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3,5-Diiodo-L-thyronine powerfully reduces adiposity in rats by increasing the burning of fats

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ABSTRACT

The effect of thyroid hormones on metabolism has long supported their potential as drugs to stimulate fat reduction, but the concomitant induction of a thyrotoxic state has greatly limited their use. Recent evidence suggests that 3,5-diiodo-L-thyronine (T_2), a naturally occurring iodothyronine, stimulates metabolic rate via mechanisms involving the mitochondrial apparatus. We examined whether this effect would result in reduced energy storage. Here, we show that T_2 administration to rats receiving a high-fat diet (HFD) reduces both adiposity and body weight gain without inducing thyrotoxicity. Rats receiving HFD + T_2 showed (when compared with rats receiving HFD alone) a 13% lower body weight, a 42% higher liver fatty acid oxidation rate, ~50% less fat mass, a complete disappearance of fat from the liver, and significant reductions in the serum triglyceride and cholesterol levels (–52% and –18%, respectively). Thyroid hormones and thyroid-stimulating hormone (TSH) serum levels were not influenced by T_2 administration. The biochemical mechanism underlying the effects of T_2 on liver metabolism involves the carnitine palmitoyl-transferase system and mitochondrial uncoupling. If the results hold true for humans, pharmacological administration of T_2 might serve to counteract the problems associated with overweight, such as accumulation of lipids in liver and serum, without inducing thyrotoxicity. However, the results reported here do not exclude deleterious effects of T_2 on a longer time scale as well as do not show that T_2 acts in the same way in humans.

Key words: thyroid hormone • mitochondria • liver • energy metabolism

Thyroid hormones [THs; thyroxine (T_4) and 3,3′,5-triiodo-L-thyronine (T_3)] exert a multiplicity of effects and are necessary for growth, differentiation, and metabolism. The first two actions predominate in the early stages of life, whereas the "metabolic effect"

predominates in the adult. Many of the effects are exerted through the positive or negative regulation of thyroid-responsive genes via thyroid hormone receptors (TRs). The TRs are members of a larger family of nuclear receptors that are generally agreed to act by binding to specific DNA elements (for reviews, see refs 1–3), although a mechanism independent of DNA binding has been suggested (4). Many nongenomic effects of THs have also been reported, and some of these are attributable to iodothyronines other than T_4 and T_3 (for reviews, see refs 5–7). Thyroid hormones are well known both to stimulate metabolism and (at the same time) to lower metabolic efficiency. This effect has long been the focus of research into the potential use of THs as drugs to stimulate weight-loss. However, the concomitant induction of a thyrotoxic state has greatly limited the use of T_3 and T_4 as weight-lowering agents. The term "thyrotoxicosis" is used here, following Braverman and Utiger (8), to mean the clinical syndrome of hypermetabolism that results when the serum concentration of free T_3 (FT_3), free T_4 (FT_4), or both is increased as a consequence of the exogenous administration of these hormones. Some signs of the thyrotoxic state are increase in heart rate, increases in thyroid and heart mass, decrease in skeletal muscle mass, and serum thyroid-stimulating hormone (TSH) levels reaching minimal values (8). In patients receiving THs, variations in the above-mentioned serum parameters (TSH, FT_3, and FT_4 serum levels) constitute the biochemical confirmation of a thyrotoxic state. Until recently, T_3 was commonly assumed to be the only active iodothyronine, with T_4 being its precursor. A growing body of evidence, however, seems to require a revision of that opinion, and it is now evident that other iodothyronines, in particular T_2, have biological effects (9–14). Indeed, a single dose of T_2 (25 μg/100 g body wt.) stimulates the resting metabolic rate (RMR) of hypothyroid rats to the same extent (~40%) as the same dose of T_3; however, the former acts via a different mechanism, one not involving the nuclear pathway (13). In addition, T_2 can induce metabolic inefficiency, possibly by stimulating energy loss via mechanisms involving mitochondrial proton leakage/redox slippage (15, 16). Such inefficiency in energy transduction should result in reduced energy storage. In view of these metabolic effects of T_2 and the very low affinity of T_2 for nuclear T_3 receptors (17), we thought it conceivable that in rats fed a high-fat diet (HFD), long-term treatment with T_2 might result in a reduced adiposity and less body weight gain without inducing a clinical syndrome related to the thyrotoxic state. To test this idea, we administered T_2 for 30 days to rats on an HFD and then assessed the thyroid state by measuring the serum levels of FT_3 and FT_4. To see if, under our conditions, T_2 exerts an effect on the mechanism underlying TSH secretion by the pituitary, we also measured TSH serum levels. In addition, we measured the adipose tissue mass, body weight gain, liver adiposity, liver fatty acid oxidation rate, and the serum levels of triglyceride, fatty acids, and cholesterol.

We also searched for the cellular biochemical pathways involved in the effects of T_2 on fatty acid oxidation. One of the major pathways leading to increased fatty acid oxidation involves activation of the carnitine palmitoyl-transferase (CPT) system by the AMP-activated protein kinase (AMPK)-acetyl-coenzymeA-carboxylase (ACC)-malonyl CoA signaling pathway (18, 19). The phosphorylated form of AMPK (P-AMPK) phosphorylates and inactivates ACC (18, 19), thus decreasing the cellular concentration of malonyl-CoA, an allosteric inhibitor of CPT1 (the outer enzyme of the CPT system), which is rate limiting for the transport of fatty acids into the mitochondrial matrix and thus for their oxidation. Decreased levels of malonyl-CoA thereby lead to an activation of CPT1 and hence to an increased transport of fatty acids into the mitochondrial matrix (20, 21). Under several physiological conditions, ACC has been shown to be subject to a regulation that is both transcriptional (22) and posttranslational via AMPK (23). Because of this, we investigated the possible involvement of such a pathway in the effects of T_2

on liver fatty acid oxidation (by measuring both CPT and ACC activity, and AMPK phosphorylation in the liver). Finally, to ascertain whether T_2 might induce a less efficient utilization of lipid substrates through a stimulation of mitochondrial uncoupling, the liver mitochondrial proton leak was measured. Indeed, during mitochondrial respiration, the transfer of protons along the respiratory chain in the inner mitochondrial membrane is coupled to the translocation (pumping) of protons from the mitochondrial matrix into the intermembrane space, thus generating an electrochemical proton gradient across the inner membrane. This gradient is used to drive the protons back into the matrix through the ATP synthase complex, thus resulting in ATP synthesis. Alternatively, the gradient can be dissipated by proton leak, which consists in a reentry of protons within the matrix that bypasses the ATP-synthase complex. The data from rats receiving HFD + T_2 were compared both with those from normal euthyroid rats receiving a standard diet and with those from rats receiving HFD alone.

MATERIALS AND METHODS

Animals and animal care

Male Wistar rats (aged 8 wk) were purchased from Charles Rivers (Lecco, Italy). They were housed in individual cages in a temperature-controlled room at 28°C (thermoneutrality temperature for rats) with a 12 h light-dark cycle. A commercial mash (Charles Rivers) was available ad libitum, and the animals also had free access to water.

At the start of the study, after 7–10 days of acclimatization (day 0), the rats were divided into three groups (each group, 12 animals). In each group, body weight was normally distributed, and group means were similar (299±5 g). The first group (referred to as N) was fed a standard diet (total metabolizable percentage of energy: 60.4 carbohydrates, 29 proteins, 10.6 fat J/J; 15.88 KJ gross energy/g); the second group (referred to as D) with an HFD (total metabolizable percentage of energy: 21 carbohydrates, 29 proteins, 50 fat J/J; 19.85 KJ gross energy/g); and the third group (referred to as DT_2) with the same HFD supplemented with a daily intraperitoneal injection of T_2 (25 μg/100 g body wt.). N and D rats were daily intraperitoneally injected with the same volume of saline. Body weight and food intake were monitored daily throughout the entire experimental period lasting 1 month. The pharmacological dose of 25 μg T_2/100 g body wt. was chosen after consideration of some data from studies in which T_3 and T_2 were used. In fact, *1*) in previous studies in which an effort was made to decrease the body fat content of ob/ob mice by administering T_3, only at 25 μg/100 g body wt. did T_3 treatment sufficiently decrease the percentage of lipid in the body (24); and *2*) chronic treatment (over 2 wk and over 3 months) of rats with doses of 25 μg T_2 /100 g body wt. did not result in any thyrotoxic effect that might be of clinical relevance (25). These considerations, together with the data present in literature so far, showing that T_2 serum levels are ~40–100 times lower than those of T_3 (26), allow us to be confident that the dose of 25 μg/100 g bdoy wt. is a pharmacological one putatively with no thyrotoxic effect. Heart rate was monitored at the beginning, in the middle, and at the end of the treatment period from the electrocardiogram obtained by means of a paper ECG recorder (Cardiotest EK 41; Hellige, Freiburg, Germany). Energy expenditure (VO_2) and respiratory quotient (RQ) were measured using an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) with an air flow of 4 Lt/min at 28°C. This was done both at the beginning and at the end of the treatment period. After 1 h, to allow for adaptation to the metabolic chamber, VO_2 and VCO_2 were measured in individual rats at 15 min intervals over a 24 h period. RQ is the

ratio of the volume of CO_2 produced to the volume of O_2 consumed (VCO_2/VO_2). The rats had free access to food (normal pellets or the HFD, as appropriate) and water during the measurements (for more details, see ref 27). Body temperature was measured using a rectal probe (307-Digital Thermometer).

At the end of the treatment, the rats were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body wt.) and then killed by decapitation. Liver, hindlimb skeletal muscle (gastrocnemius and tibialis anterior), heart, thyroid glands, and visceral white adipose tissue (epididymal or parametrial, perirenal, mesenteric, and inguinal depots) were removed. Tissues to be used for preparation of mitochondria were weighed and processed immediately; other samples were immediately frozen in liquid nitrogen and stored at –80°C before RNA extraction and histological analysis. All procedures were approved by the local and national institutional animal care and use committees.

Isolation of mitochondria

Fresh livers and hindlimb skeletal muscles were minced in ice-cold buffer consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4 and then homogenized in a Potter-Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 500 *g* for 10 min, with the resulting supernatant being centrifuged at 3000 *g* and 8000 *g* for liver and skeletal muscle, respectively, as described by Lanni et al. (28, 29). The mitochondrial pellet was washed twice and resuspended in a minimal volume of isolation medium, and kept on ice. The protein concentration was determined by the method of Hartree (30), using bovine serum albumin (BSA) as standard.

Measurement of fatty acid oxidation rate

The rate of mitochondrial fatty acid oxidation was assessed polarographically using a Clark-type electrode (31) at 30°C in a final volume of 0.5 ml of 80 mM KCl, 50 mM HEPES (pH 7.0), 1 mM EGTA, 5 mM K_2HPO_4, 1% BSA (w/v), and 2.5 mM malate in the presence of ADP (120 μg/ml). The reaction was started by the addition of palmitoyl-L-carnitine (40 μM). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Measurement of enzyme activities

To measure ACC activity, powdered frozen liver (0.1 g) was homogenized in 10 vol of 0.3 M mannitol, 100 mM Tris-HCl buffer (pH 7.4 at 4°C), 2 mM EDTA, 50 mM NaF, and 2 mM $Na_4P_2O_7$ containing 1 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 12,000 *g* for 3 min, and poly(ethylene-glycol) was added to the supernatant to a final concentration of 6% (w/v) according to Munday et al. (32). The mixture was vortex mixed, left on ice for 5 min, and then centrifuged at 12,000 *g* for 3 min. The poly(ethylene-glycol) pellet was assayed spectrophotometrically for ACC activity, as reported by Tanabe et al. (33), in the presence of 20 mM citrate.

Total CPT (CPT1 plus CPT2) activity was measured spectrophotometrically by following (at 412 nm) the kinetics of carnitine-dependent CoASH production in the presence of 5,5′-dithio-bis(2-nitrobenzoic acid) using palmitoyl-CoA as substrate, as reported by Alexson and Nedergaard (34). An Σ_{412} value of 13.6 $mM^{-1} \cdot cm^{-1}$ was applied for the calculation of total CPT activity.

Western blot analysis

Analyses were performed on liver crude lisates in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 mg/ml leupeptin, 1% Triton X-100 (all from Sigma) obtained by ultraturrax homogenizer, and centrifuged at 12,000 rpm for 10 min at 4°C. Protein levels (total AMPK and ACC) in liver lysates (30 μg of protein) were determined using 13% and 5% SDS polyacrylamide gels, respectively, and specific antibodies against AMPK (Cell Signaling Technology, Beverly, MA) and ACC (Upstate Biotech., Lake Placid, NY). Phosphorylation of the α-subunit of AMPK was determined using an antibody against phosphopeptides based on the amino acid sequence surrounding Thr 172 of the α-subunit of human AMPK (Cell Signaling Technology). UCP_3 protein levels in mitochondrial lysates (30 μg of protein) were determined using 13% SDS polyacrylamide gel and a polyclonal antibody raised against the C-terminal region of the human UCP3 protein (AB3046; Chemicon International, Temecula, CA) was used as primary antibody as described by de Lange et al. (35). Protein levels were determined, using peroxidase-linked secondary antibodies, by a chemoluminescence protein-detection method, using a commercially available kit (NEN; Life Science Products, Boston, MA).

Mitochondrial proton leak

Mitochondria (1 mg/ml) were incubated at 37°C in a medium consisting of 80 mM KCl, 50 mM HEPES pH 7.0, 1 mM EGTA, and 5 mM K_2HPO_4, with 1 μg/ml oligomycin added to block protons from reentering the mitochondrial matrix via ATP synthase. Rotenone was added (4 μM) to prevent oxidation of any endogenous NAD-linked substrates. Nigericin (80 ng/ml) was added to abolish the pH gradient. With the use of succinate as a substrate (5 mM), respiration rate and membrane potential were measured simultaneously using a Clark-type oxygen electrode and a triphenylmethylphosphonium (TPMP+)-sensitive electrode, respectively, as described by Brown and Brand (36). Potential was varied by titration with submaximal doses of malonate within the range of 0-5 mM. A TPMP binding correction factor of 0.4 was used.

RNA isolation

Total RNA was isolated from individual livers using TRIzol Reagent (Life Technologies, Gaithersburg, MD), according to the protocol supplied by the manufacturer. The quality of the RNA samples was checked on a denaturing agarose gel.

RT-PCR assays

RT-PCR assays were performed as described previously (37) with slight adaptations. One microgram of total RNA was reverse-transcribed using 100 pmol random hexamers (Invitrogen Life Technologies, Groningen, The Netherlands), 2.0 units Superscript reverse-transcriptase, 10 units RNase inhibitor, and 1 mM deoxynucleotide triphosphates (dNTPs) in reverse-transcriptase buffer (all from HT Biotechnology, Cambridge, UK). The total volume was adjusted to 20 μl with distilled H_2O, and the reaction was carried out for 1 h at 40°C. One quarter of the RT-reaction mixture was used directly for the PCR reaction in a total volume of 25 μl, containing 0.25 units of SuperTaq polymerase, 0.25 mM dNTPs, SuperTaq PCR buffer (all from HT Biotechnology), 5% (v/v) DMSO (DMSO, Sigma-Aldrich), and 0.38 pmol of the relevant

oligonucleotide primers (Sigma Genosys, Cambridge, UK). As an internal control, the same cDNAs were amplified using 40S ribosomal protein S12 (RPS12) oligonucleotide primers. The primers used had the following sequences: RPS12 sense, 5′-GCTGCTGGAGGTGTAATGGA-3′; RPS12 antisense, 5′-CTACAACGCAACTGCAACCA-3′; UCP3 sense, 5′-ATGGATGCCTACAGAACCAT-3′; UCP3 antisense, 5′-CTGGGCCACCATCCTCAGCA-3′; UCP2 sense, 5′-CCTTCTGCACTCCTGTGTTC-3′; UCP2 antisense, 5′-GTGGCCTTGAAACCAACCAT-3′; CPT1α sense, 5′-ATGTGGACCTGCATTCCTTC-3′; CPT1α antisense, 5′-CCTTCAGGAAAGGTGAGTCG-3′. Parallel amplifications (20, 25, and 30 cycles) of a given cDNA were used to determine the optimum number of cycles. For each gene under study, a readily detectable signal within the linear range was observed after 30 cycles. For the actual analysis, samples were heated for 5 min at 94°C, and then 30 cycles were carried out, each consisting of 1 min at 94°C, 1.5 min at 61°C, and 1.5 min at 72°C. This was followed by a final 10 min extension at 72°C. The quantities of the PCR products were determined in separate preparations from three rats. Separation of the PCR reaction products was performed on a 2% agarose gel containing EtBr, and the products were readily visualized. Reverse-image signals of the RT-PCR bands were quantified by means of a Bio-Rad Molecular Imager FX using the supplied software (Bio-Rad Laboratories, Hercules, CA). Expression signals of the genes were normalized to the nonregulated RPS12 signal.

Histological analyses

Sections of livers from three animals in each experimental group were fixed in formol-calcium, and 10 μm frozen sections were subsequently stained with Sudan black B for the detection of fat (38).

Serum levels of cholesterol, triglycerides, fatty acids, FT_3, FT_4, and TSH

The serum levels of cholesterol, triglycerides, and fatty acids were determined by following standard procedures. Those of FT_3, FT_4, and TSH were determined by means of radioimmunoassays. TSH was measured using materials and protocols supplied by Amersham Bioscience, with rat TSH as standard [Biotrak ^{TM} rat thyroid stimulating hormone (rTSH) [^{125}I] assay system]. FT_3 and FT_4 levels were determined using materials and protocols supplied by Byk-Sangtec Diagnostica (Dietzenbach, Germany).

TRH stimulation test

To assess the integrity of the hypothalamo-pituitary-thyroid (HPT) axis in three additional groups of animals (N, D, and DT_2), we performed a TRH stimulation test. TRH has been administered intraperitoneally at 100 ng/100 g body wt., a dose that has been reported to give a more than twofold increase in TSH at 2 min (39). Five rats from each treatment were killed at 0, 0.5, 1, and 2 h after administration of TRH, and blood was collected to perform serum TSH analysis.

Statistical analysis

Values are means ± SE. Data were evaluated by ANOVA followed by the Newman-Keuls test, with differences being considered significant when at least $P < 0.05$.

RESULTS

T_2 decreases body weight gain and adiposity without inducing a thyrotoxic state

To determine if in rats fed an HFD, long-term treatment with T_2 might result in reduced adiposity and thus less body weight gain, without inducing a clinical syndrome related to the thyrotoxic state, we administered T_2 for 30 days to rats on an HFD. In such DT_2 rats, serum levels of TSH, FT_3, and FT_4 showed no substantial differences from the normal euthyroid values (see Table 1). Although the slight decrease in FT_4 values in DT_2 rats was not significant, it might be thought to be in some way indicative of a suppression of the HPT axis. To examine this important issue, three additional groups of animals (N, D, and DT_2, n=4 for each group) were subjected to a "TRH-test." The basal TSH values were 0.58 ± 0.07, 0.53 ± 0.05, and 0.56 ± 0.06 ng/ml in N, D, and DT_2 groups, respectively. No significant difference in the response of serum TSH to TRH injection was observed among the samples from the three groups. The TSH level peaked (roughly 5-fold increases) at 30 min, reaching values of 2.81 ± 0.14, 2.64 ± 0.23, and 2.89 ± 0.18 ng/ml, respectively. It then showed a marked decline to levels ~2× basal at 60 min after the TRH injection (1.14±0.11, 1.03±0.09, and 1.16±0.09 ng/ml, respectively) and had returned to values not significantly different from basal at 2 h. These results, showing no differences between N, D, and DT_2 groups, demonstrate that the HPT axis is not disturbed in these animals. The weights of thyroid glands and heart did not change significantly from the normal euthyroid values, whereas visceral white adipose tissue and liver weights were significantly decreased (see Table 1). Moreover, no change in heart rate was observed (either in the middle or at the end of the treatment) after T_2 administration. The data reported in Table 1 are the values recorded at the end of the treatment. Over the entire period of treatment, D rats consumed ~5% more food than N rats, while DT_2 animals consumed ~6% more than the D ones (see Table 2). At the end of the treatment period, the D rats were overweight, weighing ~13% more than N rats (Fig. 1*A*). The DT_2 rats, on the other hand, were 13% lighter than the D animals (Fig. 1*B*), with the result that their body weight was not different from that of normal animals fed a standard diet (Fig. 1*A*). The DT_2 rats accumulated much less fat in their adipose tissue than the D animals (Fig. 1*C*). The visceral fat pad tissue weighed 19.3 ± 2.97 g per rat in D rats against 10.2 ± 2.4 g in DT_2 ones (P<0.05; Table 1 and Fig. 1*D*). In N rats, the corresponding weight was 9.4 ± 1.5 g (not significantly different from the DT_2 value). The lean mass was not affected by T_2 treatment. The muscle mass, in fact, did not vary after T_2 administration to both N and D rats (data not shown). Determinations made at the end of the treatment period showed that energy expenditure over a 24 h period was markedly greater in DT_2 rats (+29%) than in D ones (see Table 2). These changes resulted in an increased heat production and an increased body temperature. Indeed, the colonic temperature of DT_2 rats was slightly but significantly higher in DT_2 rats compared with both D and N (Table 2). The livers of D rats were lighter in color than the DT_2 livers, suggesting that they contained more fat (Fig. 2*A*). Stained sections showed that D livers contained abundant fat droplets, whereas these were not apparent at all in DT_2 livers (Fig. 2*B* and *C*).

T_2 increases hepatic fatty acid oxidation

We thought that an increased oxidation of fatty acids might underlie the reduced fat storage in adipose tissue in DT_2 rats. We therefore measured the fatty acid oxidation rate in the liver and in hindlimb skeletal muscle.

The fatty acid oxidation rate (reported as nmol O/min mg protein) in the liver of N rats was 61 ± 4. The rate was 30% higher in D rats, and it was further increased (+42% vs. D rats) in DT_2 rats (79±5 and 112±7, respectively; $P<0.05$ in each case). In the muscle, the fatty acid oxidation rate, which was 139 ± 13 in N rats, was almost unchanged in both D and DT_2 rats (116±15 and 128±20, respectively). To extend our insight into the lack of stimulation of fatty acid oxidation in muscle mitochondria in D and DT_2 rats, we measured CPT activity. This activity was not significantly different among the groups, although it tended to be slightly increased after T_2 treatment (22.2±2.7, 18.7±2.1, and 23.4±2.5 nmol/min mg protein for N, D, and DT_2 groups, respectively)

These results implicate the liver as a major player in the effects seen here with T_2. Such results, showing an effect of T_2 on fatty acid oxidation, are not surprising since a previous study (40) demonstrated stimulation of β-oxidation by T_2 in the whole animal by a noninvasive method: namely, $^{13}CO_2$ recovery in the breath after injection of octanoic acid-[1-^{13}C]. The decrease in RQ shown in Table 2 for D rats (vs. N rats) and the further decrease in DT_2 animals (vs. D) indicate a shift in metabolism toward an increase in the utilization of lipids as substrate. This is in line with the observed increase in the fatty acid oxidation rate.

T_2 decreases serum levels of cholesterol and triglycerides

Increased fat oxidation might result in alterations in certain serum parameters related to fat metabolism, such as the levels of cholesterol, triglycerides, and fatty acids. In fact, when compared with those in the D group, all the above-mentioned parameters were decreased in DT_2 rats (i.e., after T_2 administration; Fig. 3). The serum cholesterol level was 30% higher in D rats than in N ones, whereas in DT_2 animals it was significantly reduced (vs. the D value) to a level not significantly different from that seen in N rats. The serum triglycerides level was the most affected by T_2 administration: the level was 107% higher in D than in N rats, and T_2 administration significantly lowered this to a level similar to the N value. The serum fatty acid level was significantly higher in D rats, and administration of T_2 reduced it to a value not significantly different from that of N animals (Fig. 3).

T_2 activates CPT system and AMPK phosphorylation in liver

We next examined the biochemical mechanisms that might be involved in both the stimulation of liver fatty acid oxidation and the powerful reduction of adiposity that are induced by administration of T_2.

To ascertain whether the stimulation of fatty acid oxidation in mitochondria from both D and DT_2 rats might be dependent on the CPT system, we measured CPT activity. Although the CPT1 mRNA expression levels did not vary among the groups (data not shown), the CPT system activity was significantly greater (by 38%) in D rats than in N ones and significantly greater (by 52%) in DT_2 rats than in D rats (Fig. 4*A*).

In D rats, the total ACC activity was ~65% lower than in N rats (Fig. 4*B*) despite the phosphorylated form of AMPK being decreased (vs. N; Fig. 4*C*). In DT_2 animals, the ACC activity was not decreased further (Fig. 4*B*) despite the phosphorylated form of AMPK being increased in comparison with that in D rats (Fig. 4*C*). The decrease in ACC activity seen in D

animals (vs. N ones) was due to a reduced ACC protein content, as shown by the Western blot analysis (Fig. 4*C*). In DT_2 rats, ACC protein levels and activity were not decreased below the levels seen in D rats despite the DT_2 group exhibiting both the highest CPT activity (+52% vs. D rats) and the highest fatty acid oxidation rate (+42% vs. D rats) as well as a high level of phosphorylated AMPK (Fig. 4*B* and *C*).

T_2 activates mitochondrial proton leak in liver

We next examined whether T_2 might not only stimulate fatty acid oxidation but also induce a less efficient utilization of lipid substrates through a stimulation of mitochondrial uncoupling. We did this by evaluating the kinetic response of the proton leak to a change in membrane potential both in liver and skeletal muscle mitochondria from N, D, and DT_2 rats. To judge from our data, liver mitochondria from DT_2 rats would need to respire more than D and N rats to maintain the same membrane potential, indicating that DT_2 liver mitochondria have a higher proton conductance, although no differences in proton leak kinetics between D and N rats were observed (Fig. 4*D*). The same did not occur in skeletal muscle mitochondria as D and DT_2 skeletal muscle mitochondria have the same proton conductance (data not shown). As mitochondrial uncoupling increases the burning of fat, these results are in line with the above-mentioned effects of T_2 on adiposity and further support the liver as a principal target of T_2. The effect of T_2 on liver mitochondrial efficiency could be ascribed to an induction of the expressions of uncoupling proteins (UCP_2 and UCP_3), which have been proposed by some authors as mediators of an uncoupling effect (for reviews see refs 41 and 42). However, this appears not to be true in the case of the effect of T_2 on mitochondrial proton-leak since *1*) UCP_2 protein was found not to be expressed, even though its mRNA was present (data not shown); and *2*) UCP_3 was absent at both mRNA and protein levels (data not shown). Thus, the mechanism underlying the uncoupling effect of T_2 remains to be elucidated even though previous studies (14, 16, 43) had already shown that the addition of T_2 to a reconstituted cytochrome-*c* oxidase (COX) complex led to a decrease in the respiratory control ratio of the complex. The authors concluded that the effect of T_2 on COX results in partial uncoupling of oxidative phosphorylation and decrease H^+/e-stoichiometry, which may, at least in part, support our data.

DISCUSSION

Although it is well known that T_3 stimulates lipogenesis as well as lipolysis, the precise biological relevance of this bidirectional effect is not clearly understood. It is possible that the primary function of enhanced lipogenesis is to provide fatty acids for membrane synthesis, while the increased fat oxidation may be useful to sustain the energy cost of the various activities that are stimulated by THs (5, 6, 44, 45). The effect of T_3 on lipogenesis is due to an increased expression of genes encoding for lipogenic enzymes (5, 46, 47). Its effect on the oxidation of fats, on the other hand, seems to be secondary to a stimulation of many metabolic processes linked to mitochondrial activities (6, 45, 48, 49).

In the past, and to a large extent now, iodothyronines other than T_3 have been regarded as inactive. However, several reports indicate that there are four iodothyronines with significant, but not identical, biological activities; namely, T_4, T_3, rT_3, and T_2 (for review, see ref 5). T_2 is particularly intriguing because of its effects on metabolism. Starting more than a decade ago, when a surprising report appeared showing that T_2 resulted in a rapid stimulation (within 30 min)

of oxygen consumption in perfused livers from hypothyroid rats (10), a number of papers were published showing biological effects of T_2 (for reviews, see refs 5, 6, and 9). The results reported in the present paper are in line with the general conclusions of these previous studies. Indeed, our results show that T_2 has the potential to reduce adiposity in HFD fed rats by increasing the burning of fats without inducing thyrotoxicosis. The problem of thyrotoxicosis has strongly limited the use of THs both as weight-lowering agents and as agents able to decrease serum parameters related to lipid metabolism. The major signs related to thyrotoxicosis are an increase in heart rate, decrease in skeletal muscle mass, and a dysfunction in the feedback mechanism that regulates the endogenous production of THs. The results reported here provide clear evidence that in HFD fed rats T_2 is able to reduce both adiposity and the serum levels of fatty acids, triglycerides, and cholesterol without inducing any of these signs and without a significant effect on the thyroid axis. However, we cannot exclude that treatment with T_2 on a longer time scale may produce some of these signs or possible deleterious effects linked to oxidative damages, and these aspects, in such a case, need to be carefully checked. No data are present in the literature about the effects of T_2 in humans. Pinna et al. (50) reported that conditions in which a "low T_3 state" occurs such as nonthyroidal illness and brain tumors are characterized by higher concentrations of T_2 and concluded that such elevation of T_2 may be a compensatory/defense mechanism activated to maintain a metabolic-clinical euthyroidism in patients with reduced T_3 levels. In addition, as both central (TSH) and peripheral (CPT1 and UCP3) markers of the nuclear action of thyroid hormone are unaffected by T_2 treatment, the present data lend further support to the notion that the effect of T_2 on energy metabolism is independent of TRs [all the genes for the above markers have a thyroid-response element in their promoter (51–53)]. However, Ball et al. (17) have shown that T_2 *1*) is able in stimulating growth hormone mRNA levels in GH3 cells, even if 100-fold less potent than T_3; *2*) has almost equivalent ability as that of T_3 to down-regulate TRβ2 mRNA levels in the same cell line; and *3*) when injected into hypothyroid rats (at the same dose at which it does not produce equivalent suppression of circulating TSH) is equivalent to T_3 in inducing hepatic malic enzyme mRNA. Thus, we cannot exclude, at the moment, a potential involvement of a TRs isoform in some effects elicited by T_2.

The liver, that is a major contributor to energy expenditure (54), appears to be the principal target for T_2. Both the HFD and the T_2 treatment employed in this study induced an increase in hepatic β-oxidation, but both the entity and the biochemical mechanisms underlying these effects may differ between the two treatments. When compared with the levels in N animals, in D rats β-oxidation was 30% higher, ACC activity significantly lower (–65%), and CPT activity 38% higher, but the phosphorylated form of AMPK (P-AMPK, which is known to inhibit ACC activity under several physiological conditions) was decreased. When compared with the levels in D animals, in DT_2 rats ACC activity was not further decreased, despite further increases in β-oxidation (+42%), CPT activity (by 52%), and P-AMPK content (see Fig. 4*C*). These results apparently seem to exclude an involvement of the AMPK-ACC-CPT pathway in the observed effects of HFD and T_2 on liver fatty acid oxidation when analyzed after 30 days. The reduced ACC activity seen in D rats was likely due to a reduced ACC protein content, and this suggests that the increase in fatty acid oxidation in D rats may be due to the following malonylCoA-dependent mechanism: reduced content of ACC→reduced level of malonylCoA→ activation of CPT system. Concerning DT_2 rats, the results indicate that T_2 is able further to increase fatty acid oxidation in parallel with CPT activity without increasing the mRNA levels of CPT1 and without any further inhibition of ACC activity but with a strong activation of AMPK. In view of this, we hypothesize that T_2 might enhance fatty acid flux into mitochondria by regulating CPT1 activity

in a P-AMPK-dependent and ACC-malonyl-CoA-independent way. The possible existence of a mechanism of malonyl-CoA-independent control of hepatic CPT1 activity is supported by some recent studies (55, 56) showing that, apart from the deinhibition of CPT1 induced by depletion of intracellular malonyl CoA levels, the stimulation of hepatic fatty acid oxidation can also rely on a malonyl CoA-independent pathway involving an AMPK-mediated phosphorylation of a cytoskeletal components leading to CPT1 stimulation. The high level of the phosphorylated form of AMPK we observed in DT_2 rats (Fig. 4*C*) is consistent with this possibility.

The increases in body weight and adiposity observed in D rats, however, clearly indicate that an increase in hepatic fatty acid oxidation is not in itself sufficient to prevent fat accumulation during consumption of an HFD and that it may be a concomitant less efficient utilization of substrate (thermogenic effect) that brings about the reductions in adiposity and body weight gain observed in DT_2 animals. Indeed, as well as stimulating fatty acid oxidation in the liver, T_2 induced a less efficient utilization of lipid substrates through a stimulation of a thermogenic mechanism such as mitochondrial uncoupling (proton leak). In fact, the proton-leak [leading to a decreased ATP synthesis (57) and a greater burning of fat] was greater in DT_2 than in D rats but not different between D and N rats (Fig. 4*D*). The physiological consequence of these effects is an increased energy expenditure associate with a slight higher body temperature. This suggests that proton leak plays a determining role in the effects exerted by T_2 on the efficiency of substrate utilization and, consequently, on adiposity.

Taken as a whole, the results demonstrate that in DT_2 rats, three events [namely, *1*) a significant increase in CPT activity (+52% vs. D, but +110% vs. N); *2*) an adequate increase in β-oxidation (+42% vs. D, but +93% vs. N); and *3*) an increased mitochondrial proton leak] underlie the reductions in body-weight gain and adiposity that are induced by T_2.

In conclusion, our study shows that T_2 is able to induce in rats receiving a HFD both a reduction in fat accumulation in the rat liver and a marked reduction in adipose-tissue mass. Although the DT_2 rats consumed ~6% more food than the D rats (see Table 2), they still lost body weight (vs. the D rats). A further point to note is the T_2-induced reductions in the serum levels of triglycerides, fatty acids, and cholesterol. Further investigations will be needed before we can hope fully to explain these reductions, but the modifications observed in hepatic metabolism (stimulation of fatty acid oxidation and proton leak) already constitute a basis for the observed reductions in these parameters.

From a clinical point of view, a scenario in which there is a high level of fatty acid oxidation, reduced fat storage, considerable reductions in serum triglyceride and cholesterol levels, a reduced fat content in the liver (steatosis), and a reduced body weight gain without a reduction in calorie/fat intake is an attractive prospect for humans (especially those in the so-called developed countries who tend increasingly to follow a lifestyle that favors fat accumulation). Overweight, hepatic steatosis, and dyslipidemia are increasing problems in some countries. In fact, diet-induced nonalcohol-induced fatty liver disease (NAFLD) is now widespread in affluent societies, in which up to 24% of the general population has been estimated to have NAFLD (58). This is due to the epidemic increase in the prevalence of obesity in these societies. Although NAFLD mostly remains asymptomatic, up to 20% of affected subjects may progress to cirrhosis, and some require liver transplantation (58, 59). Although a prolonged and marked reduction in caloric intake may alleviate NAFLD, this is rarely achieved in real life. Under these

circumstances, an effective pharmacological therapy would be useful. If the results reported here hold true for humans, then pharmacological administration of T_2 might help to counteract the above problems without inducing a thyrotoxic state.

However, we wish to clearly state that here it is not excluded that T_2 could have deleterious effects on a longer time scale and it is not shown whether T_2 acts in the same way in humans.

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Table 1

Serum FT_4, FT_3, and TSH levels, together with thyroid gland, heart, WAT, and liver weights, and heart rate in N, D, and DT_2 rats

	Animal Groups		
Parameters	**N**	**D**	**DT_2**
FT_4 (ng/dl) (*n*=5)	0.80 ± 0.08	0.89 ± 0.01	0.71 ± 0.02
FT_3 (pg/ml) (*n*=5)	2.11 ± 0.27	2.03 ± 0.20	2.18 ± 0.17
TSH (ng/ml) (*n*=5)	0.60 ± 0.12	0.50 ± 0.07	0.63 ± 0.05
Thyroid gland (g) (*n*=12)	0.80 ± 0.06	0.90 ± 0.07	0.89 ± 0.14
Heart (g) (*n*=12)	1.36 ± 0.13	1.19 ± 0.11	1.27 ± 0.25
WAT (g) (*n*=12)	9.40 ± 1.5	19.3 ± 2.9*	10.2 ± 2.4
Liver (g) (*n*=12)	11.5 ± 0.70	14.5 ± 1.38*	11.3 ± 0.98
Heart rate (beats/min) (*n*=5)	311 ± 41	321 ± 46	307 ± 36

Results are means ± SE. *$P < 0.05$ vs. N and DT_2 rats.

Table 2

Pellet intake, energy expenditure, respiratory quotient, and body temperature in N, D, and DT_2 rats over a 1 month treatment period

	Animal Groups		
Parameters	**N**	**D**	**DT_2**
Pellet intake (g) (*n*=12)	782 ± 70	821 ± 82	870 ± 79
Averaged daily energy expenditure ($LtO2/Kg^{-0.67}$ min) (*n*=5)	241 ± 22	253 ± 24	326 ± 31
Respiratory quotient (*n*=5)	0.91 ± 0.01	0.79 ± 0.006*	0.75 ± 0.013*†
Body temperature (°C) (*n*=5)	37.5 ± 0.05	37.6 ± 0.1	38.3 ± 0.1*†

Results are means ± SE. *$P < 0.05$ vs. N rats ; $^{\dagger}P < 0.05$ vs. D rats.

Fig. 1

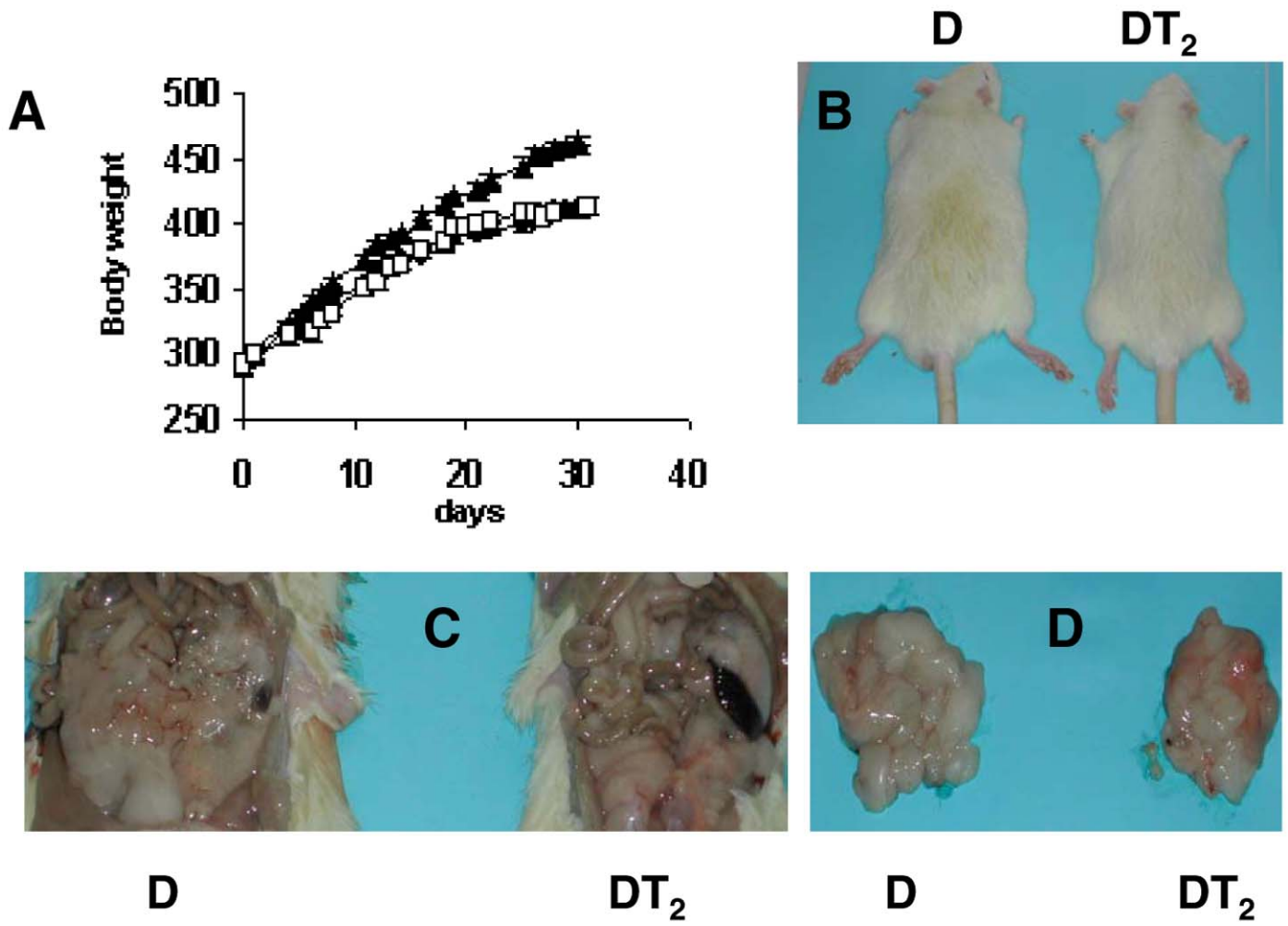

Figure 1. Body weight (growth) and adipose tissue in D and DT_2 rats. ***A)*** Averaged daily measurement of the weight (g) of rats in N (open square), D (solid triangle), and DT_2 (solid circle) groups (n=12); data are means ± SE. ***B)*** Dorsal views of D and DT2 rats. ***C)*** Abdominal views of rats, enabling visceral fat pads to be seen. ***D)*** Visceral fat pads isolated from D (19.3±2.97 g) and DT_2 rats (10.2±2.4 g).

Fig. 2

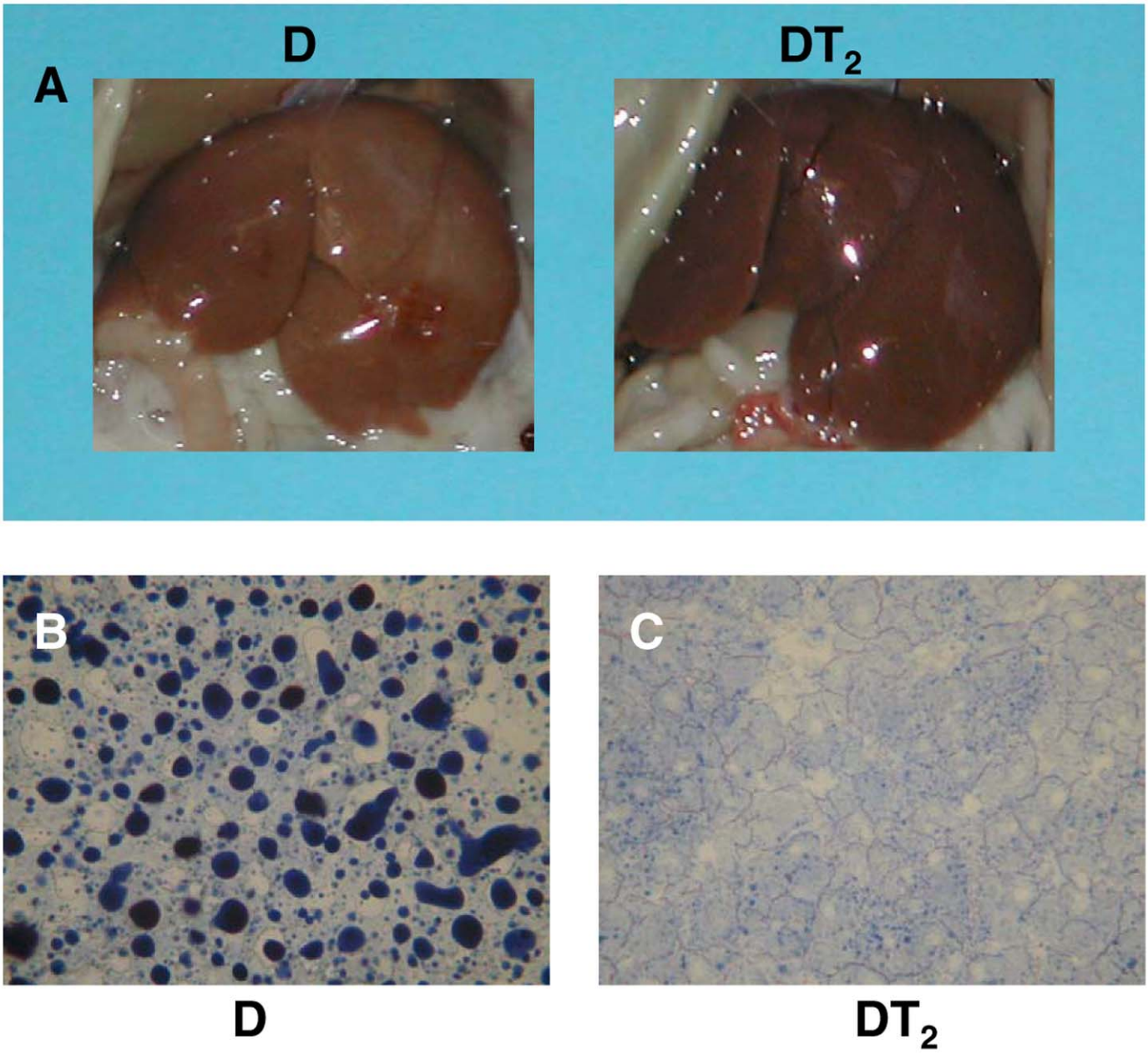

Figure 2. Histological analyses of livers obtained from D and DT_2 rats. ***A***) Livers of D and DT_2 rats. Livers of D rats (***B***) exhibited steatosis, with the presence of abundant fat droplets, while DT_2 livers (***C***) showed a complete absence of droplets.

Fig. 3

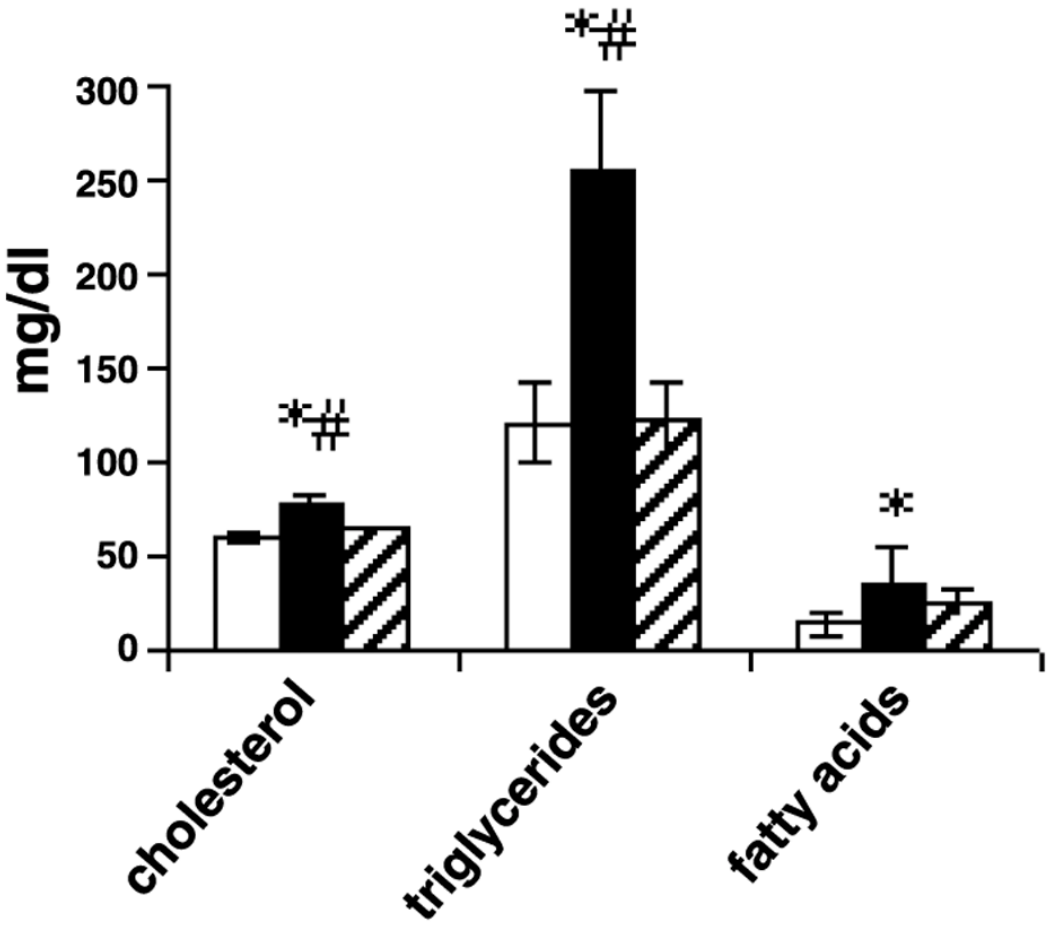

Figure 3. Serum levels of cholesterol, triglycerides, and fatty acids in N (open bars), D (filled bars), and DT_2 (hatched bars) rats (means±SE for 6 rats in each group). $^{*}P < 0.05$ compared with N value, $^{\#}P < 0.05$ compared with DT_2 value.

Fig. 4

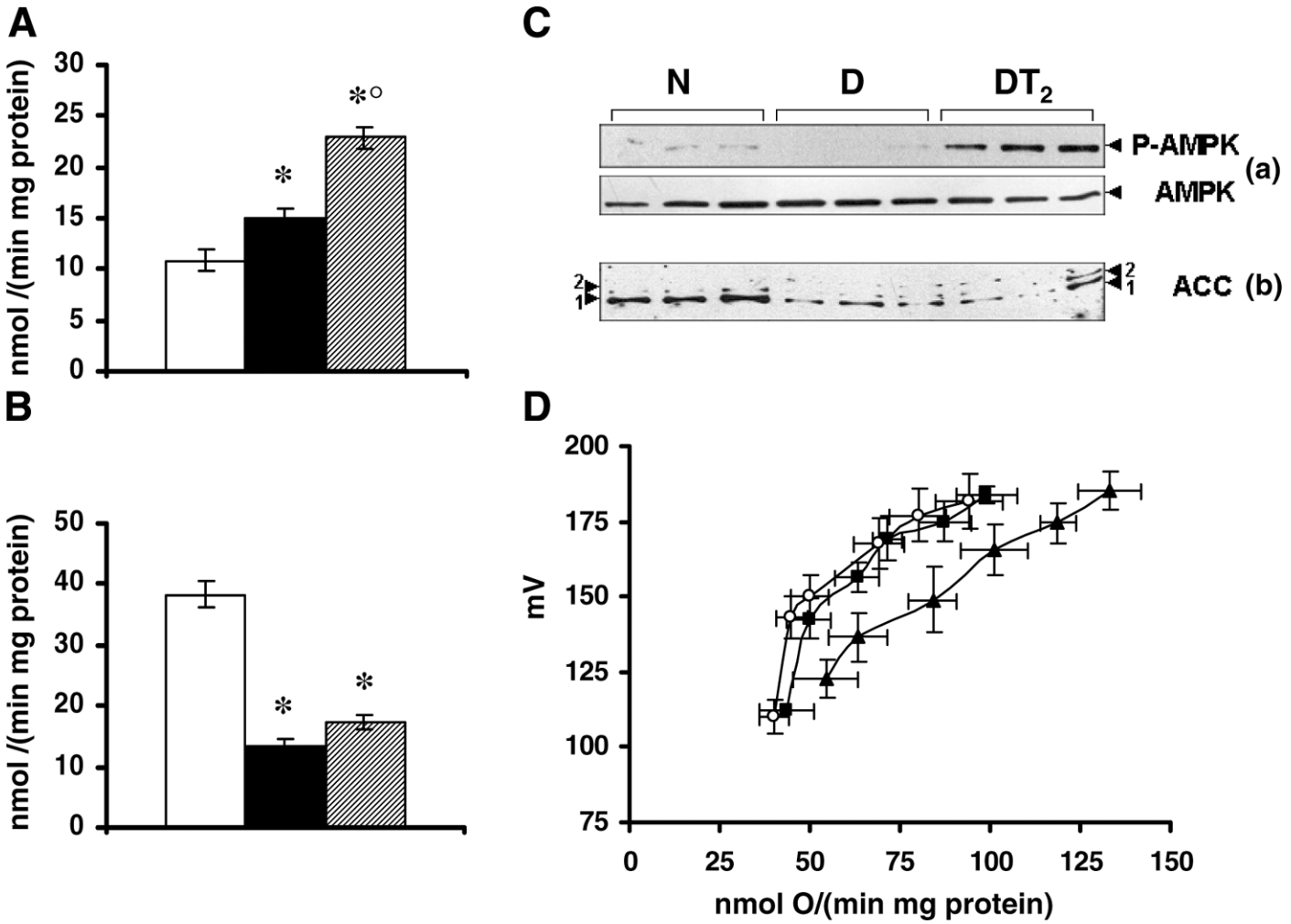

Figure 4. T_2 activates CPT system, AMPK phosphorylation, and mitochondrial proton-leak kinetics in rat liver. T_2 treatment does not further decrease ACC activity in DT_2 rat liver. ***A*)** Total CPT activity in N (open bars), D (filled bars), and DT_2 (hatched bars) rat livers. Data are means ± SE for 6 rats in each group. $^*P < 0.05$ compared with N value, $^\circ P < 0.05$ compared with D value. ***B*)** Total ACC activity in N (open bars), D (filled bars), and DT_2 (hatched bars) rat livers. Data are means ± SE for 6 rats in each group. $^*P < 0.05$ compared with N value. ***C*)** Phosphorylation and total protein levels for AMPK in liver lysates (30 μg) from N, D, and DT_2 rats (***a***). ACC protein levels in liver lysates (30 μg). Numbers and arrows indicate the 2 recognized isoforms of ACC (ACC1 and ACC2) (***b***). ***D*)** Proton-leak kinetics of liver mitochondria from N (solid square), D (open circle), and DT_2 (solid triangle) rats. Data are means ± SE for 4 rats in each group with N value, $^{\#}P < 0.05$ compared with DT_2 value.