Hormonal and Nutritional Regulation of Lipogenic Enzyme mRNA Levels in Rat Primary White and Brown Adipocytes

Hedley Christopher FREAKE and Yangha Kim MOON

1 Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269–4017, USA
2 Department of Food and Nutrition, Ewha Womans University, Seoul, 120–750, Korea

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Summary Hyroid hormone stimulates hepatic lipogenesis in the rat by increasing the expression of relevant genes, including acetyl-CoA carboxylase and fatty acid synthase. S14 mRNA, which encodes a protein thought to be involved in lipogenesis, responds in parallel. The effects of thyroid hormone on lipogenesis in white and brown adipose tissue are less clear, and may be complicated by indirect effects of the hormone. Rat white and brown preadipocytes were therefore isolated, grown to confluence, and used to test direct effects of thyroid hormone, insulin, and glucose. Lipogenesis was assessed by tritiated water incorporation, and acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and S14 mRNAs were measured by Northern analysis. Insulin (1 nM) increased lipogenesis about 9-fold in both white and brown adipocytes. Similar increases were seen in the levels of the three mRNAs. Thyroid hormone (1 µM) stimulated lipogenesis and acetyl-CoA carboxylase, fatty acid synthase, and S14 mRNA levels up to 2-fold in both types of adipocyte in the presence or absence of insulin. A high carbohydrate level (25 mM glucose) had no effect on lipogenesis compared to a low carbohydrate level (5 mM glucose) in white and brown adipocytes. There was no synergistic effect on lipogenesis by the combination of thyroid hormone and high carbohydrate level in both types of adipocytes. These experiments have shown that T₃ has small, direct stimulatory effects on lipogenesis in adipocytes. These effects are seen at a pretranslational level, through the coordinate induction of ACC, FAS, and S14 mRNAs. Although lipogenic rates were usually higher in brown adipocytes than white adipocytes, very similar patterns of regulation were seen in the two cell types. These data support the idea that the divergent results seen concerning T₃ regulation of the lipogenic pathway in both brown and white adipose tissue in vivo arise from secondary effects of the alteration of thyroid status.

Key Words lipogenesis, gene expression, hormone, glucose, adipocytes

Thyroid hormone, specifically its most active metabolite triiodothyronine (T₃), is a key regulator of lipid metabolism (1). The physiological effects of thyroid hormone are mediated by DNA-binding receptors localized in the cell nucleus (2). The complex of T₃/receptor/response element DNA can generate differential gene transcription depending on the gene and target tissue (2). In the liver, the interaction of T₃ with its receptors appears to stimulate lipogenesis by increasing the expression of genes encoding the lipogenic enzymes (1). It also increases the transcriptional rate of the S14 gene (3), which encodes a product that has long been associated with lipogenesis and, more recently, has been shown to be required for the induction of fatty acid synthesis by thyroid hormones (4).

Different lipogenic responses to thyroid hormone in adipose tissues have been reported. In epididymal fat, Diamant et al. (5), Heise et al. (6), and Gnoni et al. (7) have all reported stimulatory effects of thyroid hormone on variables associated with fatty acid synthesis, including the activities of the key enzymes, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). However, Roncari and Murthy (8) found decreased activities of ACC and FAS following T₃ treatment. Similarly, hypothyroidism has been shown to diminish (9), leave unaffected (10), or enhance (11) lipogenesis in white adipose tissue or adipocytes prepared from treated animals. The reasons for these discrepancies remain unclear. However, one possibility is that alterations in the thyroid state produce other effects that indirectly influence lipogenic activity in adipose tissues. For example, hyperthyroidism sensitizes white adipose tissue to the effects of catecholamines (12). The enhanced intracellular levels of cAMP and fatty acyl-CoA may inhibit ACC activity (13).

Brown adipose tissue (BAT) has a unique lipogenic response to the thyroid state; fatty acid synthesis in hypothyroid animals is higher than that of eu- or hyperthyroid animals (14, 15) and is actually reduced by T₃.
administration (16). However, denervation reduces the rate of fatty acid synthesis in hypothyroid BAT to lower than denervated euthyroid levels, and T₃ treatment of these animals stimulates lipogenesis and the levels of lipogenic mRNAs (16). This argues for an indirect and inverse effect of the thyroid state on BAT lipogenesis, mediated by the sympathetic nervous system, which interacts with a direct stimulatory action of T₃ in this tissue.

The existence of such secondary effects clearly shows the need for an in vitro system. Preadipocyte cell lines, which can be grown continuously and then differentiated to a mature adipocyte phenotype in vitro, have been widely used to investigate the regulation of lipogenesis in vitro. However, these lines are derived from mice, and the effects of T₃ appear to depend on which particular cell line is used. For example, Lepar and Jump found no effect of T₃ on the expression of mRNA S14 in 3T3-F442A adipocytes (17), whereas Bitzer et al. (18) reported a stimulatory effect in the closely related 3T3-L1 cells.

We have elected to use primary cell cultures from the rat. Using freshly isolated mature epididymal adipocytes, we were able to show that the rates of lipogenesis were 3-fold higher in cells taken from hyperthyroid rats as compared with eu- or hypothyroid rats (19). However, the relatively short life span of these cells made them unsuitable for the investigation of T₃ effects in vitro. Therefore, in this study, we isolated pre-adipocytes from rat white and brown adipose tissue, grew them to confluence, and then tested the responsiveness of lipogenesis and lipogenic mRNAs to hormonal manipulation in these two different types of adipocyte.

MATERIALS AND METHODS

Animals. Male Holtzman rats, initially weighing 150–175 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). They were housed individually in stainless-steel wire mesh cages on a 12/12-hour light/dark cycle (light from 0700 to 1900) at 20–22°C. The animals were given free access to rodent laboratory chow (Purina Mills, St. Louis, MO, USA). Hypothyroidism was induced by the addition of 0.025% methimazole to the drinking water for three weeks. All protocols were approved by the Institutional Animal Care and Use Committee.

Cell isolation and culture. Hypothyroid animals were used for isolating preadipocytes because they provided an increased yield of cells from BAT, though similar results were seen when cells from euthyroid animals were used. Epididymal white adipocytes and interscapular brown adipocytes were isolated as described by Nechad et al. (20) except that hypo-osmotic shock was omitted. Porcine insulin was a generous gift from Eli Lilly (Indianapolis, IN, USA). T₃ and dexamethasone were from Sigma (St. Louis, MO, USA). Fat accumulation was monitored by Oil Red O staining (21) and DNA content using the Burton reagent (22). Observations were made in triplicate and all experiments were repeated 2–5 times.

Measurement of lipogenesis. The cells were supplemented with 0.5 mCi 3H₂O (New England Nuclear, Boston, MA, USA) per dish, and labeled for 2.5 h at 37°C at the end of hormone treatment. Media were aspirated and cells were detached by the addition of 0.5 ml 200 mM EDTA for 20 min. Lipids were extracted by the method of Folch et al. (23), the organic extracts back-washed with water, and tritium content assessed by liquid scintillation counting.

Extraction of RNA and Northern blot analysis. Total RNA was isolated using the guanidium thiocyanate/acid phenol method (24) and ACC, FAS, and S14 mRNA levels were measured by Northern analysis using [³²P]-cRNA probes as previously described (25). The uniformity of loading and specificity of treatment effects were confirmed by hybridizing all membranes with a β-actin cRNA probe.

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 and t-test were employed to examine differences between individual treatment conditions. All calculations were performed using Statview software (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Growth and differentiation of preadipocytes

In preliminary experiments, culture conditions were varied to determine those optimal for observing hormonal effects on lipogenesis. Both white and brown preadipocytes reached confluence after about 5 d. Insulin, isobutylmethylxanthine (IBMX), and dexam-
Table 1. The effect of T3 and insulin on lipogenesis in white and brown adipocytes in the presence or absence of dexamethasone.

|                  | White adipocytes | Brown adipocytes |
|------------------|------------------|------------------|
|                  | nmoles H incorporated into lipid/dish/h |                  |
| Dexamethasone     |                  |                  |
| None             | 223±14           | 305±13           |
| 25 nm            | 105±3*           | 313±9            |
| T3 (1 μM)        | 297±9*           | 415±7**          |
| Insulin (1 nM)   | 971±35           | 1293±61          |
| T3+Insulin       | 1065±33          | 1492±69          |
|                  | 1482±38**        | 2022±92**        |

Confluent preadipocytes were incubated in the presence of the hormones indicated for 72 h and lipogenesis assessed by the incorporation of tritiated water as described in Materials and Methods. The results are expressed as mean±SE (n=3). Similar results were seen in two additional experiments. *p<0.05, **p<0.01 for the effect of T3; †p<0.01 for the effect of dexamethasone. All additions of insulin stimulated lipogenesis significantly (p<0.01).

Fig. 1. Time course of lipogenic induction by insulin and T3 in white and brown adipocytes. Preadipocytes were isolated and incubated as described in Materials and Methods. At confluence, white (A) and brown (B) adipocytes were treated with 25 nm dexamethasone alone (control, ○) or with the addition of 1 μM T3 (●), 1 nM insulin (△), or T3+ insulin (●). Lipogenesis was measured at the times shown using tritiated water, and is expressed as nmoles of H incorporated into lipid/h/dish. Values shown are mean±S.E. (n=3). Similar results were seen in three separate experiments. Values that show a significant effect of T3 in the presence or absence of insulin are indicated by *p<0.05, **p<0.01.

Effects of insulin and thyroid hormone on lipogenesis

Insulin (1 nM) induced fatty acid synthesis in both white and brown adipocytes within 24 h (Fig. 1). Lipogenesis continued to increase for up to 96 h in white adipocytes, but the maximum response was detected at 72 h in brown adipocytes, probably reflecting the beginning of cell detachment. Overall, insulin stimulated fatty acid synthesis about 9-fold in the presence of dexamethasone (Table 1).

T3 significantly increased lipogenesis in white adipocytes by 24 h in the presence of insulin or 48 h in its absence (Fig. 1). Lipogenesis was also stimulated in brown adipocytes, though a significant increase was not observed until 72 h of T3 treatment. The stimulation produced by T3 was always modest (1.5–2-fold) either in the presence or absence of insulin (Table 1).
Fig. 2. The effects of insulin and T₃ on ACC, FAS, and S14 mRNA levels in white and brown adipocytes. Confluent white (A) or brown (B) adipocytes were incubated for 72 h with 25 nM dexamethasone alone (C), or with 1 µM T₃ (T), 1 nM insulin (I), or both (I+T). Total RNA was extracted and 15 µg was electrophoresed through a denaturing agarose gel, transferred to a nitrocellulose membrane, and hybridized to ³²P-cRNA probes recognizing ACC, FAS, and S14 mRNA as described in Materials and Methods. The sizes of the mRNA bands were estimated with reference to an RNA ladder (Life Technologies, Gaithersburg, MD, USA) 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 S14-cRNA probe cross-hybridized with the 18S RNA, which can also been seen in the autoradiograms.

**Effects of insulin and thyroid hormone on expression of lipogenic mRNAs**

Insulin treatment provoked a substantial (8-12-fold) increase in the levels of both ACC and FAS mRNAs in the absence of T₃ (Fig. 2, Table 2). T₃ itself produced much smaller stimulation, about 2-3-fold in the absence of insulin. When both hormones were present, the effects appeared additive, such that the increment produced by T₃ was similar in the presence or absence of insulin. The responses of ACC and FAS mRNAs were similar to each other in both white and brown adipocytes. S14 mRNA also exhibited a large response to insulin, but no significant response to T₃ in the absence of insulin (Fig. 2, Table 2). When insulin was present, T₃ increased the level of mRNA-S14 1.5-2.0-fold.

**The effects of increasing glucose concentration**

The effects of T₃ on lipogenesis and lipogenic enzyme mRNAs that we observed were relatively small. It appeared possible that the glucose concentrations in the media (5 mM) might not be optimal for observing maximal stimulation by T₃. The experiments were therefore repeated in the presence of 25 mM glucose. Although this procedure did increase lipogenesis in white adipocytes in the absence of added hormones, the high glucose medium did not increase fatty acid synthesis or ACC and FAS mRNA levels in the presence of T₃ and/or insulin (Fig. 3, Table 3). However, the expression of S14 mRNA was increased by 25 mM glucose, with an increase of up to 2-fold being seen.

| Table 2. The effects of T₃ and insulin on the levels of lipogenic mRNAs in white and brown adipocytes. |
|-------------------------------------------------|
|                                  ACC mRNA  |  FAS mRNA  | S14 mRNA |
|---------------------------------|-----------|---------|
| **White adipocytes**            |           |         |         |
| Control                         | 0.77±0.05 | 0.91±0.10 | 1.01±0.09 |
| T₃ (1 µM)                       | 2.34±0.01** | 1.97±0.24* | 1.20±0.31 |
| Insulin (4 nM)                  | 8.9±1.15  | 12.2±0.63 | 8.96±0.97 |
| T₃+Insulin                     | 9.38±0.88  | 14.5±0.85  | 17.7±1.90* |
| **Brown adipocytes**            |           |         |         |
| Control                         | 0.79±0.09  | 0.93±0.12 | 1.34±0.16 |
| T₃ (1 µM)                       | 1.85±0.16** | 2.77±0.26** | 1.25±0.14 |
| Insulin (4 nM)                  | 9.29±0.77  | 7.39±0.23  | 4.59±0.89 |
| T₃+Insulin                     | 14.0±1.48* | 8.98±0.36* | 7.32±0.98* |

Confluent preadipocytes were cultured for 72 h with the additions shown. RNA was extracted and specific mRNA levels were measured by Northern analysis as described in Materials and Methods. mRNA levels are expressed as a fold of the values measured in cells prior to treatment (mean±SE, n=3). *p<0.05, **p<0.01 for the effect of T₃. All additions of insulin significantly increased lipogenic mRNA levels (p<0.01).
Fig. 3. The effects of glucose on ACC, FAS, and S14 mRNA levels in white and brown adipocytes. Confluent white (A) or brown (B) adipocytes were incubated for 72 h in the presence of either 5 mM glucose and dexamethasone (C), 1 μM T₃ (T), insulin+T₃ (I+T), or 25 mM glucose (HG) were added as shown. Total RNA was extracted and 15 μg was electrophoresed through a denaturing agarose gel, transferred to a nitrocellulose membrane, and hybridized to ³²P-cRNA probes recognizing ACC, FAS, and S14 mRNA as described in Materials and Methods.

Table 3. Effects of glucose concentration on lipogenic parameters in white and brown adipocytes.

| Treatment        | Lipogenesis | ACC mRNA nmoles H inc./h/dish | FAS mRNA nmoles H inc./h/dish | S14 mRNA |
|------------------|-------------|--------------------------------|--------------------------------|----------|
|                  | LG          | HG                             | LG                             | HG       | LG      | HG      |
| White adipocytes |             |                                |                                |          |         |         |
| None             | 92±4        | 178±4**                        |                                |          |         |         |
| T₃ (1 μM)        | 230±10      | 226±8                          | 2.71±0.43                      | 2.65±0.38 | 1.28±0.10 | 1.17±0.15 | 1.37±0.40 | 1.86±0.11 |
| Insulin (1 nm)   | 1229±62     | 1298±61                        |                                |          |         |         |
| T₃+Insulin       | 1564±53     | 1375±105                       | 8.97±0.40                      | 7.26±0.29 | 5.47±0.31 | 4.89±0.21 | 7.21±0.85 | 14.6±0.08**|
| Brown adipocytes |             |                                |                                |          |         |         |
| None             | 185±4       | 259±35                         |                                |          |         |         |
| T₃ (1 μM)        | 277±15      | 203±11                         | 1.60±0.04                      | 1.71±0.03 | 2.10±0.78 | 2.27±0.05 | 1.23±0.19 | 1.79±0.01* |
| Insulin (1 nm)   | 1444±97     | 1329±68                        |                                |          |         |         |
| T₃+Insulin       | 1566±80     | 1686±42                        | 5.22±0.10                      | 4.99±0.13 | 3.99±0.36 | 4.06±0.49 | 4.25±0.15 | 6.24±0.19**|

Confluent preadipocytes were cultured for 72 h in the presence of either 5 mM (LG) or 25 mM (HG) glucose plus or minus hormones as shown. mRNA levels are expressed as a fold of the values seen in cells incubated in 5 mM glucose without hormones. Values represent the mean±SE of three observations. Similar results were seen in a duplicate experiment. *p<0.05, **p<0.01 for the effect of glucose.

DISCUSSION

White and brown preadipocytes were successfully grown to confluence and differentiated into mature, fat accumulating cells. Their distinct phenotype was shown by the pattern of fat accumulation seen in the two types of cell, as previously reported by Nechad et al. (20). Once confluent, they maintained their metabolic activity for at least 3 d and thus constitute a stable and active system useful for studying hormonal or nutritional effects that demand long-term treatment.

A motivation for developing this system was to try and understand the discrepancies between different reports on the effects of thyroid state on lipogenic param-
duced activity of the pathway seen under hypothyroid conditions to decay. This is not surprising for the induced activity of the pathway seen under hypothryoid lipogenic activity requires the presence of the sympathetic nervous system (16). In chronically denervated animals, rates of fatty acid synthesis are low in hypothryoid BAT and then stimulated about 6-fold by chronic treatment with hyperthyroid doses of \( T_3 \). Lipogenic mRNA levels were affected in a parallel manner (16). This is consistent with the results reported here, except that the fold induction produced by \( T_3 \) in the brown adipocytes in vitro is less than half that seen in denervated tissue in vivo. The difference between the current data in cultured adipocytes and that seen in freshly isolated cells (15) may well reflect the time taken for the induced activity of the pathway seen under hypothryoid conditions to decay. This is not surprising since it occurs at the level of gene expression (16).

Lorenzo et al. (26) studied the regulation of lipogenesis in cultured fetal brown adipocytes. They reported approximately a 50% stimulation of fatty acid synthesis by \( T_3 \) treatment for 24 h in the presence or absence of insulin, quite similar to the effects seen here. Perez-Castillo and coworkers (27) investigated the regulation of S14 gene expression in brown adipocytes using very similar protocols for cell isolation. One difference was that they obtained their cells from 20-d-old euthyroid rats rather than the 10-wk-old hypothyroid animals that we used. They observed a major induction of mRNA S14 during cell differentiation, and then showed that \( T_3 \) increased this parameter 3-fold in mature cells as compared to the 1.6-fold induction seen in the current experiments. We were unable to increase those responses by substituting hormone-depleted serum (28) or omitting serum entirely.

Supplementing the media with additional glucose resulted in only minor effects on lipogenesis and lipogenic mRNA expression, and did not enhance the response to \( T_3 \) in our experiments. In hepatocytes, a similar change in media glucose concentration induced much larger changes in S14 mRNA expression. There was also a synergistic interaction between carbohydrate and \( T_3 \) in the liver, both in vivo and in vitro (29). However, the synergistic interaction was less apparent in epididymal fat, being seen with malic enzyme (30). Therefore, it can be postulated that there is tissue-specific regulation of lipogenic gene expression by glucose. The induction of S14 mRNA levels by glucose has been demonstrated in brown adipocytes in vitro (27), but only after prolonged pre-incubation of cells in low glucose media to reduce glucose to very low levels. Romero et al. (31) reported that \( T_3 \) increased glucose uptake and two glucose carriers, GLUT1 and GLUT4 transporter proteins in 3T3-L1 adipocytes. The data presented here suggest that the addition of glucose above normal levels has little effect in both white and brown adipocytes, and that media glucose is not limiting the response to \( T_3 \) in these systems.

These experiments have shown that \( T_3 \) has small and stimulatory effects on lipogenesis in adipocytes. The effects are seen at a transcriptional level through the coordinate induction of ACC, FAS, and S14 mRNAs. Although lipogenic rates were usually higher in brown adipocytes than white adipocytes, very similar patterns of regulation were seen in the two cell types. These data support the idea that the divergent results seen concerning \( T_3 \) regulation of the lipogenic pathway in both brown and white adipose tissue in vivo arise from secondary effects of the alteration of thyroid status.

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