Hepatic regulation of fatty acid synthase by insulin and T3: evidence for T3 genomic and nongenomic actions

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Radenne A, Akpa M, Martel C, Sawadogo S, Mauvoisin D, Mounier C. Hepatic regulation of fatty acid synthase by insulin and T3: evidence for T3 genomic and nongenomic actions. Am J Physiol Endocrinol Metab 295: E884–E894, 2008. First published August 5, 2008; doi:10.1152/ajpendo.90438.2008.—Fatty acid synthase (FAS) is a key enzyme of hepatic lipogenesis responsible for the synthesis of long-chain saturated fatty acids. This enzyme is mainly regulated at the transcriptional level by nutrients and hormones. In particular, glucose, insulin, and T3 increase FAS activity, whereas glucagon and saturated and polyunsaturated fatty acids decrease it. In the present study we show that, in liver, T3 and insulin were able to activate FAS enzymatic activity, mRNA expression, and gene transcription. We localized the T3 response element (TRE) that mediates the T3 genomic effect, on the FAS promoter between −741 and −696 bp that mediates the T3 genomic effect. We show that both T3 and insulin regulate FAS transcription via this sequence. The TRE binds a TR/RXR heterodimer even in the absence of hormone, and this binding is increased in response to T3 and/or insulin treatment. The use of H7, a serine/threonine kinase inhibitor, reveals that a phosphorylation mechanism is implicated in the transcriptional regulation of FAS in response to both hormones. Specifically, we show that T3 is able to modulate FAS transcription via a nongenomic action targeting the TRE through the activation of a PI 3-kinase/ERK1/2-MAPK-dependent pathway. Insulin also targets the TRE sequence, probably via the activation of two parallel pathways: Ras/ERK1/2 MAPK and PI 3-kinase/Akt. Finally, our data suggest that the nongenomic actions of T3 and insulin are probably common to several TREs, as we observed similar effects on a classical DR4 consensus sequence.

fatty acid synthase; triiodothyronine; insulin; triiodothyronine response element; phosphoinositide 3-kinase; extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase

LIPOGENESIS CONVERTS DIETARY CARBOHYDRATES to fatty acids primarily in liver (28). Insulin and triiodothyronine (T3) are involved in mediating the effects of diet on lipogenesis in vivo (34). Hepatic lipogenesis is increased in hyperthyroid states or in response to T3 injection (10, 15, 19, 24, 25, 28, 62, 71, 76, 74) and as well as in hyperinsulinemic subjects (80). In vivo, these two hormones are also involved in the long-term regulation of lipogenic enzymes activities such as fatty acid synthase (37).

Fatty acid synthase (FAS; EC.2.3.1.85) is a key enzyme in hepatic lipogenesis. In the presence of NADPH, this multifunctional enzyme catalyzes the conversion of acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids such as palmitate and stearate (92). The de novo synthesis of fatty acids in human and chicken takes place mainly in the liver (30, 58), whereas in rodents the adipose tissue is also lipogenic (30). In vertebrates, FAS is a homodimer made of two identical peptide chains of ~260 kDa (85, 91), located in the cytoplasm of the cell (31). FAS is encoded by a unique gene that generates only one mRNA in mouse (73) and two in chicken and rat, as a result of alternative splicing (3). In the liver, the activity of FAS, like most lipogenic enzymes (95), is regulated through nutrients and hormones. Starvation causes a decrease in the activity of the enzyme, and refueling restores it (3, 67).

A similar effect of refeeding is also observed on the mRNA expression level and stability, as well as on the transcription (3, 41, 48, 52, 66). It was also shown that insulin (74) and T3 (84, 95) increase the FAS mRNA expression level, whereas glucagon (50, 74, 82), medium-chain fatty acids (MCFA) (77), and polyunsaturated fatty acids (PUFA) decrease it (9, 17, 43, 65).

Insulin increases FAS transcription by modifying the binding of various transcription factors on the insulin response element (IRE) located on the promoter (74). The effect of insulin is mediated by the activation of the phosphoinositide (PI) 3-kinase/Akt pathway (93). The first IRE characterized is an E-box, which binds the ubiquitous transcription factors USF1 and USF2 (upstream stimulatory factors), located at −65 bp on the FAS promoter (69, 94). Insulin also increases transcription of FAS by inducing the binding of steroid regulatory element (SRE)-binding protein (SREBP)-1c to two different SREs, one located around −150 bp (51) and the other at −65 bp (51). Finally, insulin also regulates FAS transcription by increasing the binding of NF-Y and Sp1 to the −103-bp to −53-bp region (64).

Various studies from A. G. Goodridge’s laboratory (84, 95) showed that T3 is able to potentiate the effect of insulin on FAS transcription through an unknown mechanism. T3 is known to regulate gene transcription via the binding of hormone/receptor complexes to T3 response elements (TREs) located on the promoter of a variety of genes (96). The TREs are located, for the most part, upstream of the minimal promoter but can sometimes be located on the 3′ end of the coding sequence (8). A consensual hexameric sequence, (GA)GGTG(C/G)A, was defined. This sequence can be a palindromic (TREpal), a direct repeat (DR), or an inverted palindromic (IP) (32). These TREs bind the T3 receptors (TRs) belonging to the nuclear receptor superfamily (53). The latter can form homodimers or interact with other nuclear receptors, such as RXR (retinoid X receptor), to form heterodimers (11, 27). The heterodimers bind preferentially to DRs separated by four nucleotides (DR4) (75). This increases the transcriptional activity in response to T3 in...
HepG2 cells were purchased from ATCC (Manassas, VA). Minimum were purchased from Covigil (Sintim, Mirabel, QC, Canada). PerkinElmer (Wellesley, MA). Eggs from white Leghorn chickens Calbiochem (EMD Biosciences, San Diego, CA). 294002, PD-98059, and U-0126 inhibitors were purchased from nine, insulin, H7, and genistein were obtained from Sigma. LY-
showed that, in human fibroblasts, T3 was able to activate the membrane-bound receptors (78).

It has also been shown that SRC1, a TR coactivator, can be phosphorylated by MAP kinase through the activation of (89). It has also been shown that SRC1, a TR coactivator, can be phosphorylated in the cytosol (33) by caseine kinase II but targets of these phosphorylation events. In particular, TR can be phosphorylated by activating the transcription of target genes (42, 61, 88). The
be phosphorylated by MAP kinase through the activation of

MATERIALS AND METHODS

Materials. The restriction enzymes, the thyroxine (T4) DNA ligase, and the T4 polynucleotide kinase were obtained from New England Biolabs (Pickering, CA). The Tag polymerase was acquired from PerkinElmer (Wellesley, MA). Eggs from white Leghorn chickens were purchased from Couvoir Simentin (Mirabel, QC, Canada). HepG2 cells were purchased from ATCC (Manassas, VA). Minimum Essential Medium (MEM), Waymouth medium, 3.5,3,-triiodothyronine, insulin, H7, and genistein were obtained from Sigma. LY-294002, PD-98059, and U-0126 inhibitors were purchased from Calbiochem (EMD Biosciences, San Diego, CA). γ-32P]ATP was purchased from PerkinElmer. Eugene HD transfecting agent, CAT-ELISA kit, collagenase H, and Klenow enzyme were obtained from Roche Diagnostic (Laval, QC, Canada). Fetal bovine serum was purchased from Cansera (Etobicoke, ON, Canada). Antibodies (Akt, anti-phospho-Akt (Ser273), p42/44 MAPK, anti-phospho-p42/44 MAPK (Thr202/204)) were acquired from Cell Signaling Technology (Danvers, MA). Anti-TRα1/Trβ1 and anti-RXRα/B/γ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise stated in the text, all other chemicals were purchased from Sigma.

Plasmid constructs. The goose FAS promotor was graciously provided by Dr. A. G. Goodridge (44). This cosmid contained 46 kb of the FAS gene, including 12 kb downstream of the transcription initiation site. The first 1.6 kb of the FAS promotor were cloned into the pJFCAT1 vector, which incorporates the chloramphenicol acetyl transferase (29) reporter gene. The −902-bp to −577-bp fragment, containing the FAS TRE, was PCR amplified using specific primers containing HindIII and BamHI restriction sites at their extremities. The amplified fragment was subsequently inserted into the pBLCAT2 vector upstream of the thymidine kinase (88) minimal promotor and the CAT reporter gene. The synthetic sequence of the classical TRE DR3 (AGCTAGCTCAGGTCAAGAGTCCAGGAG) was cloned into the pBLCAT2 vector using the HindIII and SalI restriction sites.

Cell culture and transfection. Chick embryo hepatocytes (CEH) were isolated from livers of 19-day-old chick embryos (35) (protocol no. 590 approved by the University Animal Care Committee). Cells (2.5 × 10^5) were plated in 35-mm tissue dishes and cultured at 40°C under 5% CO2 in Waymouth medium supplemented with streptomycin (100 μg/ml) and penicillin (60 μg/ml). After 24 h, the medium was removed by aspiration and replaced by medium supplemented with T3 and/or insulin and incubated for different periods of time, as indicated in the figure legends. For the experiment using kinase inhibitors, the CEH were incubated with the hormones after a 30-min preincubation period with the various inhibitors (0.5% DMSO, 5 μM genistein, 25 μM H7, 50 μM PD-98059, 50 μM LY-294002, and 20 μM U-0126). The human hepatocarcinoma cells (HepG2) were cultured in MEM supplemented with streptomycin (100 μg/ml), penicillin (60 μg/ml), FBS (10%), and glutamine (4 mM final). The day before transfection, the cells were plated at 80% confluence (~6 × 10^5 cells per well). The cells were then incubated for 24 h with 7 μl of Eugene HD, 1.5 μg of the different DNA constructs tested, and 0.5 μg of pRSV-β-galactosidase in the absence of serum and antibiotics. The medium was then replaced with one containing antibiotics and serum, and hormones were added as indicated in the figure legends. After 24 h of culture, the cells were harvested and the different cellular extracts prepared.

FAS activity. FAS activity was measured by tracking the decrease of absorbance at 340 nm, which is the result of NADPH disappearance due to the conversion of malonyl-CoA and acetyl-CoA into long-chain fatty acids (36). Following hormonal stimulation, two to three plates of treated cells (~10 × 10^6 cells) were harvested in 1× PBS. After a short centrifugation, the cells were resuspended in cold homogenization buffer (0.1 M KPi, pH 7, 3 mM EDTA, pH 7, and 1 mM DTT). The cytosolic extracts were prepared through homogenization of the cells with a Dounce homogenizer. The lysates were subsequently centrifuged at 3,000 rpm for 15 min at 4°C. The FAS activities was evaluated by mixing, in a Quartz cuvette, 50 μl of cell lysate, 0.1 M KPi, pH 7, 0.0025 mM acetyl-CoA, 0.18 mM NADPH, 3 mM EDTA, and 1 mM DTT. The reaction was initialized by adding 0.1 mM of malonyl-CoA. The OD at 340 nm was then recorded for a 15 min period at 40°C in a Cary-100 spectrophotometer (Varian, Quebec, Canada).

Analysis of mRNA expression level. Total RNA was extracted from CEH as previously described (16). UV-quantified RNA were diluted in DEPC-treated water at a final concentration of 1 μg/μl. Reverse transcription (RT) was performed using the Omniscript enzyme kit of Qiagen (Montreal, QC, Canada) and oligo(dt) (Roche Diagnostics, Quebec, Canada) for 1 h at 37°C, with a 5-min inactivation step at 93°C. qPCRs were then performed using the Quantitect SYBR Green PCR Kit from Qiagen and the LightCycler device (Roche Diagnostics). The HPRT-1 gene was used as reference. The relative quantification was then performed using the ReQuant software (Roche Diagnostics). For the FAS gene, primers were defined on goose sequences: AGGAAATGAGGCTGGTGTG (sense) and CTGAGT-GCTTCAGGTTGAGT (antisense), and for the HPRT-1 gene prim-

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ers were defined on human sequences: ATGACCTCTCAACTTGG (sense) and GGCACCCTTACACCTTGT (antisense).

**Analysis of promoter activity.** HepG2 cells were lysed at room temperature in 500 μl of CAT Elisa lysis buffer (Roche Diagnostics). Protein concentration (12) and β-galactosidase activity (79) were measured by the indicated methods. CAT activity was evaluated using the CAT-ELISA kit according to the manufacturer’s instructions (Roche Diagnostics). The results were expressed as CAT activity per milligram of soluble protein and then normalized for transfection efficiency using the β-galactosidase activity.

**Gel electrophoretic mobility shift assay.** HepG2 cells were incubated for 24 h in serum-free MEM with or without 100 nM insulin, 1.6 μM T3, or both hormones, and nuclear extracts were prepared as previously described (2). A 40-bp double-stranded oligonucleotide corresponding to the TRE sequence of the goose FAS gene (TGCCCTGCCCGCCCTGTGGTAACCTCGGGACCGCGCT) was labeled with [γ-32P]ATP using the T4 polynucleotide kinase. Nuclear extract (5 μg) was incubated with 2 μl of binding buffer containing 20,000 cpm of 32P-labeled probe, 10 ng of poly(dI-dC), 1 μl of BSA, 4% (vol/vol) glycerol, and 1% (vol/vol) Ficoll. The reaction was then incubated for 15 min at room temperature. For supershift experiments, nuclear extracts were preincubated for 15 min at room temperature with 2 μg of specific antibody or with IgG. The reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel at 150 V in 25 mM Tris-HCl, 0.19 M glycine, and 1 mM EDTA. Gels were dried and visualized by autoradiography using the phosphoimager system (Molecular Imager FX; Bio-Rad, Mississauga, ON, Canada).

**Western blot.** After treatment with the test agents, for the times and the concentrations indicated in the figure legends, CEH were rinsed twice with ice-cold phosphate-buffered saline (pH 7.4) and solubilized with lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM MgCl2, 1 mM EGTA, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, tablet of EDTA free complete mini (Roche Diagnostics), 10% glycerol, and 1% Triton X-100]. Cell lysates were clarified by centrifugation at 10,000 g for 20 min at 4°C, and protein concentrations, in the resulting supernatants were determined using the Bradford method (12). Twenty micrograms of protein from cell lysates was mixed with 4 μl of 3× Laemmli sample buffer [2% SDS, 2% β-mercaptoethanol, 10% (vol/vol) glycerol, and 50 mg/ml bromophenol blue in 0.1 M Tris-HCl buffer, pH 6.8], heated at 100°C for 5 min, subjected to SDS-PAGE, and then transferred to Immobilon-P membranes (Millipore) for immunoblotting. Membranes were incubated for 1 h in blocking buffer (1× TBS + 0.1% Tween 20 TBST) containing 5% milk and then overnight at 4°C in TBST-5% BSA with the various antibodies: FAS (1:1,000), GAPDH (1:1,000), Akt (1:1,000), phospho-Akt (1:1,000), p42/44 MAPK (1:1,000), and phospho-p42/44 MAPK (1:1,000). After three consecutive washes in 1× TBST, the membranes were incubated in 1× TBST with 5% milk in the presence of an anti-rabbit IgG bound to horseradish peroxidase (1:10,000). Signals were revealed using the ECL Plus Western blotting detection reagent according to the manufacturer’s instructions (GE Healthcare, Baie d’Urfé, QC, Canada). The appropriate bands

![Fig. 1](http://ajpendo.physiology.org/) Effects of triiodothyronine (T3) and insulin on fatty acid synthase (FAS) enzymatic activity and protein and mRNA levels. A: HepG2 cells were incubated for 24 h with or without T3 and/or insulin at the concentrations indicated. Cells were lysed, and extracted proteins were resolved on 7% SDS-PAGE and immunoblotted with anti-FAS antibody. B: chick embryo hepatocytes (CEH) were incubated for 24 h with or without 1.6 μM T3 and/or 100 nM insulin, as indicated. CAT activity was evaluated as indicated in MATERIALS AND METHODS. Results are expressed as a percentage of the activity measured in the untreated sample and are the mean of ≥3 independent experiments ±SD. *P < 0.05, T3- or insulin-treated vs. untreated cells. C: HepG2 cells were treated as described above. Total RNA was extracted and FAS mRNA expression evaluated through qPCR as described in MATERIALS AND METHODS. Results are expressed as a percentage of the mRNA level measured in nontreated cells. Results represent the mean of ≥3 independent experiments ±SD. *P < 0.05 comparing T3- or insulin-treated vs. untreated cells.

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were quantified using the α-phospho-imager system (Molecular imager FX, Bio-Rad).

RESULTS

Roles of T3 and insulin on FAS enzymatic activity and protein and mRNA levels. Various studies have already demonstrated that, in liver, T3 and insulin are able to increase FAS enzymatic activity and mRNA expression (87, 88, 95). Incubation of the human HepG2 cells with 10 nM, 100 nM, or 1.6 μM T3 for 24 h significantly increases the level of FAS protein expression. Addition of 100 nM insulin also leads to a similar level of increase, whereas a combination of the two hormones is synergistic (Fig. 1). Most of the subsequent experiments were performed using 1.6 μM T3 and 100 nM insulin. In CEH, insulin and T3 are able to increase FAS enzymatic activity about threefold (Fig. 1B), and in the presence of both hormones an important synergistic effect is also observed, increasing the activity 14-fold. Similar hormonal effects are observed on mRNA expression level (2.5-fold increase with T3 or insulin and 10-fold with both hormones; Fig. 1C). Taken together, these results suggest that T3 and insulin regulate FAS expression through a pretranslational mechanism.

Effects of insulin and T3 at the transcriptional level. To evaluate whether the effects of T3 and insulin are the result of a modulation of the FAS promoter’s transcriptional activity, we cloned the proximal fragment (~1.5kb upstream of the cap site, −1450 to +133 bp) of the goose FAS promoter upstream of the chloramphenicol acetyltransferase (29) reporter gene. Subsequently, this DNA construct (TRE-TK-CAT) was transiently transfected into HepG2 cells, and the CAT activity was evaluated in the presence or not of the two hormones. As depicted in Fig. 2A, having T3 in the medium increases the transcription of the FAS gene about threefold. This suggests that a TRE is present in the first 1450 bp of the FAS promoter. By 5’ serial deletions, we localized the TRE between −902 and −577 bp (data not shown). This sequence, containing the TRE, was then inserted upstream of the minimal promoter of thymidine kinase and the CAT reporter gene and transiently transfected into the cells. When the cells were treated with T3, we observed about a threefold increase in CAT activity. Comparing the goose FAS sequence with that of different species, we were able to accurately localize the TRE between −741 and −696 bp (Fig. 2B). Astonishingly, insulin was also able to increase FAS transcription (~1.5-fold) through this TRE, and in the presence of both hormones the transcription increased more than fourfold (Fig. 2A). Interestingly, the same hormonal effects were obtained when cells were transfected with a classical synthetic TRE (DR4-TK-CAT). These results suggest that both T3 and insulin are able to increase 1) FAS transcription through a specific TRE and 2) the transcriptional activity of a classical DR4 element.

To characterize and identify the transcription factors involved in the hormonal response at the promoter level, we carried out electrophoretic mobility shift assay (EMSA) experiments. For this we used a double-stranded oligonucleotide encompassing the −741 to −696 bp sequence of the goose FAS promoter as a probe (Fig. 3A). This 32P-labeled probe was incubated with the nuclear extracts prepared from HepG2 cells treated or not with T3 and/or insulin. As indicated in Fig. 3B, in presence of nuclear extracts, a retarded band is observed in all conditions (even in the absence of hormonal treatment), suggesting that nuclear proteins are able to bind this 40-bp sequence. Following T3 or insulin treatment, binding of the nuclear proteins is increased (~1.5-fold compared with untreated cells; Fig. 3B). When both hormones were added together, the binding was even stronger (2.5-fold compared with untreated cells; Fig. 3B). By adding specific antibodies directed against TR and RXR to the nuclear extracts, we were

![Fig. 2. Localization of the T3 response element (TRE) on the goose FAS gene promoter. A: HepG2 cells were transiently transfected with various CAT DNA constructs (left, 1.5 μg/well) and pRSV-BGal (0.5 μg/well) as described in MATERIALS AND METHODS. After removal of transfection medium, hepatocytes were incubated for 24 h in MEM (open bars) or in the same medium containing 1.6 μM T3 (hatched bars), 100 nM insulin (filled bars), or both (cross-hatched bars). CAT activities were measured using a CAT ELISA kit. Results are expressed as CAT activities normalized by β-galactosidase activity/mg soluble protein, are represented as a percentage of the activity measured in the respective untreated sample, and are the mean of ±SD. *P < 0.05 vs. untreated cells. B: sequence comparison of goose FAS promoter (H: AY391824) to human (H: 250144), mouse (M: AL663090), rat (X: X54871), and chicken (C: X77339) FAS promoters. Conserved bases in the TRE between the 5 species are underlined.](image-url)
T3 and Insulin can activate specific intracellular signaling pathways leading to various phosphorylation cascades, resulting in the transcriptional modulation of numerous genes (5, 68). For that reason, CEH were incubated with a general inhibitor of either tyrosine kinase (genistein) or serine/threonine kinase (H7). The effects of these inhibitors were subsequently evaluated on the T3- and insulin-induced FAS enzymatic activity. Genistein does not statistically modify FAS activity (Fig. 4B), whereas H7 strongly decreases it and this under all hormonal treatments (Fig. 4C). The effect of those inhibitors was subsequently tested on FAS transcription by transfecting the TRE-TK-CAT construct in HepG2 cells. Similar effects of both genistein (Fig. 4D) and H7 (Fig. 4E) were observed on the FAS TRE transcriptional activity. Taken together, our results suggest that phosphorylation mechanisms involving serine/threonine kinases are required to ensure optimal effects of T3 and insulin on the regulation of FAS transcription via the TRE.

Role of the PI 3-kinase and ERK1/2 MAPK pathways in the regulation of FAS in response to T3 and insulin. It is well known that in liver both T3 and insulin are able to activate various cellular signaling pathways, such as the PI 3-kinase/ Akt and/or ERK1/2-MAPK pathways (21, 68). To evaluate the implication of such pathways in FAS regulation in response to these two hormones, we measured FAS enzymatic activity in the presence of specific pharmaceutical inhibitors for each pathway. Preincubation of T3 and insulin-treated CEH cells with LY-290042, a PI 3-kinase inhibitor, or PD-98059, a MEK1/2 inhibitor, strongly decreases FAS enzymatic activity (Fig. 5A). The inhibitors have similar or even more pronounced effects on the FAS promoter activity, generated by the TRE-TK-CAT construct transiently transfected in HepG2 cells (Fig. 5B). Interestingly, identical results were obtained when HepG2 cells were transiently transfected with the DR4-TK-CAT construct (Fig. 5C). Together, these results suggest that PI 3-kinase and/or ERK1/2 MAPK are implicated in the transcriptional regulation of gene transcription in response to T3 and insulin, and this through a TRE (FAS TRE and the classical DR4 element).

To clarify the implication of the two kinases in each hormonal effect, we measured the level of phosphorylation of ERK1/2 MAPK and Akt in response to T3 and insulin when submitted to the specific pharmacological inhibitors. As indicated in Fig. 6A, T3 increases ERK1 phosphorylation level on Thr202/204 residues. This effect is observed at both 100 nM and 1.6 μM of T3. As depicted in Fig. 6B, the maximal T3 effect is detected at 10 min, declining rapidly thereafter. The ERK2 phosphorylation level is not substantially modified by T3. Following an insulin treatment, a maximal phosphorylation of ERK1 is observed at 10 min, which is sustained up to 90 min. Similarly to T3, insulin does not modify ERK2 phosphorylation. When the cells are treated with both hormones, the stimulation profile is identical to the one observed with insulin alone.

We then evaluated the level of T3 and insulin-induced ERK1 phosphorylation in the presence of the pharmaceutical inhibitors. As depicted in Fig. 7A, the T3-induced stimulation is lost with U-0126 or PD-98059 as well as with LY-294002. However, in presence of insulin or with both hormones, only MEK inhibitors, and not LY-290402, decrease ERK1 phosphorylation (Fig. 7, B and C, respectively). It is interesting to note that,
with all hormonal stimulations, ERK1/2 phosphorylation is totally inhibited by U-0126, whereas PD-98059 did so only partially (Fig. 7, B and C). This is in agreement with the study that demonstrated that U-0126 is 100-fold more potent than PD-98059 in inhibiting ERK1/2 phosphorylation (26).

We then evaluated the effects of those hormones on the phosphorylation state of Akt on Ser473 residue (Fig. 8). As expected, the presence of insulin increase Akt phosphorylation at both 10 and 30 min, whereas no phosphorylation was detected with T3. Incubating the cells with both hormones reveals a pattern similar to that observed with insulin alone.

In conclusion, our results suggest that T3 and insulin are able to activate FAS transcription through the TRE. T3 acts on this TRE through a genomic action via the direct binding of the TR/RXR heterodimer. It also acts through a nongenomic action, involving the activation of a PI 3-kinase/ERK1/2 MAPK-dependent pathway. On the other hand, insulin modulates FAS transcription through the same TRE. However, it happens through the activation of two parallel signaling pathways: via a PI 3-kinase/Akt-dependent pathway and/or via a pathway involving ERK1/2 MAPK. Finally, we also showed that the implications of these signaling cascades are not restricted to the FAS promoter but appear to be a general phenomenon, as the same effects are observed on a classical DR4 element.

DISCUSSION

In agreement with previously published studies performed in CEH (84, 95), we showed that T3 and insulin were also able to increase FAS enzymatic activity (9.3-fold; Fig. 1A) and mRNA expression level (2.5-fold; Fig. 1C) in both CEH and HepG2 cells. Insulin alone was also identified as a potent FAS activator in mouse models (74). As demonstrated in CEH (84, 95), the presence of the two hormones synergistically stimu-
translated FAS in chicken as well as in human models (Fig. 1). It therefore appears that FAS expression is similarly regulated in different species.

Other studies suggest that the hormonal regulation of FAS is principally pretranslational, probably as a result of the transcriptional modulation of gene expression (73, 74, 84). The transcriptional regulation of FAS by insulin has been well characterized. In 3T3-L1 cells, it was shown that insulin increases FAS transcription through the activation of the PI 3-kinase/Akt-dependent pathway (93). The insulin effect is mediated through the modification of the binding of various transcription factors to insulin response elements located on the FAS promoter (74). An IRE has been identified at −65 bp on the rat FAS promoter (69, 94). It is an E-box that binds the ubiquitous transcription factors USF1 and USF2 (upstream stimulatory factors). In addition, insulin also increases mouse FAS gene transcription by inducing the binding of SREBP-1c on two different SREs located around −150 bp (51) and −65 bp (46). Finally, insulin regulates FAS transcription through the modulation of the binding of NF-Y and Sp1 on the FAS rat promoter region located between −103 to −53 bp (64).

In goose, and probably in other species, we show that insulin also modulates FAS promoter activity by targeting a TRE located between −717 and −701 bp, increasing the binding of a TR/RXR heterodimer (Fig. 3B). Interestingly, a similar phenomenon was observed with a classical DR4 element, suggesting a general effect of insulin on TREs (Figs. 2A and 5C). The action of insulin on the TRE seems to involve the activation of two distinct signaling pathways (PI 3-kinase/Akt and Ras-Raf-ERK1/2 MAPK). The activation of these two pathways may induce the phosphorylation of TR and/or various coactivators resulting in the increase binding of TR/RXR.
on the TRE (Fig. 3B). However, the exact contribution of these insulin-induced pathways on the FAS TRE remains to be elucidated.

In the present study, we also characterized the mechanism of T3 action on the FAS promoter. We localized a TRE on the goose FAS promoter between -1100 and +701 bp. This TRE is a direct-repeat sequence separated by four nucleotides (DR4). It is well conserved among species, suggesting a similar type of regulation (59). Moreover, our results show that, in the presence of insulin and T3, only a weak synergistic effect on TRE-mediated transcription is observed compared with what is seen at the mRNA level and enzymatic activity. This may indicate that the previously identified IREs located downstream on the TRE are necessary to achieve a full stimulation of FAS activity and expression.

Through mobility shift assays, we showed that a TR/RXR heterodimer binds to the TRE even without hormonal stimulation. Adding T3 and/or insulin increases the heterodimer’s binding (Fig. 3B). Many studies have shown that the TR/RXR heterodimer binds with better affinity to a DR4-type TRE (75). The four-nucleotide space helps stabilize the TR/RXR complex, leading to increased transactivation (32, 90). The binding of the complex to the TRE, in absence of hormones, could

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involve FAS basal repression, as previously described for many other genes positively regulated by T₃ (97). In this case, the TR/RXR complex would interact with TFIIB (4, 38), interfering with the formation of the transcription pre-initializing complex. This basal repression is also maintained through the association of TFIIB with corepressors (40, 54). The presence of hormone would lead to a conformational change, inducing the dissociation of corepressors, and the binding of co-activators, such as the p160/SRC protein (steroid receptor coactivator) (72), the p300/CBP complex (CREB-binding protein) (49), and PCAF (p300/CBP-associated factor) (49). The binding of co-activators on the TR/RXR complex leads to the maximum induction of gene transcription in response to T₃.

The classic genomic action of thyroid hormone is well known. Its effect is delayed, appearing only after a few hours of hormonal stimulation. Various studies reported a faster effect of T₃, in particular in the control of Ca²⁺ entry or in protein trafficking (20, 23). In fact, the research demonstrated that the hormone could act through a nongenomic action mechanism, also called extranuclear action, and involves the activation of various signaling cascades (63). Using various hormonal combinations of T₃ and insulin, our present study suggests that phosphorylation mechanisms are also involved in the transcriptional regulation of FAS via the TRE (Fig. 4A).

This was confirmed by the use of the Ser/Thr kinase inhibitor H8, another protein kinase inhibitor, decreases insulin-dependent ERK1/2 MAPK activation remains to be determined. In human fibroblasts, in the presence of T₃, the TRβ receptor is able to directly interact with p85α, the regulatory subunit of PI 3-kinase, leading to downstream activation of Akt, mToR, and directly interact with p85α, inducing an increase in the transcription of T₃-regulated genes (42, 61). A similar mechanism was described for steroid hormones.

In hepatic cells, we showed that T₃ is able to activate the ERK1/2 MAPK pathway through the activation of PI 3-kinase (Figs. 6 and 7). Recently, it has been demonstrated that T₃ activates ERK1/2 by two different mechanisms. One is independent of PI 3-kinase, activating cell proliferation, whereas the other is PI 3-kinase dependent, involving various actions such as protein trafficking (86a). The exact mechanism by which T₃ activates PI 3-kinase in liver leading to ERK1/2 MAPK activation remains to be determined. In human fibroblasts, in the presence of T₃, the TRβ receptor is able to directly interact with p85α, the regulatory subunit of PI 3-kinase, leading to downstream activation of Akt, mToR, and p70S6K (63). This cascade activates the transcription of the ZAK1/4α gene in response to T₃ (13). At the hepatic level, we showed that T₃ activates PI 3-kinase but not Akt (Fig. 8).

In general, activation of PI 3-kinase leads to Akt activation; however, PI 3-kinase needs to be recruited to the plasma membrane to adequately activate Akt (18, 70). But in the presence of T₃, TR specifically interacts with the cytosolic p85α regulatory subunit of PI 3-kinase (14). In adult rat alveolar epithelial cells, T₃ increased Na-K-ATPase activity in a transcription-independent manner via the activation of two distinct pathways: the PI 3-kinase (56) and the ERK1/2 MAPK pathway (55). They also demonstrated that T₃ stimulates the PI 3K-Akt pathway via the Src family of tyrosine kinases.

Independently of PI 3-kinase, thyroid hormones can also bind to an integrin α(V)β3 cell surface receptor, leading to the activation of ERK1/2 MAPK and its nuclear translocation (6, 21). In the nucleus, ERK1/2 MAPK phosphorylates TR (22, 81), leading to the dissociation of the SMRT and NCoR corepressors. Subsequently, this will increase the degradation of the corepressors and induce the transcription (42, 57, 89). TR phosphorylation would also help the heterodimerization with RXR, increasing the binding of the TR/RXR complex to the TRE (7, 45). The SRC-1 coactivators, which are recruited to the TR transcriptional complex, have also been described to be a target of ERK1/2 MAPK (78). In addition, the MAPK/TR complex would also be able to bind and phosphorylate the p53 (81) and STAT transcription factors (60), modulating the transcription of other target genes.

In conclusion, in the present study we have shown (Fig. 9) that, in hepatic cells, T₃ regulates the transcription of FAS through a genomic action involving the binding of a TR/RXR heterodimer to the T₃ response element. Moreover, T₃ can also act via a nongenomic mechanism, involving the activation of PI 3-kinase, which subsequently activates ERK1/2-MAPK, increasing FAS transcription. On the other hand, the binding of insulin on its membrane receptor leads to the activation of both PI 3-kinase/Akt and Ras/Raf/ERK1/2-MAPK signaling pathways, activating FAS transcription via the TRE.

GRANTS

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