Kynurenamine Metabolism in Hyperthyroidism

A BIOCHEMICAL BASIS FOR THE LOW NAD(P) LEVEL IN HYPERTHYROID RAT LIVER*

(Received for publication, August 13, 1971)

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SUMMARY

Subcutaneous administration of L-thyroxine (75 µg/100 g of body weight per day) to rats for 10 to 14 days caused a decrease in the activity of kynurenine 3-hydroxylase to about 50% of the control value. Enzymic properties of kynurenine 3-hydroxylase of mitochondrial outer membrane preparations isolated from normal and L-thyroxine-treated rats were almost identical, suggesting that L-thyroxine acted by controlling the amount rather than the structure of kynurenine 3-hydroxylase. Analyses of the time course of changes in enzyme activities following the institution and withdrawal of L-thyroxine indicated that the decreased level of kynurenine 3-hydroxylase was caused by a decreased synthesis of the enzyme, rather than an increased degradation. In contrast, the activity of kynurenine aminotransferase localized in the mitochondrial inner membrane was increased about 16 times by L-thyroxine administration. This L-thyroxine-induced rise in kynurenine aminotransferase activity was completely inhibited by ethionine or cycloheximide, suggesting that L-thyroxine induced the de novo synthesis of kynurenine aminotransferase. Kynurenine hydrolase, an extramitochondrial enzyme, was not affected however by the hormone. These enzyme patterns observed in hyperthyroid rats were consistent with the urinary levels of kynurenine and its metabolites. These results are considered in terms of the NAD(P) synthesis from tryptophan in hyperthyroidism.

In mammalian liver, L-tryptophan is metabolized to L-kynurenine via N’-formyl-L-kynurenine. L-Kynurenine thus formed may undergo three reactions, namely (a) hydroxylation to form 3-hydroxy-L-kynurenine, (b) transamination to produce kynurenic acid, and (c) hydrolytic cleavage to form anthranilate and L-alanine. The three reactions are catalyzed by kynurenine 3-hydroxylase (EC 1.14.1.2), kynurenine aminotransferase (EC 2.6.1.7), and kynurenine hydrolase (EC 3.7.1.9), respectively. In preceding papers (1-5), we have shown that the three enzymes are topographically different: kynurenine 3-hydroxylase is localized in the outer mitochondrial membrane, kynurenine aminotransferase in the inner mitochondrial membrane, and kynurenine hydrolase in the cytosol (Fig. 1). However, the control mechanism by which kynurenine is channeled into the three pathways has been completely unknown. The recent work of Okamoto indicating that the topographically different intramitochondrial enzymes are differently affected by thyroid hormone (6) may provide an important clue in understanding the regulation of kynurenine metabolism.

In this study we have determined the effect of thyroid hormone on the levels of enzymes involved in kynurenine metabolism of rat liver and the major pathway of kynurenine metabolism in hyperthyroid rats. Attempts were made to correlate these experimental observations with the NAD(P) synthesis from tryptophan in hyperthyroidism.

EXPERIMENTAL PROCEDURE

Materials—Male Wistar rats weighing from 180 to 220 g were maintained on commercial complete rat diet (laboratory chow) or ethionine diet. The latter preparation included addition of 0.5% dl-ethionine to the complete diet.

L-Kynurenine, kynurenic acid, xanthurenic acid, NADPH, α-ketoglutaric acid, pyridoxal phosphate, cycloheximide, L-thyroxine (sodium salt), and desiccated thyroid powder were products of Sigma and 3-hydroxy-L-kynurenine was a product of Calbiochem. dl-Ethionine was purchased from Nakarai

$ This investigation was supported in part by grants from the Squibb Institute for Medical Research and the Scientific Research Fund of the Ministry of Education of Japan.

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Fig. 1. Kynurenine metabolism in rat liver.
Enzyme Assay—Kynurenine 3-hydroxylase, kynurenine aminotransferase, and kynurenine hydrolase were assayed as previously described (5). The reported values represent the average of four to eight rats plus the standard error of the mean (Figs. 2 and 3).

Determination of Kynurenine, 3-Hydroxykynurenine, Kynurenic Acid, and Xanthurenic Acid in Rat Urine—An aliquot of rat urine was applied to a column of Dowex 50 and kynurenine and its metabolites were eluted according to the method of Brown and Price (8). Kynurenine and 3-hydroxykynurenine were estimated according to the method of Brown and Price (8) and Brown (9), respectively. Kynurenic and xanthurenic acids were estimated by their fluorescence characteristics in acid and alkali (10) with an Amino-Bowman spectrophotofluorometer.

RESULTS

Effects of L-Thyroxine Administration on Kynurenine 3-Hydroxylase, Kynurenine Aminotransferase, and Kynurenine Hydrolase in Rat Liver—Time courses of activities of kynurenine 3-hydroxylase, kynurenine aminotransferase, and kynurenine hydrolase as a result of daily administration of L-thyroxine are shown in Fig. 2. The kynurenine 3-hydroxylase activity of mitochondria from L-thyroxine-treated rats decreased and the maximum response occurred 10 to 14 days following treatment. The decreased level of the enzyme activity completely recovered within about 6 days after withdrawal of the hormone. In confirmation of the previous report (6) the kynurenine 3-hydroxylase activity of the mitochondrial outer membrane isolated from L-thyroxine-treated rats also decreased to about 50% of the control value. In contrast, the kynurenine aminotransferase activity of mitochondria from L-thyroxine-treated rats was about 1.4 times as great as that of mitochondria from normal rats. The maximum response occurred after 3 to 4 days and further administration of L-thyroxine revealed a tendency to decrease the enzyme activity. However, the kynurenine hydrolase activity in the cytosol of L-thyroxine-treated rats was found to be essentially unchanged. The possibility that in vivo effects of L-thyroxine were caused by the direct effect of L-thyroxine on the enzymes has been ruled out by the in vitro experiments (6).

Effects of Protein Synthesis Inhibitors on L-Thyroxine-induced Kynurenine Aminotransferase—Fig. 3 shows the effect of ethionine or cycloheximide, an inhibitor of protein synthesis, on the response of kynurenine aminotransferase to L-thyroxine. The 14 times increase in the enzyme activity, which was produced by the L-thyroxine administration, was largely abolished when the inhibitor was administered with the hormone. Ethionine or cycloheximide given alone elicited no decrease in enzyme activity. These results indicate that the thyroxine-induced increase in kynurenine aminotransferase activity was caused by a de novo synthesis of enzyme protein.

Rates of Synthesis and Degradation of Kynurenine 3-Hydroxylase—In order to interpret meaningfully the result of the influence of L-thyroxine on kynurenine 3-hydroxylase (Fig. 2), the rate constants of synthesis and degradation of the enzyme protein were measured by the time course of changes in the enzyme activity, since the concentration of an enzyme in mammalian tissues can be selectively altered by changes in the rate of either protein synthesis or protein degradation. For the calculation of rate constants, it was assumed that the rate of changes of enzyme level was the algebraic sum of a zero order rate of synthesis and a first order rate of degradation (11-13). Thus,
Effect of Activator on Kynurenine 3-Hydroxylase—It has been shown that kynurenine 3-hydroxylase preparation from rat liver mitochondria was activated with monovalent anions (14). Studies on the effect of various concentrations of potassium chloride were carried out with the enzyme preparations from both normal and L-thyroxine-treated rats (Fig. 6). In both enzyme preparations, kynurenine 3-hydroxylase was similarly activated with various concentrations of the monovalent anion and maximal activation was obtained at 10 mM for potassium chloride.

Effect of Substrate Concentration on Kynurenine 3-Hydroxylase Activity—Michaelis constants of kynurenine 3-hydroxylase were determined on the mitochondrial outer membrane preparation from L-thyroxine-treated rats. The apparent $K_m$ values of the enzyme preparation were 0.023 mM for L-kynurenine and 0.021 mM for NADPH. These were almost identical with the $K_m$ values of the enzyme prepared from normal rat liver (3, 14), indicating no difference between the catalytic properties of enzymes from the two sources.

Effect of pH on Kynurenine 3-Hydroxylase from L-Thyroxine-treated Rats—The effect of pH on the reaction rate of kynurenine 3-hydroxylase of the mitochondrial outer membrane from L-thyroxine-treated rats was studied from pH 6 to 9 with potassium phosphate buffer at pH range 6.0 to 8.5 and Tris buffer for the pH range 7.0 to 9.0. A slightly higher activity was obtained in Tris buffer than in potassium phosphate buffer at pH 8.0 to 8.5 and the maximal enzyme activity was observed between pH 8.0 and 8.2, exhibiting the same pH profile of the enzyme preparation from normal rats (14).

\[ \ln(E - E') = \ln(E_0 - E') - k't \] and \[ \ln(E_0 - E) = \ln(E_0 - E') - k't \]

where $E$, $E'$, and $E_0$ are the enzyme-specific activity at any time (0), the new steady state, and the starting steady state, respectively. A plot of $\ln(E - E' \phi)$ versus $t$ as the activity was falling to the steady state value of the high hormone level allowed the calculation of $k't$, the rate constant for the degradation reaction under conditions of high hormone (Fig. 4A). A plot of $\ln(E_0 - E)$ versus $t$ as the activity was returning to the normal steady state level allowed the determination of $k_2$, the rate constant for the degradation reaction under normal hormone conditions (Fig. 4B). It was found that the rate constants for degradation of kynurenine 3-hydroxylase under the influence of high and normal hormone levels were almost identical (Fig. 4, A and B). From the rate constants for degradation and the steady state level of the enzyme, it is possible (11-13) to calculate the rates of synthesis of the enzyme in normal and L-thyroxine-treated animals (Table I). The conclusion from these results was that the decrease in kynurenine 3-hydroxylase level after L-thyroxine administration was an effect of a decreased rate of degradation. When animals were injected subcutaneously with 60 and 80 mg per day of L-thyroxine (sodium salt), almost the same values of rate constants were obtained.

Thermal Inactivation of Kynurenine 3-Hydroxylase—Mitochondrial outer membranes, isolated from livers of both normal and L-thyroxine-treated rats, were suspended in the mannitol solution and heated at various temperatures in a constant temperature water bath. Aliquots were removed every 15 min and the residual activities of the kynurenine 3-hydroxylase were determined.

Activity—Michaelis constants of kynurenine 3-hydroxylase were determined on the mitochondrial outer membrane preparation from normal rats (14).

\[ k_2 = 0.36 \text{ day}^{-1} \]

\[ k_2 = 0.42 \text{ day}^{-1} \]

Table I

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<tr>
<th>Status</th>
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<tr>
<td>Normal</td>
<td>100</td>
<td>0.42</td>
<td>42</td>
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<tr>
<td>L-Thyroxine treated</td>
<td>52</td>
<td>0.38</td>
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Fig. 4. A, logarithmic plot of decrease in kynurenine 3-hydroxylase activity consequent to administration of hormone. Symbols (○) as in Fig. 2. B, logarithmic plot of return to the normal steady state level of kynurenine 3-hydroxylase after withdrawal of hormone. Symbols (○) as in Fig. 2 and the abscissa is days after withdrawal of hormone.

Fig. 5. Effect of heat on the stability of kynurenine 3-hydroxylase in mitochondrial outer membranes from normal and hyperthyroid rat livers. Mitochondrial outer membrane preparations, which were prepared from normal (○) and hyperthyroid (●) rat livers, were adjusted to equivalent protein concentration and heated for the time and temperature indicated. Hyperthyroid rats were prepared by subcutaneous administration of L-thyroxine-sodium at a dose of 75 μg per day per 100 g of body weight for 12 days. Residual kynurenine 3-hydroxylase activity of the outer membrane preparation was measured under the usual conditions. The enzyme activity was 26 nmoles per min per mg of protein of mitochondrial outer membrane from normal rat liver and 14 nmoles per min per mg of protein of mitochondrial outer membrane from hyperthyroid rat liver.
Urinary Levels of Kynurenine and its Metabolites—The urinary levels of kynurenine, 3-hydroxykynurenine, kynurenic acid, and xanthurenic acid in normal and hyperthyroid rats are presented in Table II. The basal urinary levels (Table II, Group IA) of kynurenine plus kynurenic acid and 3-hydroxykynurenine plus xanthurenic acid of endogenous and dietary origin were approximately 0.4 and 0.9 μmole per day per rat, respectively. In the hyperthyroid rat urine (Table II, Group IB), there were approximately 3-fold increases in the corresponding excretions of kynurenine and kynurenic acid over the same 24-hour collection period, whereas the amount of xanthurenic acid excreted was essentially unchanged and 3-hydroxykynurenine could not be detected in the urine sample. Therefore, the ratio of 3-hydroxykynurenine plus xanthurenic acid to kynurenine plus kynurenic acid was decreased to about 0.35. The experiments (Table II, Group II) using a dose of 200 mg of L-tryptophan per rat revealed that in hyperthyroid rats there were about 8-, 3-, and 2-fold increases in excretions of kynurenine, kynurenic acid, and xanthurenic acid, respectively, but the excretion of 3-hydroxykynurenine decreased. The ratio of 3-hydroxykynurenine plus xanthurenic acid to kynurenine plus kynurenic acid decreased from 0.29 of normal rats to 0.13 of hyperthyroid rats. These results indicated that hyperthyroid rats accumulated large quantities of kynurenine and kynurenic acid but 3-hydroxykynurenine was either not excreted or in only small amounts.

**DISCUSSION**

It has been reported that the concentration of pyridine nucleotides in the liver of the hyperthyroid rat is considerably lower than that of normal rats (15–18); however, the mechanism of depression of the coenzyme content has so far been unknown. Maley and Lardy found that the rates of NAD synthesis from NMN and ATP were the same in livers from the normal and the hyperthyroid rat (15). Further, Bosch and Harper indicated that the depression of the coenzyme content did not result from an increase in the rate of coenzyme destruction (18). On the other hand, several investigators suggested that thyroid hormone might exert some influence upon tryptophan metabolism: Matsumoto and Yoshimatsu (19) reported that after removal of rabbit's thyroid gland considerable kynurenine was observed in the urine. Kotake et al. (20) demonstrated that kynurenine aminotransferase activity was increased in liver homogenate of hyperthyroid rats.

The present finding establishes that the various topographically separated enzymes that participate in kynurenine metabolism of rat liver are affected differently by thyroid hormone. As a result of the daily administration of L-thyroxine, the level of kynurenine 3-hydroxylase localized in the mitochondrial outer membrane of rat liver (1-4) decreased to about 50% of the control value. On the other hand, the level of kynurenine aminotransferase localized in the mitochondrial inner membrane (5) increased to about 150%. However, kynurenine hydrolase localized in the cytosol (5) was not affected by the administration.

<table>
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<tr>
<th>Status</th>
<th>Kynurenine</th>
<th>3-Hydroxykynurenine</th>
<th>Kynurenic acid</th>
<th>Xanthurenic acid</th>
<th>Kynurenine + Kynurenic acid</th>
<th>Ratio</th>
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<td>104.7</td>
<td>19.1</td>
<td>161.0</td>
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* Ratio: 3-hydroxykynurenine + xanthurenic acid to kynurenine + kynurenic acid.
of L-thyroxine. This striking contrast suggests the possibility that thyroid hormone may play a role in the regulation of the biosynthesis of NAD(P) from tryptophan. The decreased level of kynurenine 3-hydroxylase in mitochondrial outer membrane may result in the accumulation of kynurenine. Kynurenine aminotransferase in the inner mitochondrial membrane has been found to transamine 3-hydroxy-L-kynurenine as well as L-kynurenine (5). Furthermore, malate dehydrogenase and glutamate dehydrogenase, which are thought to generate the amino group acceptors of kynurenine aminotransferase, α-keto acids, are shown to be localized in the inner mitochondrial membrane (5) and to be increased by L-thyroxine administration (6).

Clinically, hyperthyroidism exists when there is an excessive concentration of thyroid hormones in the blood. It may result from excessive administration of thyroid hormones or from an overproduction of thyroid hormones by goiters. It would therefore be possible that in hyperthyroidism the synthesis of NAD(P) from tryptophan via kynurenine proceeds poorly, some kynurenine appears to be catabolized to kynurenic acid and, if hydroxylated, to xanthurenic acid by kynurenine aminotransferase. This assumption is supported by the detection of kynurenine and its metabolites in the urine.

The experiments described in this paper offer not only the clear-cut demonstration of the regulatory significance of thyroid hormone in kynurenine metabolism of mammals but also a reasonable biochemical explanation for the low NAD(P) level in hyperthyroid animals.

Acknowledgments—Gratitude is due to Mr. Kazuichi Okawa for his able technical assistance and to Mariko Ohara for her assistance with the manuscript.

REFERENCES