Thyroid Hormone Directly Regulates Cholesterol 7-alpha-Hyroxylase (CYP7A) by Binding to the CYP7A Promoter

BACKGROUND AND OBJECTIVE

For over 70 years the inverse relationship between serum thyroid hormone (T3) and cholesterol levels has been known, but no mechanism behind this phenomenon has been established (1). In hyperthyroidism serum cholesterol levels decline. Conversely, in hypothyroidism serum cholesterol levels are elevated (hence the inverse relationship). The Osborne lab is investigating key target genes, including the Low-Density Lipoprotein Receptor (LDLR) gene and Cholesterol 7-alpha-hydroxylase (CYP 7A) gene, to ascertain a mode of action behind the aforementioned relationship. Both of these genes are either directly or indirectly activated by thyroid hormone.

Thyroid hormone works at the level of transcription by binding to its receptor, the thyroid hormone receptor (TR) (1). Thyroid hormone, by virtue of its chemical structure, is able to penetrate the phospholipid bilayer and bind to its receptor located within the cell. There are four variants of the thyroid hormone receptor: alpha-1, alpha-2, beta-1, and beta-2. TR beta-1 is the dominant receptor in the liver (4). Furthermore, TR beta-1 has been identified as the mediator of T3 action on CYP7A and consequently as a major regulator of cholesterol metabolism in vivo (4). TR beta knockout mice exhibit a reduced T3 dependent stimulation of CYP7A activity and mRNA level, but the same result was not observed for TR alpha knockout mice. Our lab will be using TR beta-1 in transfection studies as explained in the next section.

T3 binds to TR which then complexes with RXR and the TR/RXR heterodimer, the liganded complex binds to DNA sites in target genes. A multi-step process is then initiated which ultimately results in gene activation (1).

Our proposed mechanism can be divided into two parts: regulation of cholesterol at the level of uptake and excretion. A previous study by the Osborne Lab suggests that cholesterol uptake is indirectly regulated by T3. More specifically, it was demonstrated that the gene encoding sterol regulatory element binding protein 2 (SREBP-2), a major direct transcriptional regulator of genes involved in cholesterol uptake, was directly regulated by T3. It was further proposed that the SREBP-2 activation, in turn, activated LDLR which resulted in increased LDL-cholesterol uptake and a decrease in serum cholesterol levels (1). However, this explanation is only half the picture. We further propose that T3 regulates serum cholesterol levels at the level of cholesterol catabolism into bile acids in the liver.

The liver enzyme, cholesterol 7-alpha-hydroxylase (CYP7A), catalyzes the first and rate-limiting step in the conversion of cholesterol to bile acids (2). Regulation of CYP7A occurs at the level of transcription. In previous studies it was indicated that taurocholate, a bile acid, represses the CYP7A activity, both *in vivo* and in primary hepatocytes in culture. Northern blot analysis of RNA isolated from primary hepatocytes, cultured in the absence or presence of 50 μM taurocholate, demonstrated that the addition of taurocholate led to an eightfold decrease in the level of CYP7A mRNA. Since the CYP7A mRNA levels declined, it was concluded that bile acids do in fact regulate CYP7A at the level of transcription (2). Furthermore, the results of a different study suggested that T3 activates CYP7A at the transcriptional level however, no thyroid hormone receptor element (TRE) was established in that particular study (3). We hypothesize that: one, the CYP7A promoter is activated by T3, and two, a thyroid hormone receptor element (TRE) is located between .342 kilo-base pairs and 7.5 kilo-base pairs upstream of the CYP7A +1 start site of the gene. This hypothesis implies the following mechanism: if CYP7A is in fact directly

regulated by T3 then in hyperthyroid conditions more cholesterol is converted to bile acids, which are excreted in the feces and serum cholesterol levels decline.

APPROACH

To date, we have already constructed .342 kb, 3.8 kb, and 7.5 kb CYP7A promoter constructs and we have transfected each promoter into HepG2 cells. We will be subsequently creating different length CYP7A constructs. As of now, the region of the promoter we will be cloning is not known. However, subsequent constructs will most probably contain segments of the promoter ranging from 342 to the more upstream 5' boundary at 3.8 kb. Cloning will require plasmids, restriction enzymes to cut plasmids, and calcium chloride to induce plasmid uptake in *E. coli* cells. Once the appropriate constructs are prepared, they will be transfected into HepG2 (liver) cells.

HepG2 cells will be analyzed by standard methods we routinely use successfully already. The cells will be maintained in minimum essential medium supplemented with 10% fetal bovine serum at 37 C and 5% CO2. One day before transfection, cells will be seeded in 6-well plates at 350,000 cells/well. After incubation for 16 hours, the HepG2 cells will be transfected with CYP7A promoter constructs (the nature of the construct will vary depending on the particular experiment) fused with the luciferase reporter gene, TR-beta (2 μg) expression vector, RXR-alpha (0.5 μg) expression vector, beta-galactosidase expression vector as an internal control for transfection efficiency. For the purpose of control not all groups will contain TR-beta or RXR-alpha; but rather, some groups will contain one or the other, both, or none. Appropriate amounts of Salmon sperm DNA will be added to equalize total amounts of DNA in each sample. After 4-6 hours, the cells will be treated with 10% glycerol with an incubation time of three minutes in order to induce exogenous DNA uptake. The cells will then be washed three times with phosphate-buffered saline and re-fed with serum-free minimum essential medium in the presence or absence of T3. The cells will then be incubated between 40-48 hours prior to being harvested. Once harvested, luciferase and beta-galactosidase assays will be performed using standard protocols.

OUTCOMES AND PREDICTIONS

The results, thus far, indicate that there is a relatively higher level of activation in the 3.8 kb and 7.5 kb CYP7A promoter constructs when T3 and TR/RXR are included with the promoter construct. The 7.5 kb CYP7A promoter has consistently showed greater levels of activation than the 3.8 kb construct. In contrast, the construct retaining only 342 bps of 5' flanking DNA was not activated by T3 in two transfection experiments (see figure 1). We will create new constructs to further define the TR responsive region. Starting with the construct containing 3.8 KB of 5' flanking DNA we will make serial truncations and analyzed them by the assay procedures describe above. In subsequent studies, different constructs will be created depending on the results of subsequent transfection experiments. With the results of our transfection experiments thus far in mind, I am certain our ultimate results will also indicate that CYP7A is activated directly by T3. However, finding the location of thyroid hormone receptor binding site is the more challenging aim of the project.

The Osborne lab has already obtained data suggesting that thyroid hormone regulation and cholesterol metabolism are linked through SREBP-2. The data suggested that in the presence of high T3 levels (hyperthyroidism), the TR/RXR heterodimer activates SREBP-2 which in turn results in LDL receptor activation. In the presence of a greater number of LDLR units, more cholesterol is taken up by cells and therefore serum cholesterol levels decline. However, as mentioned above, this mechanism is only half the story.

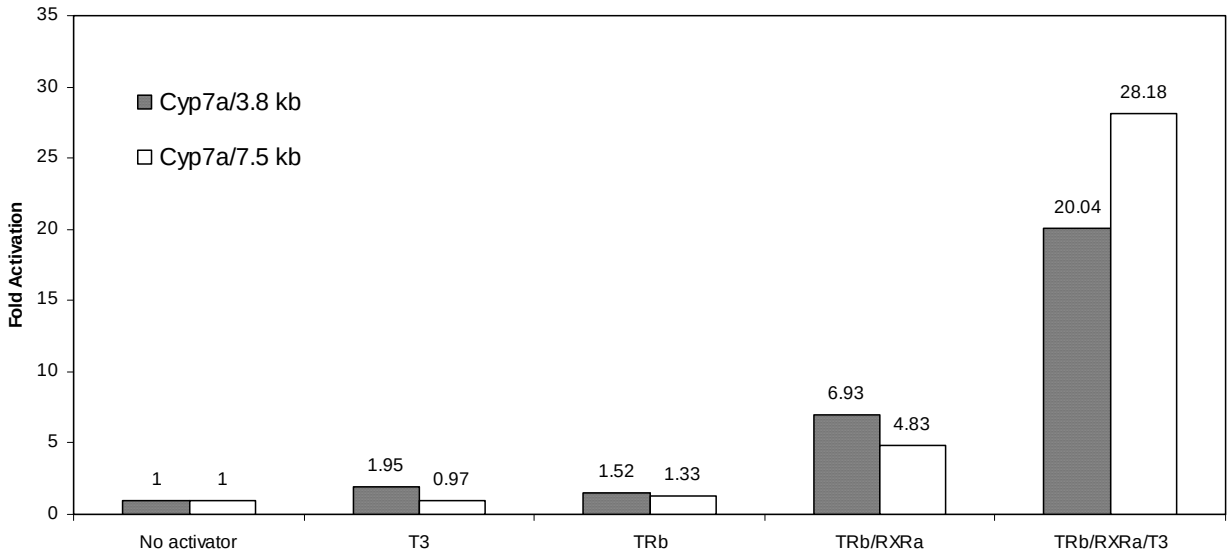

Figure 1. Two CYP7A promoter constructs (3.8 kb and 7.5 kb) fused with the luciferase reporter gene were assayed. A beta-galactosidase assay was also performed for normalization. Then the fold activation for each construct under the indicated conditions was plotted.

CYP7A activation also occurs in the presence of T3 and therefore the results of my project may complement the results of the other Osborne lab project. If the mechanism behind the inverse relationship of serum thyroid hormone and cholesterol levels is ascertained, then it would of benefit to patients currently suffering from thyroid hormone and cholesterol deficiencies. Once a mechanism behind a biochemical phenomenon is known, pharmaceuticals can be designed to target key genes in the pathway.

SPECIFIC RESPONSIBILITIES

1. Prepare CYP7A constructs using restriction enzymes
2. Clone CYP7A promoter constructs
3. Perform CYP7A transfection with HepG2 cells and subsequent cell harvesting
4. Perform luciferase and beta-galactosidase assays after cell harvesting
5. Input data

TIMELINE

September 15 to October 15 – Performed first round of transfections plus assays
October 15 to November 15 – Will prepare and clone second set of CYP7A constructs
November 15 to December 15 – Will perform second round of transfections plus assays
The above three month procedure may be repeated one or two more times with a different set of CYP7A promoter constructs each time.

Note: Data will be ready to be presented by early May in time for the Symposium.

REFERENCES:

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2. Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G., Mager, W.H. (1993). Transcriptional Regulation of the Gene Encoding Cholesterol 7-Alpha- Hydroxylase in the Rat. *Gene* **130**, 217-223.

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Itemized Budget

Thyroid Hormone Directly Activates Cholesterol 7-alpha-Hydroxylase (CYP7A) by Binding to the CYP7A Promoter

Item	Cost
Luciferase Assay Kit	$750
Restriction Enzymes and Poligonucleotides	$250

The luciferase assay kit is necessary to determine the levels of CYP7A activation
The restriction enzymes are required to create the desired CYP7A constructs.

Total Expenses:
$1000