For more than a century, thyroid hormones (THs) have been known to exert powerful catabolic effects, leading to weight loss. Although much has been learned about the molecular mechanisms used by TH receptors (TRs) to regulate gene expression, little is known about the mechanisms by which THs increase oxidative metabolism. Here, we report that TH stimulation of fatty acid β-oxidation is coupled with induction of hepatic autophagy to deliver fatty acids to mitochondria in cell culture and in vivo. Furthermore, blockade of autophagy by autophagy-related 5 (ATG5) siRNA markedly decreased TH-mediated fatty acid β-oxidation in cell culture and in vivo. Consistent with this model, autophagy was altered in livers of mice expressing a mutant TR that causes resistance to the actions of TH as well as in mice with mutant nuclear receptor corepressor (NCoR). These results demonstrate that THs can regulate lipid homeostasis via autophagy and help to explain how THs increase oxidative metabolism.
Thyroid hormone stimulates hepatic lipid catabolism via activation of autophagy

Rohit Anthony Sinha,1 Seo-Hee You,2 Jin Zhou,1 Mobin M. Siddique,1 Boon-Huat Bay,3 Xuguang Zhu,4 Martin L. Privalsky,5 Sheue-Yann Cheng,4 Robert D. Stevens,6 Scott A. Summers,1,6 Christopher B. Newgard,6 Mitchell A. Lazar,2 and Paul M. Yen1,6

1Cardiovascular and Metabolic Disorders Program, Duke-NUS Graduate Medical School, Singapore. 2Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, The Institute for Diabetes, Obesity, and Metabolism, Perelman School of Medicine at the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. 3Department of Anatomy, Yong Loo Lin School of Medicine, Department of Anatomy, National University of Singapore, Singapore. 4Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA. 5Department of Microbiology, UCD, Davis, California, USA. 6Sarah W. Stedman Nutrition and Metabolism Center, Departments of Medicine and Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA.

For more than a century, thyroid hormones (THs) have been known to exert powerful catabolic effects, leading to weight loss. Although much has been learned about the molecular mechanisms used by TH receptors (TRs) to regulate gene expression, little is known about the mechanisms by which THs increase oxidative metabolism. Here, we report that TH stimulation of fatty acid β-oxidation is coupled with induction of hepatic autophagy to deliver fatty acids to mitochondria in cell culture and in vivo. Furthermore, blockade of autophagy by autophagy-related 5 (ATG5) siRNA markedly decreased TH-mediated fatty acid β-oxidation in cell culture and in vivo. Consistent with this model, autophagy was altered in livers of mice expressing a mutant TR that causes resistance to the actions of TH as well as in mice with mutant nuclear receptor corepressor (NCoR). These results demonstrate that THs can regulate lipid homeostasis via autophagy and help to explain how THs increase oxidative metabolism.

Introduction

Thyroid hormones (THs) have been known to stimulate basal metabolic rate for over a century (1, 2). Subsequent studies showed that THs induced energy expenditure in response to increased caloric intake (3). Later, several intracellular processes were shown to be involved in the calorigenic effects of THs. These included increased ATP expenditure due to increased Na+/K+-ATPase activity to maintain ion gradients in various tissues (4, 5) as well as reduced efficiency of ATP synthesis, particularly through the induction of uncoupling proteins (UCPs), which cause proton leakage in the electron transport chain of the mitochondria of target tissues (6, 7). However, despite these advances in our understanding of THs on cellular metabolism, none of these proposed mechanisms appears to be dominant. Currently, little is known about other mechanisms that might be utilized by THs to regulate energy consumption within the cell. This is particularly true for the events involved in the delivery of fatty acids to mitochondria, a necessary step in converting stored intracellular triglyceride fuel into ATP.

The active form of TH, 3,3′,5-triiodo-L-thyronine (T3), is a critical regulator of cellular and tissue metabolism throughout the body. It controls gene expression in target tissues by binding to its cognate nuclear receptors (TRα and TRβ), which are ligand-inducible transcription factors. In the presence of T3, TR receptors (TRs) bind to TH response elements in the promoters of target genes and form coactivator complexes containing histone acetyltransferase activity to activate transcription (8). In the absence of T3, TRs recruit corepressors such as NCoR and silencing mediator of retinoid and thyroid receptors (SMRT), which together with transducin β-like protein 1 (TBL1) and histone deacetylase 3 (HDAC3) form a complex with histone deacetylase activity on the promoters of target genes that repress basal transcription (9). At the metabolic level, T3 exerts strong effects on hepatic carbohydrate and lipid metabolism during both anabolic and catabolic states. Lipid synthesis and storage are regulated by T3 via increased expression of lipogenic genes such as fatty acid synthase (FAS), Thrsp (Spot 14), and acetyl-CoA-carboxylase (ACC1) (10, 11). In addition, prolonged T3 treatment promotes the catabolism of fatty acids by increasing the expression and activity of Cpt1α, a rate-limiting enzyme for transport and β-oxidation of fatty acids in the mitochondria (12). Thus, catabolism of fatty acids is a cardinal metabolic feature of prolonged hyperthyroidism (13).

T3 stimulates the shuttling of free fatty acids for delivery into the mitochondria (14). While this process is well described, the T3-regulated cellular pathways that lead to the generation of free fatty acids from stored lipid droplets in liver are not very well understood. Recently, autophagy of lipid droplets, termed “lipophagy,” has been shown to be a major pathway of lipid mobilization in hepatocytes (15–17), and its inhibition has been linked to development of fatty liver and insulin resistance (18–20).

Here, we show that T3 induces lipophagy in cultured liver cell lines. TH also induces hepatic autophagy in vivo coupled with ketogenesis and a lipolytic metabolomic profile. Moreover, TH stimulation of autophagy and lipid metabolism is TR dependent and modulated by NCoR corepressor activity. Our findings suggest that T3 plays an important role in the regulation of hepatic autophagy, which is a critical step for the physiological mobilization and metabolism of fatty acids.

Results

TH (T3) induces autophagy in TR-expressing HepG2 cells. To address the role of TH in hepatic autophagy in human liver, we studied the well-characterized HepG2 cell line, which retained many liver-spe-
specific metabolic functions (21). Since HepG2 cells have very low levels of TR proteins (22, 23), we also studied HepG2 cells expressing hTRα1 or hTRβ1 (HepG2/TRα, HepG2/TRβ). These cells previously were shown to be transcriptionally responsive to T3 in transfection and microarray studies (24).

The phosphatidylethanolamine-conjugated form of LC3, LC3-II, is present in autophagosomes and thus is a commonly used marker of autophagy activation. We found that T3 significantly increased LC3-II levels in HepG2/TRα cells (Figure 1, A and B). However, T3 did not induce autophagy in WT HepG2 cells, suggesting that detectable TR expression was necessary for this effect to occur (Figure 1, A–C).

T3 induction of autophagy was rapid, as it occurred as early as 24 hours (Figure 1, D and E) and was observed at T3 concentrations as low as 0.1 nM (Figure 1, F and G), with an ED50 in the nanomolar range consistent with the known binding affinity of TR (25).

Since it is possible that either induction of autophagy or inhibition of autophagosome clearance could account for the increase in LC3-II levels (26), we measured autophagic flux by comparing the generation of LC3-II by T3 alone or in combination with chloroquine (CQ). CQ inhibits the acidification within lysosomes and endosomes and thus blocks the turnover and degradation of autophagosomes that fuse with them. CQ and T3 treatment significantly increased LC3-II levels compared with T3 alone in cells (Figure 2, A–C). This accumulation of undegraded LC3-II after CQ treatment suggested that T3 induction of LC3-II was due to increased autophagic flux. We also confirmed the increase in autophagic flux using tandem red fluorescent protein–GFP–tagged (RFP-GFP–tagged) LC3 plasmid (27), which showed an increase in the number of both autophagosomes (yellow dots) and autolysosomes (red dots) following T3 treatment (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI60580DS1). The presence of the red dots indicates that LC3 is present within lysosomes, since autophagosomes are able to fuse with lysosomes due to autophagic flux. We also tested the ability of T3 to promote autophagy in several other hepatic cell lines, AML-12, Hep3B, and Huh7, that contain endogenous TRs at detectable protein levels (23, 28, 29). We observed that T3 significantly increased LC3-II levels in these cells, further confirming that T3 is proautophagic in hepatic cells (Figure 2, D and E).

T3 induction of autophagy was also detected by the increased staining of LC3-II as punctate dots that are characteristic of autophagosomal membranes in the cytoplasm (Figure 3A). T3 also increased cytoplasmic acidine orange (AO) staining, indicative of acidic vesicular organelle formation that is characteristic of
autophagy and autolysosomal activity (Figure 3B and ref. 30). Additionally, T3 stimulated cytoplasmic accumulation of monodansylcadaverine (MDC), which marks autolysosomes and lysosomes (ref. 31 and Figure 3B). These results demonstrating increased lysosomal acidification by T3 are consistent with earlier findings that showed that T3 induced lysosomal activity in mice (32).

**T3 promotes lipophagy in hepatic cells.** To further characterize the autophagy induced by T3, we performed transmission EM of HepG2/TRα cells. This revealed an increased number of autophagosomes and lysosomes as well as increased formation of large lipid droplets in T3-treated cells (Figure 4, B–F). The latter likely are due to the rapid induction of fatty acid synthesis by T3 (Supplemental Figure 2) that occurs before the switch to fatty acid oxidation seen after chronic T3 treatment (13). Higher magnification images revealed sequestration of lipid within double membranous autophagosomes and autolysosomes that are characteristic of lipophagy (Figure 4, D–F). Moreover, increased staining of lipophilic BODIPY dye was observed after T3 treatment, and its overlap with LC3 confirmed induction of lipophagy by T3 (Supplemental Figure 2). These results demonstrate that T3-mediated induction of hepatic lipophagy is not dependent upon the TR isoform being expressed in hepatic cells.

**Figure 2**

*T3 stimulates autophagic flux and also induces autophagy in multiple hepatic cell lines. (A–C) Immunoblot and densitometric for LC3-II showing autophagic flux using HepG2/TRα cells treated with 1 μM T3 and 50 μM CQ for 72 hours (*n* = 4; *P* < 0.05). (D and E) Immunoblot analysis of LC3-II levels in AML-12, Hep3B, and Huh7 cells upon 1 μM T3 treatment for 72 hours showing increased autophagy (*n* = 3; *P* < 0.05). Results are expressed as mean ± SEM.

**T3 promotes hepatic autophagy in vivo.** In order to assess the role of T3 in the regulation of autophagy in vivo, we injected C57BL/6 mice with T3 (10 μg/100 g BW) daily for 3 days. In these hyperthyroid mice, T3 increased hepatic LC3-II and decreased p62 protein levels in mouse livers, indicative of autophagy (Figure 5, A–D). The downregulation of p62 protein was likely secondary to increased autophagic flux, as p62 gene expression was unchanged (data not shown).

EM images showed increased hepatic lipid–containing autophagosomes and lysosomes, with a concomitant increase in the number of hepatic mitochondria in T3-treated mice, suggesting mitochondrial biogenesis and an increase in oxidative phosphorylation (Figure 5, E–I, and ref. 33). We also found a significant increase in the expression of PGC1α mRNA (2.87 ± 0.2-fold), which is consistent with the increase in mitochondrial number (data not shown).

**T3-regulated autophagy is TR dependent.** To further study the role of TR in T3-mediated autophagy, we used a well-characterized mouse model (34) in which the normal TRβ allele is replaced by the TRβPV mutant allele originally identified in a patient with resistance to TH (RTH) (35). The encoded mutant receptor has markedly decreased T3 binding and transcriptional activity while maintaining its ability to bind to the thyroid hormone response elements (TRE) and thus functions as a dominant negative on WT TR (34). These TRβPV mice have defective β-oxidation of lipids and develop fatty livers (36). Remarkably, TRβPV mice had reduced LC3-II levels compared with WT euthyroid littermate controls despite having elevated circulating serum T3 levels (ref. 34 and Figure 6A). The TRβPV mice also had increased hepatic p62 accumulation, similar to the levels observed in hypothyroid WT mice (Figure 6B). These data strongly suggest that the induction of liver autophagy by T3 is mediated by TR and not via pathways involving other cellular proteins (37).

autophagy and autolysosomal activity (Figure 3B and ref. 30). Additionally, T3 stimulated cytoplasmic accumulation of monodansylcadaverine (MDC), which marks autolysosomes and lysosomes (ref. 31 and Figure 3B). These results demonstrating increased lysosomal acidification by T3 are consistent with earlier findings that showed that T3 induced lysosomal activity in mice (32).

**T3 promotes lipophagy in hepatic cells.** To further characterize the autophagy induced by T3, we performed transmission EM of HepG2/TRα cells. This revealed an increased number of autophagosomes and lysosomes as well as increased formation of large lipid droplets in T3-treated cells (Figure 4, B–F). The latter likely are due to the rapid induction of fatty acid synthesis by T3 (Supplemental Figure 2) that occurs before the switch to fatty acid oxidation seen after chronic T3 treatment (13). Higher magnification images revealed sequestration of lipid within double membranous autophagosomes and autolysosomes that are characteristic of lipophagy (Figure 4, D–F). Moreover, increased staining of lipophilic BODIPY dye was observed after T3 treatment, and its overlap with LC3 confirmed induction of lipophagy by T3 (Supplemental Figure 2). These results demonstrate that T3-mediated induction of hepatic lipophagy is not dependent upon the TR isoform being expressed in hepatic cells.

**T3 promotes hepatic autophagy in vivo.** In order to assess the role of T3 in the regulation of autophagy in vivo, we injected C57BL/6 mice with T3 (10 μg/100 g BW) daily for 3 days. In these hyperthyroid mice, T3 increased hepatic LC3-II and decreased p62 protein levels in mouse livers, indicative of autophagy (Figure 5, A–D). The downregulation of p62 protein was likely secondary to increased autophagic flux, as p62 gene expression was unchanged (data not shown).

EM images showed increased hepatic lipid–containing autophagosomes and lysosomes, with a concomitant increase in the number of hepatic mitochondria in T3-treated mice, suggesting mitochondrial biogenesis and an increase in oxidative phosphorylation (Figure 5, E–I, and ref. 33). We also found a significant increase in the expression of PGC1α mRNA (2.87 ± 0.2-fold), which is consistent with the increase in mitochondrial number (data not shown).

**T3-regulated autophagy is TR dependent.** To further study the role of TR in T3-mediated autophagy, we used a well-characterized mouse model (34) in which the normal TRβ allele is replaced by the TRβPV mutant allele originally identified in a patient with resistance to TH (RTH) (35). The encoded mutant receptor has markedly decreased T3 binding and transcriptional activity while maintaining its ability to bind to the thyroid hormone response elements (TRE) and thus functions as a dominant negative on WT TR (34). These TRβPV mice have defective β-oxidation of lipids and develop fatty livers (36). Remarkably, TRβPV mice had reduced LC3-II levels compared with WT euthyroid littermate controls despite having elevated circulating serum T3 levels (ref. 34 and Figure 6A). The TRβPV mice also had increased hepatic p62 accumulation, similar to the levels observed in hypothyroid WT mice (Figure 6B). These data strongly suggest that the induction of liver autophagy by T3 is mediated by TR and not via pathways involving other cellular proteins (37).
research article

**T3-induced lipophagy regulates β-oxidation of fatty acids in hepatic cells.** Singh et al. previously showed that lipophagy was causally linked to β-oxidation of fatty acids in hepatocytes (15). To determine whether lipophagy stimulated by T3 also induced fatty acid oxidation, we used autophagy-related 5 (ATG5) siRNA to inhibit autophagy in HepG2/TRα cells treated with oleic acid to maximize β-oxidative flux. We then measured the levels of the end product of fatty acid β-oxidation, β-hydroxybutyrate, in the medium. These cells showed a significant increase in β-hydroxybutyrate release following T3 stimulation when transfected with a control siRNA; however, this increase was completely abolished in the presence of ATG5 siRNA (Figure 7). The effect of ATG5 knockdown on autophagy also was confirmed by the expected changes in LC3-II levels (Figure 7), indicating that autophagy plays a major role in T3-induced β-oxidation of fatty acids in hepatic cells.

**T3-mediated autophagy is tightly coupled with β-oxidation of fatty acids in vivo.** We next investigated the contribution of autophagy in T3-mediated fatty acid β-oxidation in the livers of living mice. Mice were subjected to tail-vein injection of ATG5 siRNA, which led to a marked reduction in hepatic ATG5 protein (Figure 8A) and mRNA (data not shown) relative to mice injected with control siRNA after 72 hours. T3 treatment caused increased LC3-II levels in livers of mice injected with control siRNA and increased serum hydroxybutyrate levels (Figure 8, A–C). In contrast, livers of T3-treated mice injected with ATG5 siRNA did not exhibit increased autophagy or increased circulating levels of β-hydroxybutyrate (Figure 8, A–C). Taken together with our previous findings in hepatic cells, these results show that T3-mediated β-oxidation of fatty acids is tightly coupled with the effect of the hormone in inducing autophagy in liver. These findings also suggest that autophagy is an important physiological mechanism for T3 stimulation of β-oxidation of fatty acids.

**NCoR modulates T3-mediated autophagy and lipid catabolism in liver.** TH action involves TR interaction with nuclear receptor corepressor (NCoR) (38–40). We examined the role of NCoR in T3-mediated autophagy in vivo by studying mice (NCoR DAdm mice) expressing a mutant NCoR that is expressed at normal levels but does not bind to HDAC3, a critical component of the corepessor complex (40). In both the WT and NCoR DAdm mice, a progressive increase was observed in LC3-II levels in the hypothyroid, euthyroid, and hyperthyroid mice. However, LC3-II levels were higher in the hypothyroid NCoR DAdm mice than in the WT littermates (Figure 9, A and B). In addition, although T3 stimulated LC3-II levels in both NCoR DAdm and WT mice, the LC3-II levels were attenuated in the NCoR DAdm mice compared with WT mice (Figure 9, A and B). These findings suggest a role for NCoR in the induction of autophagy by T3.

Both T3 and NCoR modulate the hepatic acylcarnitine profiles. In order to better understand how T3 effects on autophagy have an impact upon β-oxidation of fatty acids in the liver, we analyzed the levels of a broad array of acylcarnitines in livers of WT and NCoR DAdm mice under hypothyroid, euthyroid, and hyperthyroid conditions by tandem mass spectrometry. The levels of various acylcarnitines reflected the levels of cognate acyl-coAs generated by oxidation of fatty acids and other fuels within the mitochondria. Our studies showed that medium-chain acylcarnitines (C8, C10) increased by similar amounts in both euthyroid and hyperthyroid mice compared with hypothyroid mice (control and TH vs. Per/MMI) (Figure 9C). Additionally, NCoR DAdm mice had concentrations of medium-chain acylcarnitines similar to those of WT mice under all 3 conditions (Figure 9C). On the other hand, long-chain acylcarnitines (C16, C18) increased in hyperthyroid mice compared with euthyroid and hypothyroid mice, and the increase in long-chain acylcarnitines was sharply attenuated in hyperthyroid NCoR DAdm mice (Figure 9D). Finally, short-chain acylcarnitines such as acetyl (C2) and propionyl (C3) were largely unaffected by thyroid status, but were sharply reduced in NCoR DAdm mice only under hyperthyroid conditions (Supplemental Figure 4A). The overall picture that emerges is one of TH-induced activation of autophagy/ lipophagy, increased delivery of long-chain fatty acyl CoAs/acylcarnitines to the mitochondria, and increased rates of fatty acid oxidation. The graded increase in medium-chain acylcarnitines under hypothyroid, euthyroid, and hyperthyroid conditions may indicate increased substrate entry into the β-oxidative pathway. In hyperthyroid mice, the rate of delivery of acylcarnitines may saturate the β-oxidative system, contributing to the accumulation of long-chain acylcarnitines under these conditions. These effects were attenuated in hyperthyroid NCoR DAdm mice, perhaps due to a decrease in autophagy-mediated lipolysis in these animals.

The expression of hepatic lipase (LIPC) was significantly downregulated in hypothyroid WT and NCoR DAdm mice, whereas it did not significantly change in hyperthyroid mice (Supplemental Figure 4, B and C). ATGL levels were significantly downregulated. 

Figure 3

**T3 stimulates autophagosomal and lysosomal activity in hepatic cells.** (A) LC3 immunostaining. Punctate staining shows autophagosome formation in cells treated with 1 μM T3 for 72 hours. (B) Lysosomal acidification visualized using MDC and AO in HepG2/TRα cells treated with 1 μM T3 for 72 hours.
only in hypothyroid NCoR DADm mice, whereas they remained unchanged in WT mice. Cpt1α mRNA levels remained largely unchanged, whereas ACO levels were decreased in hyperthyroid WT mice (Supplemental Figure 4, B and C).

Microarray pathway analysis confirms that T₃ stimulates lipid catabolism pathways in WT but not NCoR DADm mice. We also performed microarray analyses of hepatic target genes induced by T₃ in WT and NCoR DADm mice. Interestingly, pathway analysis of the differentially expressed genes showed that T₃ induced pathways of lipid and amino acid catabolism pathways (Supplemental Figure 5, A and B). Moreover, these effects were dependent on NCoR-HDAC3 recruitment, as the effects of hyperthyroidism on the pathways regulating lipid and amino acid catabolism were not ranked in NCoR DADm mice (Supplemental Figure 5, A and B).

Discussion
TH is well known as a metabolic regulator of energy expenditure through activation of β-oxidation of fatty acids in mammals (41). However, the precise mechanism of this effect has never been revealed. Here we have demonstrated an action of T₃ in promoting lipophagy in both human hepatic cells in vitro and mouse liver in vivo. This lipophagy was coupled with clear effects of T₃ stimulation in altering the levels of a broad array of hepatic lipid-related metabolites, consistent with a key role of T₃ as important regulator of the delivery of fatty acids to mitochondria and their metabolism.

Autophagy is a stress-induced catabolic process involving lysosome fusion that is conserved in all eukaryotes. During periods of starvation, autophagy degrades cytoplasmic materials to produce amino acids and fatty acids that can be used to synthesize new proteins or generate ATP for cell survival (42). Derangement of autophagic response has been implicated in several pathologic hepatic conditions, such as ischemia, reperfusion, viral infections, acute injury, α1-antitrypsin deficiency, hepatocellular carcinoma, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) (20, 43, 44). Hormonal regulation of hepatic autophagy by glucagon and insulin has been described previously (43). Recently, a previously unknown function for autophagy leading to the degradation of intracellular lipid droplets was described in hepatocytes and termed “lipophagy.” This process is now believed to provide substrate for β-oxidation of fatty acids (15).

T₃ induces lipophagy in hepatocytes. Several different lines of investigation presented here demonstrate T₃-mediated autoph-
agy, including immunodetection of LC3-II and p62, lysosomal staining showing increased acidification, tandem RFP-GFP-LC3 fluorescence, and EM evidence of lipophagy. Interestingly, T3-treated cells exhibited an increased number of large lipid droplets in the cytosol compared with a few small lipid droplets in the untreated cells. It is likely that T3 initially increased accumulation of triglycerides due to its known early effect on lipogenesis (13). Indeed, we found the levels of key target lipogenic enzymes such as FAS and ACC were slightly increased by T3 at 24 hours after treatment (Supplemental Figure 2). Furthermore, LC3-II itself may be a critical regulator of lipid droplet formation in hepatocytes (45). The dual ability of T3 to form lipid droplets and lipid-containing autophagosomes could serve as a dynamic process for fuel delivery while concomitantly reducing the amount of toxic free fatty acids in the cells. This may be even more relevant at the physiological level, since fatty acids that are generated by T3-stimulated lipolysis in adipose tissue must also be metabolized by the liver. Thus, the ability of T3 to regulate lipogenic enzymes such as FAS and ACC were slightly increased by T3 at 24 hours after treatment (Supplemental Figure 2). Furthermore, LC3-II itself may be a critical regulator of lipid droplet formation in hepatocytes (45). The dual ability of T3 to form lipid droplets and lipid-containing autophagosomes could serve as a dynamic process for fuel delivery while concomitantly reducing the amount of toxic free fatty acids in the cells. This may be even more relevant at the physiological level, since fatty acids that are generated by T3-stimulated lipolysis in adipose tissue must also be metabolized by the liver.

**Figure 5** T3 induces hepatic autophagy in vivo. (A and B) Immunoblot and densitometric analysis of LC3-II levels in euthyroid and hyperthyroid mice injected with 10 μg T3/100 g BW for 3 days (n = 3; *P < 0.05). (C and D) Immunoblot and densitometric analysis of p62 levels in euthyroid and hyperthyroid mice (n = 3; *P < 0.05). Results are expressed as mean ± SEM. EM showing livers obtained from control (E) and hyperthyroid (F–I) mice injected with 10 μg T3/100 g BW for 3 days. White arrowheads in F denote increased number of mitochondria in hyperthyroid mouse liver, and white arrows show autolysosomes. (G) Magnified image of the boxed area in F showing double-membrane lipid droplets inside autolysosomes. (H) Autophagic vesicle containing several lysosomes (white arrows) and lipid droplets (black arrow). (I) Autophagosome inside a large lipid droplet in hepatocyte from hyperthyroid mouse liver. Scale bars: 2 μm (E and F); 0.2 μm (G and H); 0.1 μm (I).
eral groups have reported cytosolic TR interaction with different protein kinases (37). However, our finding that the repression of autophagy observed in hypothyroidism did not occur in NCoR DADm mice argues for a nuclear effect of the TH via its TR.

**NCoR-HDAC3 interaction regulates T3-mediated autophagy and lipid metabolism in vivo.** In the absence of TH, TR interacts with corepressors such as NCoR and SMRT that recruit HDACs to the promoters of target genes (8). NCoR is believed to preferentially control repression by unliganded TR in vivo. Consistent with this model, coexpression of dominant negative corepressors can interfere with the basal repression of target genes in mouse livers (38–40). Since transcriptional repression by unliganded TR-NCoR complex involves recruitment of HDAC3, several TR-responsive genes are modestly derepressed in the hypothyroid state in the livers of NCoR DADm mice, demonstrating an important role of the NCoR-HDAC3 interaction in basal repression (40). Similar findings have been reported in other dominant negative mutant NCoR mouse models (38, 39). Interestingly, we found that the repression of autophagy observed in hypothyroidism did not occur in NCoR DADm mice. Thus, it is possible that T3-regulated genes involved in autophagy that normally are repressed in hypothyroid WT mice may be derepressed in NCoR DADm mice. This is further supported by recent findings showing increased autophagy in hepatic cancer cells upon HDAC inhibition (47). In this connection, we also have observed that the HDAC inhibitor, trichostatin A, can induce autophagy in HepG2 cells (R.A. Sinha and P.M. Yen, unpublished observations).

Medium- and long-chain acylcarnitine levels in both groups of hyperthyroid mice correlated well with the LC-III increase observed in the hypothyroid mice, although the increase in long-chain acylcarnitines was sharply attenuated in the NCoR DADm mice. It is possible that the decreased autophagy and β-oxidation of fatty acids in the NCoR DADm mice may predispose them to develop hepatosteatosis. In this connection, a recent study showed that disruption of SMRT and nuclear receptor interaction led to insulin resistance and fatty liver (48). Similarly, the diabetes- and obesity-regulated gene (*DOR*), which is also a nuclear cofactor of TRs expressed abundantly in metabolically active tissues, has been shown to induce autophagy in drosophila and mammals (49). Thus, transcriptional cofactors, in addition to nuclear hormone receptors, may contribute to the development and phenotype of metabolic diseases and serve as potential therapeutic targets.

Although T3 has been known to induce lipolysis, the mechanism by which it occurs is poorly understood. Our data suggest that T3 increases the delivery of fatty acids to the mitochondria for β-oxidation via its induction of autophagy. Indeed, our cell culture and in vivo studies with ATG5 siRNA to knock down ATG5 expression and block autophagy showed that autophagy is critical for β-oxidation of fatty acids in the liver. These data and the observed increase in both long- and medium-chain acylcarnitines in our metabolomic studies suggest that lipolysis may be the key regulatory mechanism for increased β-oxidation of fatty acids early after T3 treatment. In this connection, we did not observe any significant increase in the levels of hepatic lipases (*LIPC* and *ATGL*) or *Cpt1α* and *ACO* after acute T3 treatment (14 hours), but it remains possible that additional mechanisms may contribute to the more prolonged effect of T3.

Our findings on T3 regulation of lipolysis and β-oxidation of fatty acids may have relevance for patients with advanced stages of nonalcoholic steatohepatitis (NASH) in which activation of transcription by T3 may be impaired in the liver (50). Indeed, it has been reported that TH deficiency is associated with increased incidence of NAFLD (51). However, it should be noted that many pathways in addition to autophagy contribute to hepatic lipid metabolism and the development of NAFLD (52).

Although our study has mostly focused on the protective aspects of T3-induced hepatic autophagy, prolonged autophagy can eventually lead to cell death (53). In particular, autophagic cell death may contribute to the hepatic damage and resultant liver failure sometimes observed during the rare clinical condition of extreme

---

**Figure 6**

In vivo regulation of hepatic autophagy is TR dependent. (A) Immunoblot showing LC3-II levels in WT euthyroid and TRPV/PV mice. (B) Immunoblot showing LC3-II and p62 levels in (2 to 3 months old) euthyroid mice, hypothyroid mice (fed with a low-iodine diet supplemented with 0.15% propylthiouracil [Harlan Teklad] for 35 days, and TRPV/PV mice.

**Figure 7**

Autophagy mediates fatty acid oxidation and ketosis by T3 in hepatic cells. HepG2/TRα cells were transfected with either control siRNA or ATG5-specific siRNA. Cells then were cultured with oleic acid (0.5 mM) in the absence or presence of T3 for the next 48 hours before harvesting. (A) β-Hydroxybutyrate concentrations in culture medium were measured along with (B) ATG5 and LC3-II protein levels, which were measured by Western blotting (n = 3/each group). Results are expressed as mean ± SEM.
hyperthyroidism or “thyroid storm” (54). Therefore, it is likely that there is an optimal intrahepatic TH concentration that mediates autophagy to enable the liver to clear and metabolize cellular fuel stores in an efficient manner.

In conclusion, we have described a cellular action of TH to induce autophagy in mammalian hepatic cells both in vitro and in vivo. Our results underscore the importance of T3 in not only regulating eumetabolic status, but also mobilizing and metabolizing stored lipids in the liver to provide the necessary fuel to maintain that state. These findings raise the possibility that T3 or its analogs, through their proautophagic action, may be useful in the treatment or prevention of NAFLD and its associated complications.

Methods

Reagents. T3, monodansylcadaverine (MDC), AO, oleic acid, anti-p62 antisera, and DAPI were from Sigma-Aldrich. Antibodies recognizing human LC3, actin, cleaved caspase-3, and GAPDH were from Cell Signaling Technology. Culture medium and serum were from Invitrogen. GFP-RFP-LC3 and stored at –80°C until analysis. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred immediately onto polyvinylidene difluoride membranes (Bio-Rad) using 1× Towbin buffer (25 mmol/l Tris, pH 8.8, 192 mmol/l glycine, 25% v/v glycerol, 10% v/v 2-mercaptoethanol, and 0.01% w/v bromophenol blue), and samples were heated to 100°C for 5 minutes and stored at –80°C until analysis. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred immediately onto polyvinylidene difluoride membranes (Bio-Rad) using 1× Towbin buffer (25 mmol/l Tris, pH 8.8, 192 mmol/l glycine, 25% v/v glycerol, 10% v/v 2-mercaptoethanol, and 0.01% w/v bromophenol blue). Cells were lysed using mammalian lysis buffer (Sigma-Aldrich). Total RNA (1 µg) was reverse-transcribed by the iScript Select cDNA Synthesis Kit (170–8896) (Bio-Rad) in accordance with the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) in accordance to the manufacturer’s instructions. Actin levels were taken for normalization and fold change was calculated using 2-ΔΔCt. Primer sequences are provided in Supplemental Table 1.

MDC, AO, and BODIPY 493/503 staining. Cells were grown on glass coverslips and treated with 1 µM T3 for 72 hours. Thereafter, the cells were incubated with 0.05 mM MDC or 1 µg/ml AO (Sigma-Aldrich) for 15 minutes, fixed for 30 minutes at room temperature at 37°C, and immediately observed under fluorescence microscope. BODIPY 493/503 (Invitrogen) was dissolved in ethanol to give a stock of 1 mg/ml. Then 4% PFA-fixed cells were stained for 15 minutes at 1:1000 dilution, washed with PBS, and observed under fluorescence microscope. BODIPY 493/503 (Invitrogen) was dissolved in ethanol to give a stock of 1 mg/ml. Then 4% PFA-fixed cells were stained for 15 minutes at 1:1000 dilution, washed with PBS, and observed under fluorescence microscope.
15% v/v methanol). Membranes were blocked in 5% milk and subsequently were incubated in 1% w/v bovine serum albumin in PBST with specific antibodies overnight at 4°C. Membranes were washed 3 times in TBST and subsequently incubated with species-appropriate, peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) for 1 hour. Blots were washed 3 times with TBST and once with TBS without Tween and developed using an enhanced chemiluminescence system (GE Healthcare). Densitometry analysis was performed using ImageJ software (NIH).

Figure 9
NCoR modulates T3-mediated autophagy and acylcarnitine levels in vivo. (A and B) Immunoblot and densitometric analysis of LC3-II levels in WT and NCoR DADm mice. Female WT and NCoR DADm mice (19 weeks old) were used for the experiment. Fresh drinking water containing 1% perchlorate and 0.05% methimazole was provided daily for 2 weeks to induce hypothyroidism. Fourteen hours before they were killed, all animals were given a subcutaneous injection of vehicle (0.9% saline in 100 μl volume for control and hypothyroid groups or 40.0 μg/100 g T4 with 4.0 μg/100 g T3 for hyperthyroid group). *P < 0.05; n = 3 animals in each group. (C) Metabolomic analysis of medium-chain acylcarnitines in the above-described animal groups (n = 5; *P < 0.05). (D) Metabolomic analysis of long-chain acylcarnitines in the above-described animal groups (n = 5, *P < 0.05; **P < 0.01; ***P < 0.001). Results are expressed as mean ± SEM.
As recommended by recent reviews on autophagy detection methodology (56–59), we generally used LC3-II/actin ratio as an index of autophago- somes formation; however, LC3-II/LC3-I ratio also showed in some of the same figures. Both methodologies gave similar results even though the magnitude of the ratios may be different.

**Immunofluorescence studies.** After treatment, cells were washed in PBS and then fixed with 4% paraformaldehyde for 15 minutes at room temperature. Fixed cells were washed with PBS, permeabilized in 100% methanol for 10 minutes at -20°C, washed in PBS, and blocked in blocking buffer for 1 hour at room temperature. Cells subsequently were incubated with anti-LC3 antibody overnight at 4°C. After 3 TRST washes, cells were incubated with Alexa Fluor-anti-rabbit antibody (Invitrogen) for 2 hours at room temperature and then washed 3 times in TBST. Coverslips were mounted on slides using Vectashield anti-fade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were observed under fluorescence or confocal microscopes. For autophagic flux analysis, tandem RFP/GFP-tagged LC3 plasmid (a gift from T. Yoshimori, Osaka University) was transfected into HepG2/TrxR cells with Lipofectamine 2000 Transfection Reagent (Invitrogen). Cells were visualized after 48 hours treatment with T3 (1 μM) using an LSM710 Carl Zeiss confocal microscope.

**EM.** Cells were seeded onto 4-chambered coverglass (Nalgene-Nunc) at a density of 2 x 10^4 cells/ml (14,000 cells/well). After 72 hours, cells were fixed with 2.5% glutaraldehyde and washed 3 times with PBS. Subsequent post-fixation with 1% osmium tetroxide was followed by dehydration with an ascending series of alcohol before embedding samples in Araldite. Ultrathin sections were cut and doubly stained with uranyl acetate and lead citrate. Images were acquired using the Olympus EM208S transmission electron microscope.

**Knockdown of ATG5 by siRNA and β-hydroxybutyrate measurement.** Stealth siRNA duplex oligoribonucleotides targeting ATG5 (Invitrogen) were resuspended to make a 20-μM solution following the manufacturer’s instructions. Transfections were carried out in HepG2/TrxR cells using 10 nM of both ATG5 and negative control siRNA (Stealth RNAi siRNA; Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) following the reverse transfection protocol for HepG2 cells. Conditions were optimized with varying ratios of Lipofectamine and RNA as well as different time intervals after the transfections, as determined by immunoblot analysis of ATG5 protein levels. After 24 hours of transfection, cells were subjected to T3 (1 μM) and oleic acid (0.5 mM) treatment and consequent β-hydroxybutyrate release in the medium after 48 hours of treatment using a β-hydroxybutyrate assay kit (Abcam).

**Metabolomic analysis.** Hepatic acylcarnitines were measured in liver extracts by previously described methods (60, 61). Briefly, proteins were removed by methanol precipitation and analytes esterified with hot, acidic methanol (acylcarnitines) or n-butanol (amino acids). Analysis employed tandem MS with a Quattro Micro instrument (Waters Corporation), and quantification of the “targeted” intermediary metabolites was achieved by addition of stable-isotope internal standards. Total ketone levels were measured in plasma as described (62).

**In vivo ATG5 siRNA treatment and measurement of ketone bodies.** Male C57BL/6 mice (4 to 6 weeks old) receiving T3 (10 μg/100 g BW i.p.) were coadministered 40 μg of ATG5 (5’-ACCGGAACUCAGUUGAAU-3’) or Negative Control siSTABLE siRNA (D-001700-01; Dharmacon) every 24 hours for 3 days via hydrodynamic tail-vein injection protocol using Mirus Bio TransIT-QR Delivery Solution. The non-T3-treated control mice and a group of T3-treated mice (n = 4–5 per group) received an equivalent amount of Negative Control siSTABLE siRNA in TransIT-QR Delivery Solution (Mirus Bio). Serum ketone levels were measured using a β-hydroxybutyrate assay kit (Cayman Chemical).

**Liver microarray and pathway analysis.** Total RNA was extracted from the liver using the RNeasy Tissue Mini Kit (QiAGEN) according to the manufacturer’s instructions. Preparation of RNA for hybridization to Affymetrix MoGene 1.0 ST (Affymetrix) and scanning of the arrays were performed by the University of Pennsylvania Microarray Facility (http://www.bioinformatics.upenn.edu/index.html) according to the manufacturer’s instructions. Robust multiarray averaging (RMA) signal extraction, normalization, and filtering were performed by the University of Pennsylvania Microarray Facility Bioinformatics Group (http://www.ncbi.upenn.edu/) using Partek Genomics Suite (Partek). An adjusted P value based on the false discovery rate (FDR) was used for filtering. Pathway analysis was done using DAVID Bioinformatics Resources 6.7 (63).

**Calculations and statistics.** Individual culture experiments were performed in duplicate or triplicate and repeated 3 independent times using matched controls; the data were pooled. Results were expressed as mean ± SEM. The statistical significance of differences (P < 0.05) was assessed by 2-tailed t test.

**Study approval.** All mice were maintained according to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 1.0.0. Revised 2011), and experiments were approved by the IACUCs at the University of Pennsylvania, the National Cancer Institute, and the Duke-NUS Graduate Medical School.

**Acknowledgments.** The authors would like to thank Benjamin A. Bikman, Chui Sun Yap, Brijesh Singh, Guan Yuyuand and Sherwin Ying Xie (Cardiovascular and Metabolic Disorders Program, Duke-NUS Graduate Medical School), and Mei Wang (Cancer Stem Cell Program, Duke-NUS Graduate Medical School) for their helpful advice and constructive criticism. This work was supported by grant NIH DK 43806 (to M.A. Lazar), the Nuclear Receptor Signaling Atlas grant NIH U19DK/HL/ES 62434 (to C.B. Newgard and M.A. Lazar), and an American Diabetes Association (ADA) mentored research fellowship (to S.H. You and M.A. Lazar). This work also was supported by Duke-NUS Graduate Medical School Faculty Funds (to P.M. Yen and S.A. Summers) sponsored by the Ministry of Health, Ministry of Education, and Ministry of Trade, Singapore, and A*StaR.

Received for publication November 28, 2011, and accepted in revised form April 26, 2012.

Address correspondence to: Paul M. Yen, Duke-NUS Graduate Medical School, Laboratory of Hormonal Regulation, CVM Program, 8 College Road, Singapore 018987. Phone: 65.6516.7332; Fax: 65.6516.7396; E-mail: paul.yen@duke-nus.edu.sg. Or to: Mitchell A. Lazar, University of Pennsylvania, 3400 Civic Center Blvd., Bldg. 421, Translational Research Center, 12-102, Philadelphia, Pennsylvania 19104, USA. Phone: 215.898.0199; Fax: 215.898.5408; E-mail: lazar@mail.med.upenn.edu. Or to: Christopher B. Newgard, Duke University Medical Center, Duke Independence Park Facility, 4321 Medical Park Drive, Durham, North Carolina 27704, USA. Phone: 919.668.6059; Fax: 919.477.0632; E-mail: newga002@mc.duke.edu.


