J. Food Sci. Nutr.
Vol. 3, No. 4, p. 362~367 (1998)

Effect of Thyroid Hormone on Lipogenesis in Rat White and Brown Adipocytes Culture System

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Abstract

Thyroid hormone (T_3) stimulates hepatic lipogenesis by increasing expression of genes, including acetyl-CoA carboxylase and fatty acid synthase. S14 protein, which is thought to be involved in lipid metabolism, appears to respond in parallel. Effects of T_3 on lipogenesis in white and brown adipose tissue are less clear, and may be complicated by indirect effects of the hormone. We developed an adipocytes system where the indirect effects of thyroid hormone are abolished and direct effects of T_3 on lipogenesis could be tested. Fat accumulation was measured by Oil-Red O staining. Insulin clearly enhanced fat accumulation by 2-fold. Isobutylmethylxanthine (IBMX) appeared to inhibit insulin-stimulated fat accumulation. Dexamethasone increased insulin-stimulated fat accumulation about 1.3-fold. Confluent adipocytes were cultured in serum-free medium or medium containing 10% fetal calf serum or 10% fetal calf serum stripped of thyroid hormone and lipogenesis, assessed by the incorporation of ^3H_2O, was measured. Medium without serum or supplemented with T_3-depleted serum did not amplify the stimulatory effect of T_3 on lipogenesis compared to medium containing 10% fetal calf serum. Dexamethasone alone led to a decrease in lipogenesis of about 50% in white adipocytes and 25% in brown adipocytes. However, dexamethasone amplified the lipogenic response to T_3 by about 30% in white adipocytes and 60% in brown adipocytes. T_3 (1 μM) stimulated lipogenesis and acetyl-CoA carboxylase and fatty acid synthase mRNA levels up to 2-fold in both types of adipocytes. It seems that these adipocyte systems are a useful model to study the effects of hormones on lipogenic gene expression as well as lipogenesis.

Key words: thyroid hormone, lipogenesis, white and brown adipocytes

INTRODUCTION

Both white and brown adipose tissues are the major sites for *de novo* lipogenesis. The primary function of white adipose tissues (WAT) is storage of triglycerides. The principle role of brown adipose tissue (BAT) is oxidation of fat stores to generate heat; fatty acids are the chief substrates for this process (1). The rate of synthesis of long chain fatty acids in these tissues is under complex hormonal and nutritional control.

Thyroid hormone, especially its most active metabolite triiodothyronine (T_3), is a key regulator of lipid metabolism (2). The physiological effects of thyroid hormone are thought to be mediated by DNA-binding receptors localized to the cell nucleus. The complex of T_3/receptor/DNA can generate differential gene transcription, depending on the gene and target tissue (3). In liver, interaction of T_3 with its receptors appears to stimulate lipogenesis by increasing expression of the genes encoding lipogenic enzymes (2). Specific nuclear thyroid hormone binding receptors are also found in rat adipocytes (4) as well as the preadipocytes cell line (5), allowing the possibility of similar regulation of lipogenic genes in adipose tissue. However, the lipogenic responses to thyroid hormone in these tissues are different from that of liver and not clear. In white adipose tissues, Diamant et al. (6) showed that T_3 increased activities of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and glucose-6-phosphate dehydrogenase. However, Roncari and Murthy (7) reported decreased activities of ACC and FAS as well as lipogenesis, following T_3 treatment. Freake et al. (8) showed that T_3 stimulated epididymal fat lipogenesis to a similar extent as in liver. Moreover, in a subsequent report using similar rats, they found no significant differences in epididymal fat lipogenesis with thyroid hormone treatment (9). Brown adipose tissue showed a unique lipogenic pattern; fatty acid synthesis in hypothyroid rats was higher than that of eu- or hyperthyroid rats (10). The ambiguity of T_3 effect on lipogenesis was postulated partially by the indirect effects of thyroid hormone to these tissues *in vivo*, e.g., T_3-stimulated catecholamine action in white adipose tissue (11) and sympathetic nervous system (SNS)-mediated lipogenic responses in brown adipose tissue (10).

Studies of hormone action, particularly in intact animals, are complicated by the reciprocal effects of the so-called "counter-regulatory" hormones that are secreted in response to hormone-induced effects (12). In contrast, cell culture systems allow exact control of the cellular environment. It may be kept relatively constant, and reagents can be added directly at a wide range of concentrations. Specially primary cell cultures can duplicate the situation observed *in vitro* compared with the established cell lines which show changes in cell phenotype. It is possible, using primary cell culture system, to investigate the effects of T_3 and other factors singly or together without the complicating changes in substrate hormonal environments that occur *in vivo*. The role of T_3 in the lipogenic regulation

in WAT and BAT can be compared with this system.

The established 3T3 cells, derived from disaggregated 17~19 days old Swiss mouse embryo, are capable of spontaneously differentiating into adipocytes after they have become density/growth-arrested. During differentiation, 3T3 cells accumulate triglyceride, acquire the morphological appearance of mature adipocytes, and exhibit high levels of lipogenic enzymes including FAS, and glycerol-3-phosphate dehydrogenase (13,14). A variety of agents have been identified which can accelerate the differentiation process of 3T3 cells. These include insulin (13), dexamethasone (15), isobutylmethylxanthine (IBMX) (16), and prostglandin E (16). One of the most efficient means to induce lipogenesis in 3T3 cells is treatment of the confluent monolayer of cells with a combination of insulin, dexamethasone, and IBMX (15).

To define the regulatory system of T_3 *in vivo*, we developed a primary adipocyte system where the indirect effects of thyroid hormone are abolished and then tested the responsiveness of lipogenesis and mRNAs of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), and S14 to hormonal manipulation of white and brown adipocytes in rat.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, initially weighing 150~175 g, were used in all experiments. They were housed individually in stainless steel wire mesh cages on a 12/12 hour light cycle (light from 07:00 h to 19:00 h) at 20~22°C. The animals were given free access to rodent laboratory chow (Purina Mills, MO). Hypothyroidism was induced by the addition of 0.025% methimazole to the drinking water for three weeks.

Cell isolation and culture

Epididymal white adipocytes and interscapular brown adipocytes were isolated as described by Nechad et al. (17) except hypo-osmotic shock was omitted. Epididymal fat pads and interscapular brown adipose tissue were carefully dissected out under sterile conditions and digested at 37°C for 45~60 minutes with 0.15% (w/v) collagenase (CLS 1, Worthington Biochemicals, NJ) in isolated buffer (123 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 1.5% (w/v) BSA (fraction V, Intergene, NY), and 100 mM HEPES, pH 7.4). After digestion, the tissue remnants were removed by filtration through a nylon mesh. The preadipocytes were pelleted by centrifugation at 700×g for 5 min and were resuspended in 10 ml DMEM culture medium (Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, MD), 100 mM HEPES, 50 IU penicillin, and 50 μg streptomycin per ml). The cells were grown at 37°C in an atmosphere of CO_2 in air with 95% humidity. The medium was first changed the day after plating, and then every second day. Thereafter, at confluence, the adipocytes were treated with hormone. The porcine insulin used was a generous gift from Eli Lilly (Indianapolis, IN). T_3 was from Sigma (St. Louis, MO).

Oil Red O staining

Insulin (4 nM), dexamethasone (25 nM), and IBMX (100 μM) were added to the medium when cells were confluent. Incubation was continued for 72 h further. Cells were washed in 5 ml of PBS twice, frozen in situ on dishes at -20°C for 20 min, and then thawed at room temperature for 10 min. The cells were dehydrated in 60% isopropanol, and stained with 0.25 ml of Oil Red O reagent saturated with 60% isopropanol (v/v) for 10 min. Dishes were then rinsed with 60% isopropanol, and dried. Fat accumulation was observed with an inverted microscope (Inverted Microscope SO41, OLYMPUS TOKYO) and scored as percentage of area stained (18).

Depletion of thyroid hormone from fetal calf serum

AG 1X-2 resin (chloride form, BIO-RAD) was washed 3 times with distilled water. Fetal calf serum was incubated with resin (5 g/100 ml serum) with a magnetic stirrer for 4~5 h at room temperature and centrifuged at 1,000×g for 10 min. The serum was then sterilized by filtration and stored at -20°C (19).

Measurement of lipogenesis

The cells were supplemented with 0.5 mCi ^3H_2O (New England Nuclear, MA) per dish, and labeled for 2.5 h at the end of hormone treatment. Media were aspirated and cells were detached by addition of 0.5 ml 200 mM EDTA. Lipids were extracted by the method of Folch et al. (20), the organic extracts was back-washed with water, and tritium content was assessed by liquid scintillation counting.

DNA assay

Fat cell number was estimated by measuring the DNA content, assuming 6.7 pg of DNA per cell. Cells were detached by addition of 1 ml of 200 mM EDTA, 5 ml of ethanol : 1.5 M CH_3COONa (3 : 1) was added, and centrifuged at 2,500×g for 20 min. The pellet was washed twice with ethanol : ethyl ether (3 : 1), and washed again with 5 ml of 0.5 N $HClO_4$. The pellet was incubated in the 24 μl of 2% BSA (w/v) and 1 ml of 0.4 M $HClO_4$ at 70°C for 30 min. After cooling the sample, 625 μl of Burton reagent (21) was added and incubated overnight at room temperature. The sample was spun at 7,000×g for 10 min and filtered through 0.2 μm syringe filter (Nalge Co.) to remove fine fat droplets. The concentration of DNA was assayed by measuring the absorbance at 600 nm with a spectrophotometer.

Extraction of RNA and Northern blot analysis

Total RNA was isolated using the guanidium thiocyonate/acid phenol method (22) and ACC. FAS and S14 mRNA levels was measured by Norther analysis, using [^{32}P]-cRNA probes, as described (23). Uniformity of loading and specificity of treatment effects were confirmed by hybridizing all membranes with a β-actin cRNA probe (Ambion, TX).

Data analysis

One and two way analysis of variance (ANOVA) were used to test for differences in experimental groups. Scheffe's

least significant difference test was employed to examine differences between individual treatment conditions.

RESULTS AND DISCUSSION

Differentiation of preadipocytes

Insulin, dexamethasone, and isobutylmethylxanthine (IBMX), which are routinely used for induction of differentiation in 3T3 cells, were investigated for their effects on the differentiation of white and brown preadipocytes (Table 1). Fat accumulation after supplement of these agents for 72 hr was measured by Oil-Red O staining. The response patterns of fat accumulation to these agents were similar in both types of adipocytes. Insulin clearly enhanced fat accumulation by 2-fold. IBMX appeared to inhibit insulin-stimulated fat accumulation, although not significantly. Dexamethasone increased insulin-stimulated fat accumulation about 1.3-fold.

One of the most efficient means to induce a adipogenesis in the 3T3 cell is treatment of the confluent monolayer of cells with a combination of insulin, dexamethasone, and IBMX (15). The known pharmacological effect of IBMX is inhibition of cAMP phosphodiesterase (16). Elks and Manganiello (18) suggested that inhibition of cAMP phosphodiesterase activity and subsequent changes in cellular cAMP level might play an important role in the mechanism whereby IBMX enhanced differentiation. However, in our experiments, IBMX inhibited insulin-stimulated fat accumulation in both types of primary adipocytes suggesting that the effect of IBMX on lipogenesis was not the same in 3T3 cells and primary adipocytes. The different differentiation mechanism between 3T3 cells and primary adipocytes showed a limitation in use of established cell lines for the investigation of adipocyte regulation.

Development of the adipocyte system

Serum effects

Most studies dealing with hormone action in cell culture use serum-free medium when the hormone treatment is begun (24,25). Serum has endogenous hormones and unknown factors which can influence cellular function. In particular, if the serum contains the same hormone under investigation, the serum may mask the real effect of treatment. However, it is also necessary to consider how long the cells can maintain a healthy status without serum. In liver *in vivo*, fatty acid synthesis was stimulated after a lag time of 12~16 hr and reached a plateau after 4~5 days following thyroid hormone treatment of hypothyroidism animals (8). It may, therefore, be necessary to incubate cells for relatively long period to investigate the mechanism of thyroid hormone action. The incubation period in serum-free media used by other investigations was relatively short, less than 48 hr (24,25).

To investigate the masking effects of serum on T_3, confluent adipocytes were cultured for 72 hr in serum-free medium, medium containing 10% new-born calf serum (CS) or 10% calf serum stripped T_3 by resin treatment ($-T_3$ CS). The effects of T_3 on lipogenesis, assessed by the incorporation of ^3H_2O, were then measured (Table 2). The lipogenic values were significantly higher in adipocytes cultured in serum-supplemented medium than that of adipocytes cultured in serum-free medium. Moreover, in serum-free condition, the adipocytes were not well-differentiated over the 72 hr incubation period, starting to detach from the dish. Cells cultured in serum-free medium or T_3 stripped medium did not exhibit a higher lipogenic response to T_3 compared with that of cells cultured in 10% serum in either types of adipocyte. The endogenous concentration of T_3 in serum supplemented to medium is about 0.1 nM which corresponds to the levels found in hypothyroid serum *in vivo* (26). Therefore, it can be postulated that the level of thyroid hormone in medium may not be high enough to induce lipogenesis in adipocytes. For subsequent experiments, 10% newborn calf serum was added under all cell culture conditions.

Dexamethasone

The effects of dexamethasone on lipogenesis were examined. Confluent adipocytes were treated with different combinations of hormones for 72 hr and lipogenesis, assessed by the incorporation of ^3H_2O, was measured (Table 3). Dexamethasone alone, led to a decrease in lipogenesis of about 50% in white adipocytes and 25% in brown adipocytes. However,

Table 1. Effect of differentiating agents on fat accumulation in white and brown adipocytes

Differentiating agents	% of Area stained	
	White adipocytes	Brown adipocytes
None	30±2.2	35±2.0
Insulin	66±4.1[a]	79±3.1[a]
Insulin+IBMX	57±2.4	70±4.1
Insulin+Dexamethasone	77±2.4[b]	90±2.0[b]
Insulin+IBMX+Dexamethasone	64±2.5	81±2.4

When adipocytes were reached at confluent monolyer, 4 nM insulin, 100 μM IBMX, and 25 nM dexamethasone were added as shown. The results are expressed as a percentage of area stained by Oil-Red O. Values shown are mean±S.E. (n=4). Similar results were obtained in one additional experiment. Statistical significance was indicated by [a]$p<0.01$ for the effect of insulin, and [b]$p<0.05$ for the effect of dexamethasone.

Table 2. Effect of serum on the induction of lipogenesis by T_3 in white and brown adipocytes

	White adipocytes (nmoles H incorporated into lipid/dish/h)			Brown adipocytes (nmoles H incorporated into lipid/dish/h)		
	None	CS	$-T_3$ CS	None	CS	$-T_3$ CS
Control	209±12	260±14.2	304±11.4[a]	321±18.2	267±5.5	450±8.7[a]
T_3	191±20	314±10[a,*]	359±20[a]	210±12	376±16[a,*]	383±11[a,*]
T_3/C	0.91	1.2	1.17	0.65	1.4	0.85

When adipocytes were reached at confluent monolyer, they were treated with or without 1 μM T_3 for 72 h in different conditions of serum: serum-free (None), 10% new-born calf serum (CS), and 10% T_3-stripped serum ($-T_3$ CS) in the presence of 4 nM insulin. The results are expressed as mean±S.E. (n=3). Statistical significance was indicated by [a]$p<0.05$, for the effect of serum; [*]$p<0.05$, for the effect of thyroid hormone.

dexamethasone amplified the lipogenic response to T_3 by about 30% in white adipocytes and 60% in brown adipocytes. Since the effects of T_3 on lipogenesis were amplified by the presence of dexamethasone, 25 nM of dexamethasone was added to the medium in subsequent experiments. The DNA content did not differ per dish in the different groups.

Although the kind of cell culture used was different, Shapiro et al. (27) demonstrated that thyroid hormone induced growth hormone synthesis by approximately 5~20 fold and glucocorticoid increased this response 2~6 times further in GH_1 cells. In our experiments, dexamethasone alone either inhibited or had no effect on the level of ACC and FAS mRNAs. In combination with T_3, however, dexamethasone amplified the effect of T_3 on the expression of ACC and FAS mRNAs (unpublished data). Dexamethasone did not affect the level of S14 mRNA, which encodes a protein thought to be involved in lipid metabolism, in both types of adipocyte. However, in 3T3- F442A adipocytes, dexamethasone significantly stimulated expression of S14 mRNA (28). These different effects of T_3 on the lipogenic gene expression may be attributed to the difference between primary and established adipocytes. From our results, it appears that dexamethasone is useful to observe the effects of thyroid hormone on lipogenesis. Further studies are necessary to define the mechanism of interaction between T_3 and dexamethasone on lipogenesis.

Effects of thyroid hormone on lipogenesis in adipocytes

The kinetics of lipogenic induction were studied over a period of 96 hr to test the lifespan of cells and the response of the adipocyte systems to a well described regulation of fatty acid synthesis by insulin (Fig. 1). A significant ($p<0.01$) increase in the rate of lipogenesis was obtained within 24 hr after insulin treatment in both types of adipocytes. This lipogenic effect of insulin was maintained linearly up to 72 hr of incubation time in both types of adipocyte (Fig. 1). In white adipocytes, T_3-treated values significantly increased over control values at 24 hr in the presence of insulin or 48 hr in the absence of insulin, and this effects persisted until 96 hr (Fig. 1). A significant lipogenic induction in brown adipocytes was detected 72 hr after T_3 treatment (Fig. 1).

In white adipose tissue, lipogenesis was not affected by thyroid hormone status, suggesting that lipogenesis is regulated, perhaps via the byproducts of thyroid hormone-stimulated lipolysis *in vivo* (9). In our white adipocytes, however, lipogenesis was positively regulated by thyroid hormone. In brown adipose tissue, lipogenesis was enhanced in hypothyroidism as compared to eu- or hyperthyroidism (10). Our results showed that in brown adipocytes, where the influence of the SNS is abolished *in vitro*, lipogenesis was significantly stimulated by thyroid hormone. Lorenzo et al. (24) demonstrated a 1.4-fold enhancement of lipogenesis using primary fetal brown adipocytes. Thus, it is postulated that the response of T_3 on lipogenesis may be different between *in vivo* and *in vitro* studies.

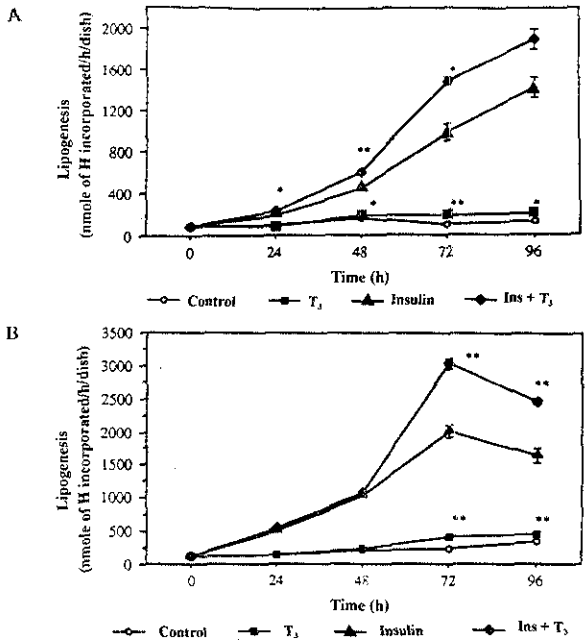

Fig. 1. Time course of lipogenic induction by T_3 in white and brown adipocytes. At confluence, white (A) or brown (B) adipocytes were treated with 25 nM dexamethasone (control, ○) with the addition of 1 μM T_3 (■), 1 nM of insulin (△) or both (◆). Lipogenesis was measured, using tritiated water, at the shown time and is expressed as nmole of H incorporated into lipid per h per dish. Values shown are mean±S.E. (n=3). Similar results were seen in two separate experiments. Values that show a significant effect of T_3 in the presence or absence of insulin are indicated by $^*p<0.05$, $^{**}p<0.01$. Insulin addition stimulated lipogenesis significantly at 24 h and all subsequent time points compared to control.

In white adipocytes, thyroid hormone increased the levels of ACC and FAS mRNA by 3- and 2-fold, respectively. The amount of S14 mRNA was increased by T_3 only in the presence of insulin (Fig. 2). In brown adipocytes, T_3 increased the levels of ACC and FAS mRNA by 2.3- and 3-fold, respectively. In the presence of insulin, T_3 enhanced the levels of ACC, FAS, and S14 mRNAs by 1.5-, 1.2-, and 1.6-fold, respectively (Fig. 2). Insulin enhanced the levels of ACC, FAS, and S14 mRNAs by 12-, 13-, and 9-fold in white adipocytes and 12-, 8-, and 3.5-fold in brown adipocytes, respectively. However, insulin did not amplify the T_3 effects on lipogenic mRNA levels in both types of adipocytes (Fig. 2). It can be postulated that the response of lipogenesis by T_3 occurred at the level of gene expression, as shown by alteration in the levels of ACC,

Table 3. Effect of thyroid hormone on lipogenesis in white and brown adipocytes in the presence or absence of dexamethasone

	White adipocytes (nmoles H incorporated into lipid/dish/h)		Brown adipocytes	
Dexamethasone	None	25 nM	None	25 nM
Control	223±14	105± 3*	305±13	229±6*
T_3 (1 μM)	297± 9[a]	186±14[b*]	313± 9	415±7[b*]
T_3/C	1.33	1.77	1.03	1.81

The results are expressed as mean±S.E. (n=3). Similar results were seen in two additional experiments. Statistical significance was indicated by [a]$p<0.05$, [b]$p<0.01$ for the effect of thyroid hormone; *$p<0.01$ for the effect of dexamethasone.

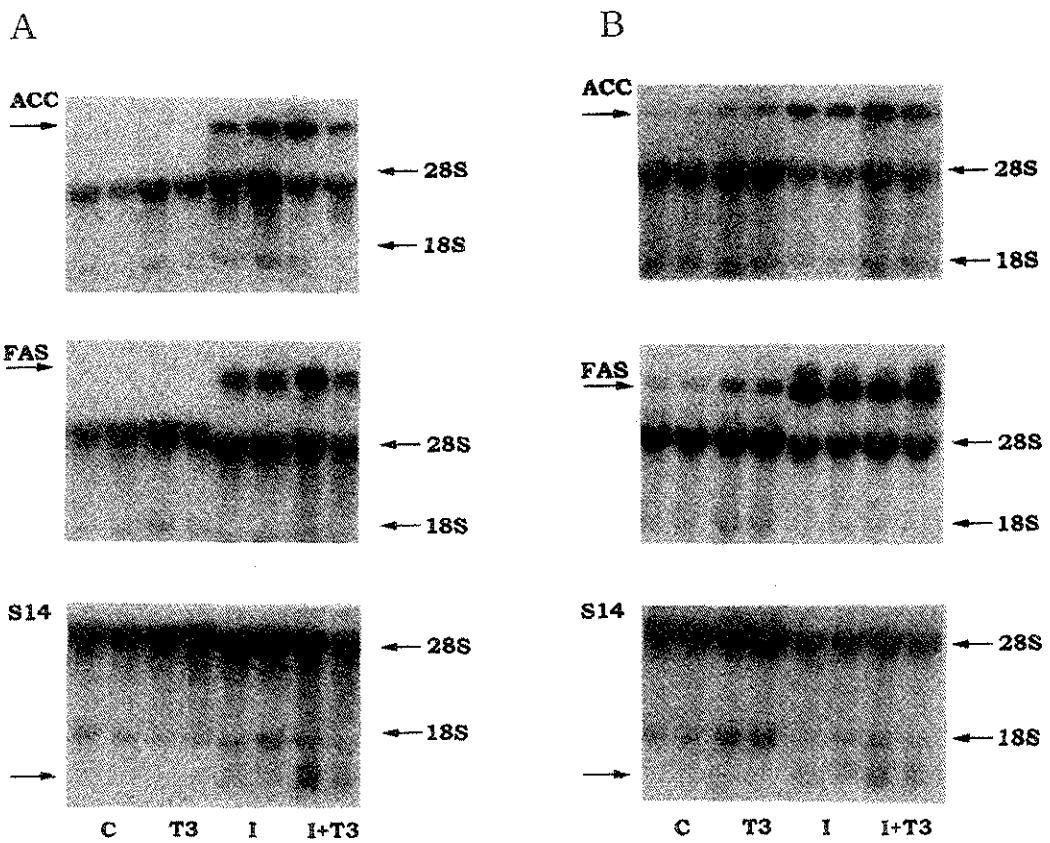

Fig. 2. Effect of T3 and insulin on the levels of lipogenic mRNAs in white and brown adipocytes. Confluent white (A) and brown (B) adipocytes were cultured for 72 h alone (C), with 1 μM T3 (T3), 4 nM insulin (I) or both (I+T3). Total RNA was extracted and ACC, FAS, and S14 mRNA levels were determined by Northern blot analysis. The size of the mRNA bands, indicated by the arrows, were estimated with reference to an RNA ladder at approximately 8 kb for ACC mRNA, a doublet of 8~8.5 kb for FAS and a doublet of 1.3~1.5 kb for S14. The migration of 28S and 18S rRNA are indicated by the arrows.

FAS, and S14 mRNA. The changes seen in these mRNAs were quite sufficient to account for the alterations in lipogenesis.

However, the effects of T_3 on lipogenesis and gene expression were relatively small when compared to those observed in the liver (9). In rat primary fetal hepatocytes, T_3 increased both lipogenesis and FAS activity by 1.4-fold (24). Those results imply that there is a marked difference in the relative lipogenic effect of T_3 between *in vivo* and *in vitro*. It remains to be determined whether the modest effects of T_3 seen in adipocytes represent a limitation of the *in vitro* system or a tissue specific difference between adipose tissue and liver.

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(Received October 9, 1998)