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Cytosolic Action of Thyroid Hormone Leads to Induction of Hypoxia-Inducible Factor-1α and Glycolytic Genes

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Thyroid hormone (TH) effects are mediated through T_3, which regulates gene expression by binding to the nuclear TH receptors, TRα and TRβ. Using microarrays and real-time PCR we found mRNAs of the following genes increased in response to T_3 in a TRβ-specific manner: the transcription factor hypoxia-inducible factor (HIF)-1α, its target genes glucose transporter (GLUT)1 and platelet-type phosphofructokinase (PFKP), and the monocarboxylate transporter (MCT)4. The products of these genes have important roles in cellular glucose metabolism. HIF-1α expression and activity can be regulated through phosphatidylinositol-OH-3-kinase (PI3K) and MAPK signaling; thus the possibility of alternative, nonnuclear pathways of TH action was raised. We examined the involvement of these pathways in mediating TH effects by treating human skin fibroblasts with 2 nM T_3 in the absence or presence of either the PI3K inhibitor LY294002 or the MAPK inhibitor PD98059. T_3 induced HIF-1α mRNA by 2.7-fold (±0.4; $P < 0.013$). This increase was completely abrogated by LY294002 (1.1 ± 0.1; nonsignificant = 0.57), but preserved in the presence of PD98059 (2.2 ± 0.2; $P < 0.009$). Western blotting confirmed these results at the protein level, indicating dependency on the PI3K pathway. The same pattern of response was observed for GLUT1, PFKP, and MCT4 expression. To examine whether HIF-1α is directly induced, we used the translation inhibitor cycloheximide (CHX). T_3 induction of HIF-1α mRNA was not affected by CHX, whereas T_3 effect on GLUT1, PFKP, and MCT4 mRNA was completely abrogated by CHX. These results demonstrate that cytosolic activation of the PI3K signaling pathway has a role in TH-mediated direct (HIF-1α) and indirect (GLUT1, PFKP, MCT4) gene expression, and possibly provides a link between TH and cellular glucose metabolism in human fibroblasts. (*Molecular Endocrinology* 19: 2955–2963, 2005)

THYROID HORMONES (THs) are essential for normal development, growth, and metabolism (1). Their effects are mediated principally through T_3, which regulates gene expression by binding to the TH receptors, TRα and TRβ. Using microarrays and real-time PCR, we studied the effect of T_3 on gene expression in primary cultures of skin fibroblasts obtained from two normal subjects (2). To ensure that the observed effects were TRβ specific, we examined in parallel the effect of T_3 on fibroblasts from two patients with resistance to thyroid hormones (RTH). Among the 91 genes up-regulated by T_3 in the normal fibroblasts was the hypoxia-inducible factor (HIF)-1α (2). Although not studied in detail initially, it showed no change with T_3 treatment in the fibroblasts from the two patients with RTH, thus demonstrating TRβ-dependent induction.

HIF-1α is a key mediator of angiogenesis and metabolic adaptation to hypoxia in tumors and is responsible for increased expression of glycolytic enzymes and glucose transporters. The finding of increased HIF-1α mRNA in response to 2 nM T_3 in normoxic, normal cells (see also *Results*) suggested that HIF-1α induction by TH has a physiological role. Furthermore, we found four of its known target genes to be induced by TH, namely the glucose transporter (GLUT)1 and the glycolytic enzymes enolase 1α, triosephosphate isomerase, and phosphoglycerate kinase 1 (2). Two other genes found to be TH responsive seem to complete the picture. These were the platelet-type phosphofructokinase (PFKP), a key enzyme of glycolysis and possibly also an HIF-1α target gene, and monocarboxylate transporter (MCT)4 [formerly MCT3, also known as solute carrier family 16 member 3 (SLC16A3)], a widely expressed lactate transporter (3,

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Abbreviations: BTEB, Basic transcription element-binding protein; CHX, cycloheximide; DHT, dihydrotestosterone; EGF, epidermal growth factor; GLUT, glucose transporter; HIF, hypoxia-inducible factor; MCT, monocarboxylate transporter; MEK, MAPK/ERK-kinase; mTOR, mammalian target of rapamycin; n.s., nonsignificant; PI3K, phosphatidylinositol-OH-3-kinase; PFK, phosphofructokinase; PFKP, platelet-type phosphofructokinase; RTH, resistance to thyroid hormone; TH, thyroid hormone; TR, TH receptor; TRE, TH response element.

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4). The group of genes presented above is of special interest because their products have important roles in cellular glucose metabolism, including glucose uptake (GLUT1), glycolysis (PFKP), and lactate export (MCT4). The effect of TH seemed to be mediated through HIF-1α, because at least GLUT1 and the glycolytic genes are HIF-1α target genes.

HIF-1α is a component of the basic helix-loop-helix transcription factor HIF-1, which consists of two subunits, α and β. In human cells, including skin fibroblasts, two HIF-1α isoforms are generated by alternative splicing. Both isoforms dimerize with the β-subunit, are similarly regulated, and have biological activity (5). Whereas HIF-1β is constitutively expressed, the HIF-1α subunit is tightly regulated by cellular oxygen levels (6, 7) and, especially in normoxic conditions, by growth factors and hormones such as epidermal growth factor (EGF), IGF-1, insulin, androgens, and nitric oxide (8, 9). Normoxic up-regulation of HIF-1α seems to be controlled by signal transduction pathways, especially the phosphatidylinositol-3-OH-kinase (PI3K) and the MAPK pathways (9, 10).

T_3 is best characterized as a ligand of the TRs, which act as nuclear ligand-dependent transcription factors. However, it has been demonstrated recently by us and by others that, in addition to this nuclear action, T_3 can activate both the PI3K (11, 12), and the MAPK pathway (13, 14). Because HIF-1α expression and activity are regulated through the PI3K and MAPK pathways, and TH has been now found to activate these pathways, it is tempting to speculate that the TH-induced increase in HIF-1α mRNA levels is a direct consequence of PI3K and/or MAPK activation in the cytosol.

Another possible mechanism for HIF-1α induction by TH, which needs to be considered, is the induction of unknown gene(s) through classical stimulation of nuclear transcription. Such gene(s) would contain TH response elements (TREs), and their product(s) would, in turn, activate PI3K or MAPK. HIF-1α induction will then be secondary to nuclear action of TH and require prior protein synthesis. Such a mechanism has been suggested for HIF-1α protein induction by another hormone, dihydrotestosterone (DHT) (15). DHT induces EGF (16), and the secreted EGF is thought to stimulate the EGF receptor in an autocrine loop, leading to PI3K activation and HIF-1α protein increase, even though no HIF-1α mRNA increase had been detected by Northern blot.

The aim of this study was to explore how TH leads to induction of HIF-1α and the genes involved in glucose uptake (GLUT1), glycolysis (PFKP), and lactate export (MCT4). We wanted to know whether HIF-1α induction is an indirect consequence of nuclear action of TH on a TRE, similar to what had been proposed for DHT, or is the consequence of a nontranslational, TH-mediated activation of cellular signaling pathways.

RESULTS

Stimulation of HIF-1α mRNA by T_3

The effect of T_3 on HIF-1α mRNA in cultured human skin fibroblasts, measured by real-time PCR, is shown in Fig. 1. An increase in HIF-1α mRNA was observed 3 and 24 h after addition of 2 nM T_3 in fibroblasts obtained from normal subjects (1 and 2) but not in those from the patients with RTH. These results indicate that this T_3 action is TRβ dependent and are consistent with results obtained using another mutant TRβ, G345R (12).

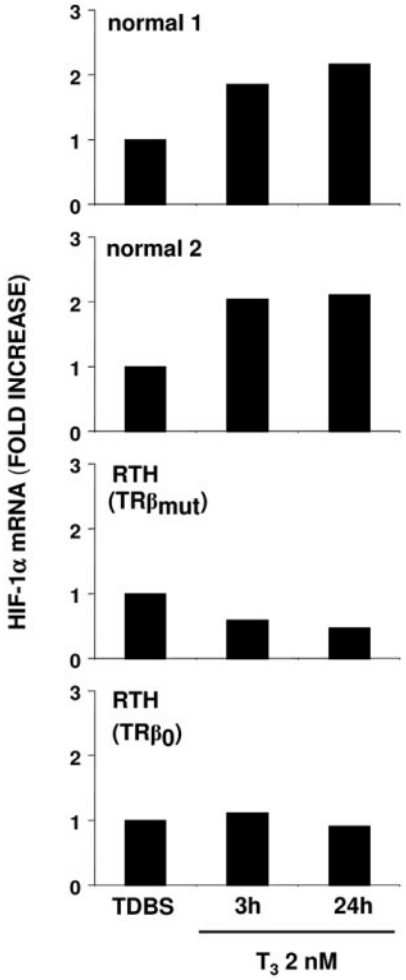

Fig. 1. Effect of T_3 on HIF-1α mRNA Expression in Human Skin Fibroblasts

After culture for 48 h in DMEM supplemented with 10% TH-depleted bovine serum (TDBS), fibroblasts were treated with 2 nM T_3 for 3 and 24 h. The relative amount of mRNA, as measured by real-time PCR and compared with that in fibroblasts cultured in the absence of T_3, is given as fold increase on the *ordinates*. Responses were observed in fibroblasts from two normal individuals (*top panels*) but not in two individuals with RTH due to TRβ gene mutation A317T (heterozygous, $TR\beta_{mut}$) and complete deletion (homozygous, $TR\beta_0$) (*bottom panels*).

T_3-Dependent Induction of HIF-1α mRNA and Protein Is LY294002 Sensitive

To test whether the PI3K pathway is involved in mediating the T_3 effect on HIF-1α expression, we treated normal fibroblasts (subject 2) with T_3 in the absence and presence of the PI3K inhibitor LY294002. To examine the influence of the MAPK pathway, we also measured the effect of T_3 in the presence of the MAPK/ERK-kinase (MEK) 1 and 2 inhibitor PD98059. HIF-1α mRNA levels, measured by real-time PCR and expressed as fold-change relative to those in cells grown in T_3-depleted serum, are shown in Fig. 2A. Consistent with the previous results shown in Fig. 1, T_3 treatment increased HIF-1α mRNA levels (2.7 ± 0.4; $P < 0.05$). This effect of T_3 was completely abrogated by LY294002 [1.1. ± 0.1; $P = 0.57$ (n.s.)], indicating that the stimulatory effect of TH on HIF-1α mRNA involves PI3K. In the presence of PD98059, HIF-1α mRNA increase was preserved with a 2.2-fold increase

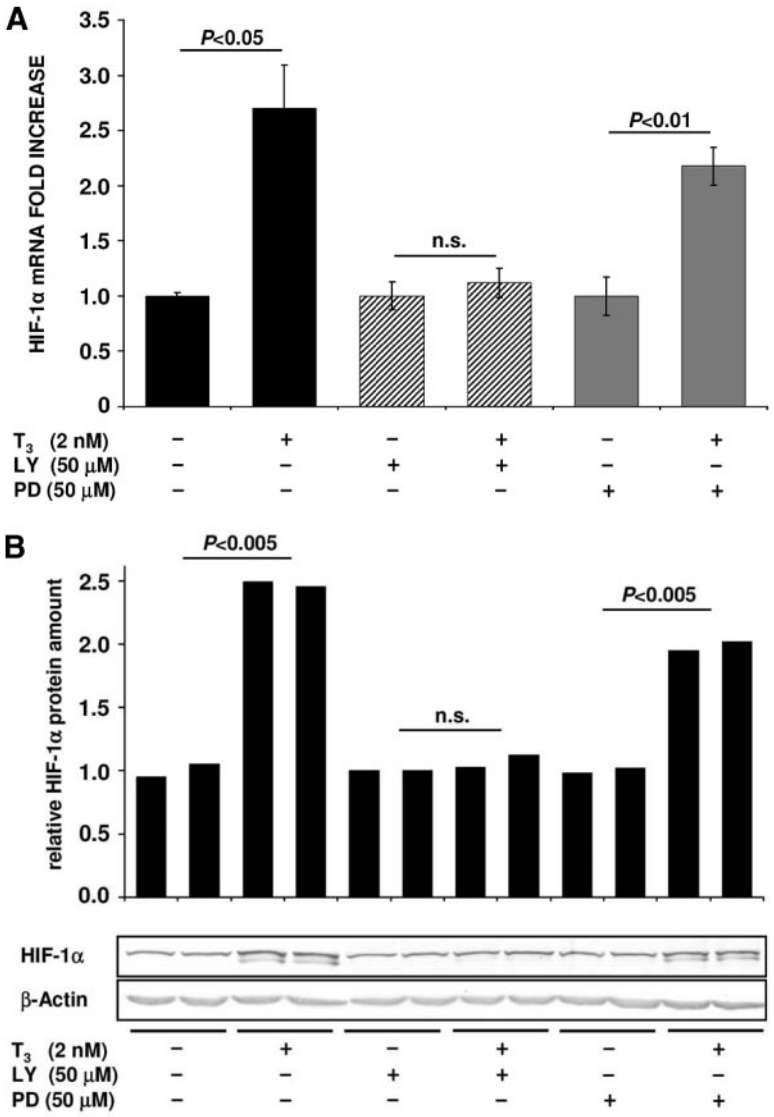

Fig. 2. Effect of T_3, the PI3K Inhibitor LY294002, and the MAPK Inhibitor PD95058 on HIF-1α mRNA and Protein Levels

Fibroblasts (normal subject 2) were cultured for 2 d in DMEM supplemented with 10% TH-depleted bovine serum. They were then treated with 2 nM T_3 in the absence or presence of either LY294002 (LY, 50 μM) or PD98059 (PD, 50 μM), added 1 h before the addition of T_3. Cells were harvested 24 h later for RNA extraction or whole-cell lysate preparation. A, The relative amount of HIF-1α mRNA, as measured by real-time PCR and compared with that in fibroblasts cultured in the TH-depleted medium, is plotted in the *ordinates* as fold increase. Data are expressed as mean ± SE (n = 3 for each treatment). B, Whole-cell lysates, obtained from two separately treated culture dishes for each treatment, were subjected to Western blotting with anti-HIF-1α and anti-β-actin. The results of quantification, measured by scanning and expressed relative to that in fibroblasts cultured in TH-depleted medium, are shown *above* the blot. They include both HIF-1α bands, the *lower band* seen most clearly after T_3 stimulation.

(±0.2; $P < 0.01$), the same as without PD98059 ($P = 0.29$). Activation of the MAPK pathway therefore seems not to be involved in T_3-dependent HIF-1α mRNA increase.

To examine whether the T_3 effects on mRNA expression are also present at the protein level, we determined the presence of the HIF-1α protein by Western blot. In accordance with the real-time PCR results, T_3 produces also an increase in both variants of HIF-1α protein (Fig. 2B). Stimulation was on the average 2.5-fold for both bands (larger variant alone: 1.8-fold). Moreover, the response was completely abrogated to 1.1-fold (both variants) by pretreatment with the PI3K inhibitor LY294002, but not by pretreatment with PD98059 (2.0-fold and 1.6-fold). Loading was controlled by probing the same membrane for β-actin, and no differences were observed among the samples tested. Inhibition of the T_3-induced increase in HIF-1α mRNA and protein expression by LY294002 indicates a T_3 effect on HIF-1α through the activation of the PI3K pathway. These results were reproduced with fibroblasts from normal subjects 1 and 3.

T_3-Dependent Induction of GLUT1, PFKP, and MCT4, But Not Basic Transcription Element Binding Protein (BTEB)1 mRNA Is LY294002 Sensitive

Blocking the PI3K pathway with LY294002 also inhibited the T_3 effect on GLUT1, PFKP, and MCT4 expression, whereas T_3 effect on these genes was preserved when MAPK pathway was inhibited with PD98059 (Fig. 3). T_3 produced an increase in the GLUT1 mRNA level (3.9 ± 0.2; $P < 0.001$). This effect was abrogated by LY294002 (1.0 ± 0.2; $P = 0.98$ n.s.) but not by PD98059 (2.1 ± 0.2; $P < 0.01$). The T_3-induced increase in PFKP mRNA (2.0 ± 0.1; $P < 0.001$) was inhibited by LY294002 (1.2 ± 0.05; $P = 0.13$ n.s.), but not PD98059 (1.8 ± 0.2; $P < 0.05$). Similarly, the T_3-induced increase (2.3 ± 0.4; $P < 0.05$) of MCT4 mRNA was inhibited by LY294002 (0.9 ± 0.1; $P = 0.45$ n.s.), whereas the increase was preserved in the presence of PD98059, although not statistically significant (1.8 ± 0.5; $P = 0.17$ n.s).

Previously, we found BTEB1 to be TH responsive in a TRβ-dependent manner in human fibroblasts (2). BTEB1 has a well-studied TRE and is directly induced by nuclear action of TH in tadpoles (17). BTEB1 induction is therefore independent of PI3K and MAPK and could serve as a control. BTEB1 induction by T_3 was almost 4-fold (3.9 ± 0.6; $P < 0.01$). As expected, this increase was not inhibited by pretreatment with either LY294002 (3.1 ± 0.2; $P < 0.005$) or PD98059 (3.1 ± 0.3; $P < 0.005$) (Fig. 3).

The observations regarding PFKP mRNA were confirmed at the protein level and showed the same pattern of change: 3.7-fold increase after T_3 treatment, which was reduced by LY294002 pretreatment (1.3-fold), but preserved at 2.4-fold after pretreatment with PD98059 (Fig. 4).

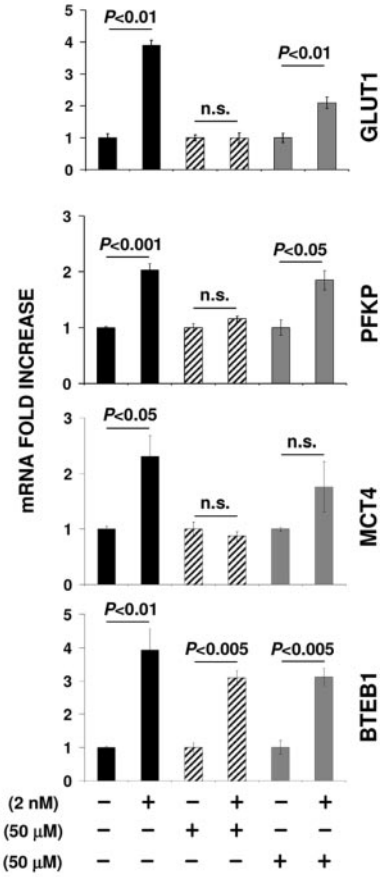

Fig. 3. Effect of T_3, the PI3K Inhibitor LY294002, and the MAPK Inhibitor PD95058 on GLUT1, PFKP, MCT4, and BTEB1 mRNA Levels

Fibroblasts (normal subject 2) were cultured in DMEM supplemented with 10% TH-depleted bovine serum for 48 h. They were then treated for 24 h with 2 nM T_3 alone, or together with either LY294002 (LY, 50 μM) or PD98059 (PD, 50 μM) 1 h before the addition of T_3. Cells were harvested 24 h after T_3 treatment for RNA extraction. The relative amount of mRNAs, as measured by real-time PCR and compared with that in fibroblasts cultured in TH-depleted medium, is plotted on the ordinates as fold increase. Data are expressed as mean ± SE (n = 3 for each treatment).

T_3-Induced HIF-1α mRNA Increase Is Translation Independent

Next, we examined whether the T_3/TRβ-dependent induction of HIF-1α mRNA is mediated by a direct activation of the PI3K pathway. An indirect effect would involve expression of a target gene, transcribed and translated after nuclear action of T_3/TRβ on a TRE.

Fibroblasts from a normal individual (subject 3) were cultured for 48 h in T_3-depleted medium followed by the addition of 2 nM T_3 for 24 h in the absence or presence of the inhibitor of protein synthesis, cycloheximide (CHX, 10 μg/ml). A significant increase in HIF-1α mRNA, measured by real-time PCR, was again observed ($P < 0.001$), which was not abolished by

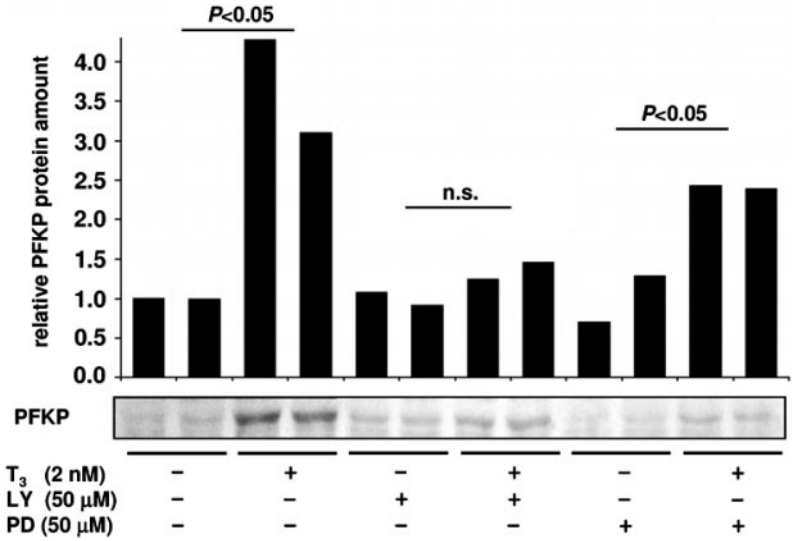

Fig. 4. Effect of T_3, the PI3K Inhibitor LY294002, and the MAPK Inhibitor PD95058 on PFKP Protein Levels

Fibroblasts (normal subject 2) were cultured for 2 d in DMEM supplemented with 10% TH-depleted bovine serum. They were then treated with 2 nM T_3 in the absence or presence of either LY294002 (LY, 50 μM) or PD98059 (PD, 50 μM), added 1 h before the addition of T_3. Cells were harvested 24 h after T_3 treatment. Whole-cell lysates, obtained from two separately treated culture dishes for each treatment, were subjected to Western blotting with anti-PFKP. The results of quantification, measured by scanning and expressed relative to that in fibroblasts cultured in TH-depleted medium, are shown *above* the blot.

CHX treatment (Fig. 5A). BTEB1 also showed the expected T_3-induced and translation-independent increase in mRNA (Fig. 5A). We conclude that T_3-induced HIF-1α mRNA expression is direct because it does not require protein synthesis.

CHX Abrogates the mRNA Expression of GLUT1, PFKP, and MCT4

In the experiment described in the preceding paragraph, we also examined the effect of CHX on the following mRNAs, all shown to be induced by T_3: GLUT1, PFKP, and MCT4. Because GLUT1 was known to be a target gene of HIF-1α, it was not surprising that its induction by T_3 was abrogated by pretreatment with CHX (Fig. 5B). This also held true for the induction of PFKP and MCT4 (Fig. 5B).

Complete abrogation of the T_3-induced mRNA increase by CHX establishes GLUT1, PFKP, and MCT4 as genes indirectly induced by TH with the requirement of prior protein synthesis.

DISCUSSION

Until recently, TH-mediated changes in gene expression were thought to be primarily, if not solely, initiated by the nuclear TR binding to a TRE in the promoter of a direct target gene. Work from only a few laboratories has suggested that TH may also exert a nongenomic mechanism of action (12–14, 18–20).

Using the same human fibroblast primary cultures, we recently demonstrated a new mechanism of TH action that takes place in the cytosol and involves the association of the TRβ with the p85α subunit of PI3K (12). This leads to the phosphorylation and activation of Akt/protein kinase B, phosphorylation of mTOR (mammalian target of rapamycin) and its substrate $p70^{S6K}$, and ultimately induction of ZAKI 4α mRNA and protein. TRβ binding to PI3K was ligand independent, but activation of PI3K required the presence of T_3. Activation of PI3K was not affected by CHX pretreatment, but ZAKI 4α induction was sensitive to CHX, showing that it is an indirectly induced gene, depending on *de novo* synthesis of a yet unknown protein. The physiological consequence of ZAKI 4α stimulation by TH is still unclear (21). The present study demonstrates that this new mechanism of TH-mediated gene induction applies to more genes and can have direct and indirect effects on gene expression and possibly provides a link between TH and glucose metabolism at the cellular level.

HIF-1α mRNA was increased by TH. This effect was observed in fibroblasts from three normal subjects, but not in those from patients with RTH caused by either a dominant-negative TRβ mutation or TRβ gene deletion, demonstrating the necessary involvement of the TRβ. Inhibition of PI3K by LY294002 completely blocked the T_3 effect on HIF-1α mRNA and protein, indicating that activation of the PI3K pathway is required for the TH effect on HIF-1α expression.

Theoretically, PI3K could be activated by the product of other TH-responsive genes, and HIF-1α induction could be secondary to the classical nuclear action

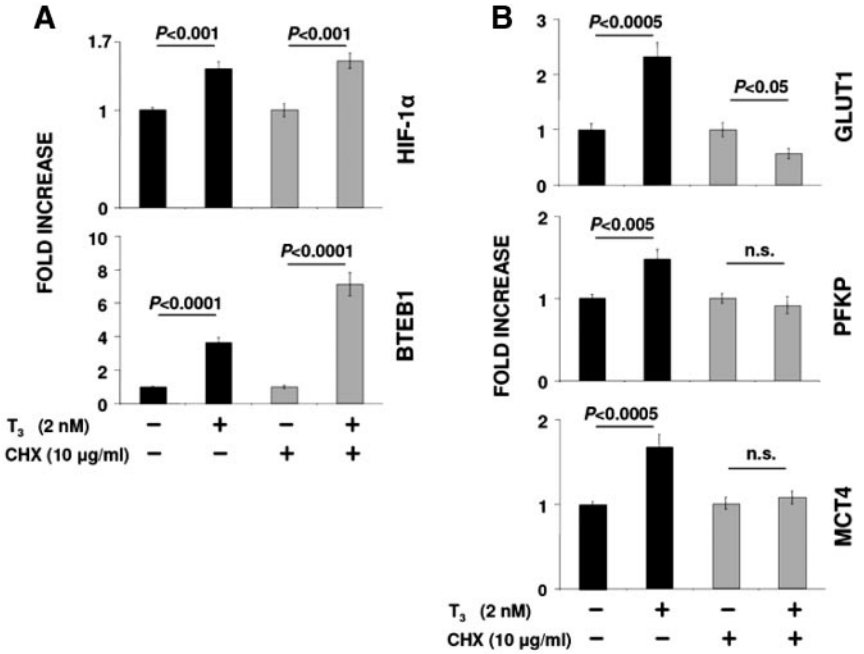

Fig. 5. The Effect of T_3 on HIF-1α, BTEB1, GLUT1, PFKP, and MCT4 mRNA Levels in the Absence and Presence of the Protein Synthesis Inhibitor, CHX

Human skin fibroblasts (normal subject 3) were cultured in DMEM with 10% TH-depleted bovine serum for 48 h. They were then treated with 2 nM T_3 without or with pretreatment with CHX (10 μg/ml) for 1 h. Cells were harvested 24 h after T_3 treatment. The relative amount of mRNAs, as measured by real-time PCR and compared with that in fibroblasts cultured in the TH-depleted medium, is plotted on the *ordinates* as fold increase. Data are expressed as mean ± SE (n = 8 for each treatment) A, T_3-mediated mRNA induction of HIF-1α and BTEB1 is preserved in the presence of CHX. B, T_3-mediated mRNA induction of GLUT1, PFKP, and MCT4 is completely abrogated by pretreatment with CHX.

of TH. If this were the case, induction of HIF-1α mRNA should be blocked by the inhibition of new protein synthesis. However, this did not occur with CHX treatment. Taken together, these results suggest that HIF-1α is induced directly by TH, an effect mediated by PI3K.

HIF-1α induction via the PI3K pathway in normoxia or in response to TH is mostly thought to result from an increase in the rate at which HIF-1α mRNA is translated into protein (22, 23). We previously found an increase in HIF-1α mRNA in response to TH by both microarrays and real-time PCR in primary cultures of fibroblasts from two normal individuals (2), which could be demonstrated again in this study using the same fibroblasts and those from a third normal subject. TH, therefore, seems to lead to increased HIF-1α protein synthesis by increased transcription rather than posttranscriptional mechanisms.

Stimulation of the other TH responsive genes, namely GLUT1, PFKP, and MCT4, was also inhibited by blocking PI3K, indicating their dependence on the PI3K pathway. However, contrary to HIF-1α, their mRNA induction is sensitive to CHX. They are, therefore, genes that are indirectly responsive to TH and require the prior expression of other proteins that can act as transcription factors. One such transcription factor is HIF-1, of which HIF-1α is the regulated subunit. Thus it is logical to postulate that GLUT1, a known HIF-1 target gene (24, 25), is stimulated by T_3 via HIF-1.

Functional phosphofructokinase (PFK) is a tetramer formed by association of the products of three separate gene loci, which encode muscle-type, liver-type, and platelet-type subunits. PFKP expression has been found in human fibroblasts (26) and is sometimes referred to as fibroblast-type PFK. The phosphofructokinase L gene contains an HIF-1 binding site that mediates hypoxia-induced transcription in HepG2 cells (27), and muscle-type PFK was augmented by hypoxic stimulus in human muscle (28). Thus it is possible that the T_3 effect on PFKP also involves HIF-1.

In addition, a search with the Conserved Transcription Factor Binding Site Finder (CONFAC, http://morenolab.whitehead.emory.edu/cgi-bin/confac/login.pl) (29) reports a possible HIF-1 binding site (5′-TACGTG-3′) in the promoter of both the human PFKP gene and its mouse ortholog, as it did also for the known HIF-1 target genes GLUT1, triosephosphate isomerase, phosphoglycerate kinase 1, and enolase 1α, but not for the lactate exporter MCT4. MCT4, for example, could be induced by increased accumulation of lactate as a consequence of increased glycolysis. Figure 6 provides a pictorial overview of TH-mediated direct (HIF-1α) and indirect (GLUT1, PFKP, MCT4) gene expression through the T_3-mediated activation of the PI3K pathway. It is also possible that other, as of yet

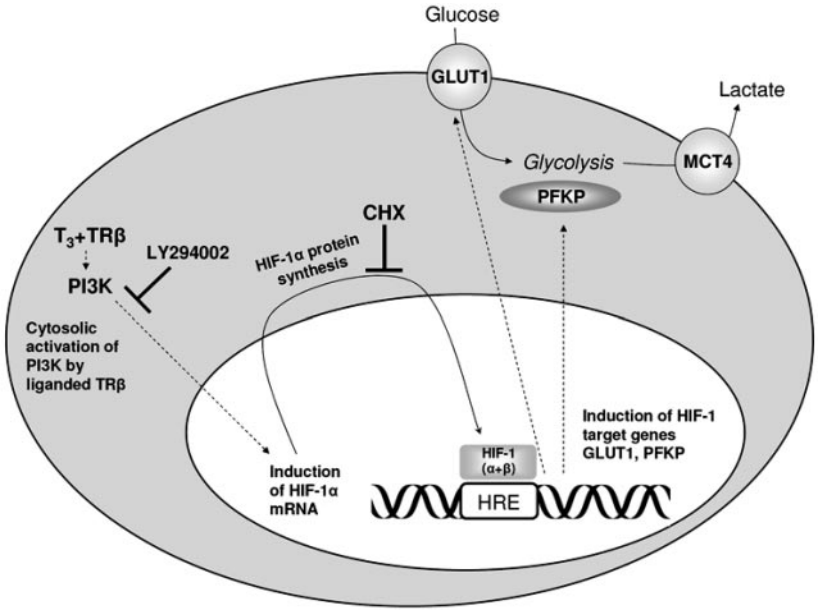

Fig. 6. Induction of HIF-1α, GLUT1, and PFKP by TH

Activation of the PI3K in the cytosol by the liganded TRβ leads to direct, translation-independent increase of HIF-1α mRNA. This can be prevented by the PI3K inhibitor LY294002. After translation, HIF-1α (together with the constitutively expressed HIF-1β comprising the transcription factor HIF-1) seems to induce GLUT1 and PFKP by binding to hypoxia-response elements (HREs) in their promoters. These are indirectly TH-responsive genes, because their mRNA induction is sensitive to the translation inhibitor CHX. The roles and interactions of the glucose transporter GLUT1, the glycolysis enzyme PFKP, and the lactate transporter MCT4 are shown. Note that for simplicity the TH transmembrane transport and the generation of T_3 by iodothyronine deiodination are not shown.

unknown, T_3 responsive transcription factors are involved in induction of these genes.

The PI3K pathway is not the only cellular signaling pathway activated by TH or regulating HIF-1α. The MAPK pathway can also stimulate HIF-1α synthesis (9, 10, 22). Davis and colleagues (13, 30) have provided ample evidence for activation of the MAPK pathway by TH, which was also observed in the human fibroblasts used in the current study (Davis, Faith B., personal communication). To examine whether this mechanism played a role in the mediation of TH effect on the expression of these genes, we also studied HIF-1α mRNA and protein and GLUT1, PFKP, and MCT4 mRNAs in the presence of the MAPK pathway inhibitor PD98059, but could not detect inhibition of the T_3 effect. The MAPK pathway, therefore, seems not to be involved in the expression of these genes in human skin fibroblasts.

Early studies have demonstrated a stimulating effect of T_3 on glucose consumption in cultured cells. In GH_1 cells, glucose utilization rates increased with increasing hormone concentrations (31). This effect could not be explained solely by the rate of cell growth. A similar effect was observed in cultured human skin fibroblasts (32). The initially cytosolic action of TH leading to induction of HIF-1α and, subsequently, induction of GLUT1, PFKP, and MCT4 could be a link between TH and glucose metabolism in human fibroblasts.

MATERIALS AND METHODS

Antibodies and Reagents

The monoclonal mouse HIF-1α antibody was purchased from BD Transduction Laboratories (Lexington, KY) and the polyclonal rabbit PFKP antibody was obtained from Abgent (San Diego, CA). The polyclonal rabbit β-actin antibody and the horseradish peroxidase-linked antirabbit and antimouse antibodies were purchased from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) reagents were obtained from PerkinElmer (Boston, MA). The PI3K inhibitor LY294002 and the MEK-1/MEK-2 inhibitor PD98059, both purchased from Cell Signaling Technology, were diluted to 50 mM and stored at −20 C. CHX (Sigma Chemical Co., St. Louis, MO) was diluted to 10 mg/ml and stored at −20 C.

Cell Culture and Treatments

Human skin was obtained by punch biopsy from three normal individuals (normal 1, 2, and 3) and two patients with RTH due to TRβ gene mutations, one heterozygous for a dominant-negative TRβ mutation A317T ($TR\beta_{mut}$) and the other homozygous for TRβ gene deletion ($TR\beta_0$). The study was approved by the Institutional Review Board of the University of Chicago. Primary cultures of fibroblasts were grown in DMEM (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum as previously described in detail (33). At confluency, the medium was replaced with one containing TH-depleted bovine serum, produced by treatment of fetal bovine serum with anion exchange resin (34). T_3 was added 48 h later to a final concentration of 2 nM. The inhibitors LY294002 (50 μM), PD98059 (50 μM), and CHX (10 μg/ml) were added to the medium 1 h before T_3 treatment.

The experiments were terminated 24 h later by either RNA extraction for real-time PCR or cell lysis for Western blotting. All experiments were carried out at early cell passages (<10).

Isolation and Reverse Transcription of RNA

The medium was removed and the dish was washed twice with Hank's buffered saline solution (Invitrogen, Carlsbad, CA). Total RNA was extracted using phenol/guanidine isothiocyanate (TRIZOL, Invitrogen). RNA was reverse transcribed with the Superscript III RNase H Reverse Transcriptase Kit (Invitrogen) using 2 μg of total RNA and 100 ng of random hexamers.

Real-Time PCR

Quantification of mRNAs was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), using SYBR Green I as detector dye. The reaction mixtures contained 25 μl iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA), 0.3 μmol/liter of each primer, 10 ng template cDNA, and nuclease-free water to a final volume of 50 μl. The oligonucleotide primers were designed to cross introns, and their sequences are available upon request. The reaction conditions were 95 C for 2 min followed by 40 cycles of 95 C for 15 sec and 60 C for 45 sec. Expression of genes was calculated relative to that in untreated cells and normalized for 18S rRNA, measured under the same conditions with TaqMan Ribosomal RNA Control Reagent (Applied Biosystems/Roche, Branchburg, NJ) primers, using the $2^{-\Delta\Delta CT}$ method (35).

Western Blot Analysis

Human skin fibroblasts were washed with cold Hank's buffered saline solution and lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton, 5 mM EGTA, and 1.5 mM $MgCl_2$. Cellular debris were removed by centrifugation at 14,000 rpm for 15 min at 4 C. Protein concentration was determined by Bradford's method using BSA as standard. Samples containing 50 μg protein were applied to 7.5% SDS-PAGE. The separated proteins were then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences Corp., Piscataway, NJ). Membranes were blocked overnight with 5% skim milk in PBS with 0.25% Tween 20 (Bio-Rad) and probed with anti-HIF-1 α or anti-PFKP diluted to 1:250. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked second antibody and treatment with ECL reagents. To control for loading, the membrane was stripped with Western blot recycling kit (Alpha Diagnostic International, San Antonio, TX), probed with anti-β-actin at 1:1000 dilution and developed again. Quantification of the scanned bands was performed on a PC using the public domain ImageJ program (available at Research Services Branch, National Institutes of Health at http://rsb.info.nih.gov) and expressed as fold change relative to that in cells grown in TH-depleted medium.

Data Analysis

Real-time PCR results are expressed as mean ± SE, and statistical analysis was done by ANOVA.

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