs. saline-treated animals by ANOVA. This experiment was performed twice.

DISCUSSION

The present investigation provides three new findings that shift the current paradigm about thyroid-adrenergic synergism and adaptive thermogenesis. First, brown adipocytes of Dio2$^{-/-}$ mice respond to sympathetic stimulation with respect to lipolysis and the activation of three cAMP-responsive genes (Figs. 1–3, Tables 1 and 2). Second, the D2-mediated adaptive increase in T3 production is not

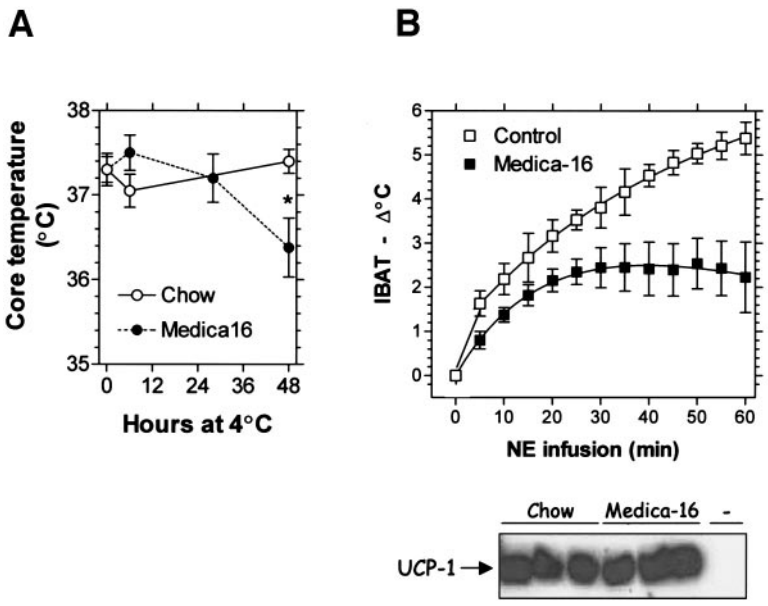

FIG. 5. Core temperature and IBAT thermal response to NE in MEDICA-16–fed WT mice. MEDICA-16 was added to the diet (0.25%), and after 5 days, animals were moved to 4°C; control animals were fed regular diet. *A*: Colonic temperatures were measured at the indicated times. *B*: IBAT temperatures were measured in anesthetized animals during infusion with NE; before NE infusion, all animals were cold-exposed for 12 h and moved to room temperature for the next 24 h. Values are the mean ± SD of four to five animals; *$P < 0.01$. Below is the Western analysis (performed as in ref. 13) of mitochondrial UCP-1 in the BAT of representative animals studied in *B*. −, a negative control. The experiments were performed twice.

critical for NE-induced increase UCP-1 gene expression (Fig. 3, Table 2). Third, the compensatory increase in adrenergic stimulation of Dio2$^{-/-}$ brown adipocytes causes intense lipolysis and prevents the normal increase in BAT lipogenesis observed during cold exposure, rapidly exhausting the availability of fatty acids (Fig. 4). Data obtained in animals that were fed an inhibitor of two key lipogenic enzymes (MEDICA-16) indicate that the de novo synthesis of fatty acids is a critical determinant of BAT thermogenesis (Fig. 5). Its suppression by increased NE stimulation (Fig. 3) explains the impaired adaptive thermogenesis and hypothermia in cold-exposed hypothyroid or Dio2$^{-/-}$ mice (13).

There is no question that hypothyroidism decreases sympathetic responsiveness in a number of tissues and cells, including heart and white and brown adipocytes, all of which produce less cAMP when incubated with a variety of adrenergic stimulators (7,8). Hypothyroid brown adipocytes, for example, produce ~10-fold less cAMP in response to NE or forskolin, supporting the connection between adrenergic insensitivity and cold-induced hypothermia (39). However, using electron microscopy and measuring the TG content and the activity of HSL, UCP-1, and other cAMP-dependent genes, we found that the poor adrenergic responsiveness is easily bypassed and even overcompensated by an increase in sympathetic activity in hypothyroid or Dio2$^{-/-}$ brown adipocytes (Figs. 1–3, Tables 1 and 2). Remarkably, despite reduced in vitro responsiveness to adrenergic stimulators (13), UCP-1 mRNA levels are three- to fourfold higher in the BAT of cold-exposed Dio2$^{-/-}$ animals than in the BAT of cold-exposed WT animals (Fig. 3). This is unexpected, particularly in light of previous observations that the normal increase in UCP-1 gene transcription and mRNA levels observed during acute cold exposure is blunted in hypothyroid rats (14,15) and mice (16) or by treatment with the D2 inhibitor iopanoic acid (40), and is restored by T3 in a dose-dependent manner until full occupation of TR is attained (14,41). These data indicate that the basal TR saturation of ~50% provided by serum T3 is sufficient to confer even super-normal adrenergic responsiveness to the UCP-1 gene. This minimizes the role of adaptive D2 activation in the control of UCP-1 activation in euthyroid mice. In addition, these data constitute the first evidence that decreased sympathetic responsiveness does not cause impaired adaptive thermogenesis in hypothyroid or Dio2$^{-/-}$ animals, indicating that other, as-yet-unrecognized T3-dependent mechanisms must play a pivotal role. This may also apply to other systems, e.g., heart and white adipose tissue, where there is thyroid-adrenergic synergism.

BAT lipogenesis is upregulated during cold acclimatization (38). The present studies show that this is preceded by an acute lipolytic phase during which lipogenesis is markedly inhibited (Fig. 4). The electron microscopy studies as well as the activity of key rate-limiting enzymes indicate that, in normal animals, lipolysis peaks at 12 h of cold exposure, a time at which lipogenesis is minimal and intracellular lipid droplets are not detectable. These events are rapidly followed by the surge in the activity of key lipogenic enzymes and the overall rate of lipogenesis, reaching levels three- to fourfold above normal between 72 and 96 h of continued cold exposure (Fig. 4). This is also illustrated by the reorganization of the lipid inclusions in the cytoplasm of the brown adipocytes (Fig. 1).

Two important physiological events explain the shift in predominance from lipolysis to lipogenesis, namely *1*) a reduction (although not to normal) in the BAT sympathetic stimulation (Fig. 3) and *2*) an increase in the BAT T3 as a result of D2-mediated local conversion of T4 to T3 (5).

That Dio2$^{-/-}$ brown adipocytes of cold-exposed animals enter and remain in the acute lipolytic phase without ever presenting the lipogenic surge (Figs. 2 and 4) indicates that an optimal balance between lipolysis and lipogenesis can be achieved successfully only as a result of the adaptive D2-catalyzed T3 production in these cells. Both the fall in sympathetic activity (Fig. 3) and the stimulation of lipogenesis (Fig. 4) are absent in the Dio2$^{-/-}$ BAT and therefore depend on the D2-mediated development of tissue-specific thyrotoxicosis during cold exposure. As a result, the brown adipocytes of these animals remain locked in the initial high NE-turnover phase in which lipolysis predominates and lipogenesis is suppressed, exhausting the

supply of fatty acids and limiting both the usefulness of β-oxidation as an energy source and the uncoupling process. In fact, the data indicate that sustained NE stimulation suppresses ACC and Spot-14 expression even in animals that had received a bolus injection of T3 to saturate TR.

Support for this hypothesis was obtained in experiments in which MEDICA-16 was used to block lipogenesis. On the basis of the profile of lipogenesis stimulation during cold exposure in normal animals, we anticipated that a blockade in this pathway would impair adaptive thermogenesis between 24 and 48 h. In fact, MEDICA-16–fed mice developed hypothermia after being in the cold for 48 h (Fig. 5*A*). Although MEDICA-16 blocks lipogenesis systemically, our direct measurements of IBAT temperature during NE infusion indicate that the blockade of fatty acid synthesis impairs BAT thermogenesis (Fig. 5*B*).

In conclusion, our data indicate that the decreased adrenergic responsiveness of Dio2$^{-/-}$ brown adipocytes (13) is successfully bypassed and even overcompensated by an increase in the SNS tonus, leading to an exaggerated and sustained lipolytic response, overexpression of cAMP-dependent genes, and suppression of genes encoding key lipogenic enzymes. Exhaustion of fatty acids in brown adipocytes rather than decreased sympathetic responsiveness is the cause of impaired adaptive thermogenesis and hypothermia in cold-exposed hypothyroid and Dio2$^{-/-}$ animals.

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