On the Role of NADPH and Glutathione in the Catalytic Mechanism of Hepatic Thyroxine 5'-deiodination

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Abstract

A possible metabolic linkage between hepatic thyroxine (T4) 5'-deiodination and the NADPH-glutathione (GSH) cycle was studied in rat liver. Supplementation of 1 mM NADPH to stocked liver homogenates in vitro produced 4-fold increase in 3, 5, 3'-triiodothyronine (T3) formation from T4, whereas the effect of 1 mM FMN, FDN, NAD, NADH, or GSH was relatively small. An exponential dose-response relation was obtained between NADPH and T3 generated. The dose-dependent increase in T3 formation on GSH was eliminated in the presence of 1 mM MADPH, and the additive effect of GSH to NADPH was not apparent in comparison with NADPH alone. Inhibition of T3 generation by graded doses of methylene blue was not affected by the presence of 5 mM GSH. Furthermore, metabolic changes in the hexose-monophosphate shunt were produced in male Wistar rats aged 5 w by treating them with fasting-refeeding (FF group), with the administration of insulin and glucose (IG group), with propylthiouracil (PTU group) and with T4 (T4 group). All these treatments significantly reduced hepatic T4 5'-deiodinase activity (P<0.01-0.001 vs control), while glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GSSG-R) activities were increased. Between generated T3 and G6PD or GSSG-R activity, an inverse correlation was noted (r=-0.802 and -0.933, P<0.001). No consistent relation was found between T4 5'-deiodinase activity and GSH or non-protein SH contents. The addition of 1 mM NADPH and GSH to the homogenates of FF, T4 and the control group stocked for 4 w at -20°C, restored T4 5'-deiodinase activity from a level of 10% to 60% of the initial value, whereas the activity remained depressed in PTU (19%) and the IG group (37%). These results indicate that both GSH and NADPH are important cofactors of the T3 generating system, but NADPH is more rate-limiting and its effect appears to be rather direct, not mediated by GSH formation. It is possible that T4 5'-deiodinase may be one of the NADPH-dependent enzymes.

The extrathyroidal conversion of thyroxine (T₄) to 3, 5, 3'-triiodothyronine (T₃) has been established as the major pathway of T₃ production (Surk et al., 1969. Braverman et al., 1970. Pittman et al., 1971). Little is known, however, of the regulatory mechanism of this reaction in vivo. Thyroid hormone analogues and antithyroid drug were shown to be inhibitory to the system (Oppenheimer et al., 1972. Visser et al., 1975), but these cannot be regarded as physiological factors except 3, 3', 5'-triiodothyronine (rT3) (Heyma et al., 1980). The importance of the sulfhydryl (SH) group and glutathione (GSH) was stressed in vitro studies (Visser et al., 1976. Chopra, 1978. Balsam et al., 1979) and metabolic linkage
between the NADPH-GSH cycle and T₄ conversion to T₃ was suggested (Harris et al., 1979). Recent observation, however, did not substantiate the essential role of GSH or the nonprotein SH group in vivo (Gavin et al., 1980). These controversies prompted us to reevaluate the effect of nicotinamide adenine nucleotides and GSH on T₄ 5'-deiodination in vitro and in vivo. Because of the technical difficulty in measuring these nucleotides directly in biological materials (Buch et al., 1967), we employed stimulatory manipulation of the hoxose-monophosphate shunt in vivo, estimating changes in the activity of glucose-6-phosphate dehydrogenase (G6PD) (EC 1, 1, 1, 49), glutathione reductase (GSSG-R) (EC 1, 6, 4, 2) and the GSH content as the parameters of NADPH-generating system. G6PD activity has been known to be stimulated by fasting-refeeding (Anderson and Hollifield, 1966. Szepesi et al., 1971), high carbohydrate diet or insulin administration (Rudack et al., 1971). On the other hand, T₄ or T₃ administration induced T₄ converting enzyme in thyroidectomized rats (Grussendorf and Hufner, 1977), whereas propylthiouracil (PTU) consistently inhibited the conversion of T₄ to T₃ (Oppenheimer et al., 1972). Accordingly, a possible metabolic linkage between the two systems was examined in rats with these treatments.

Materials and Methods

Male Wistar rats aged 5 w, 115-140 g in weight were provided with commercial rat chow and water ad libitum except where noted. Four experimental groups and control, each consisting of 5 rats, were selected. The PTU group was given a 0.1% PTU solution as drinking water throughout the experimental period. T₄ group was injected with 10 µg/rat of T₄ subcutaneously 3 times on alternate days and sacrificed on the at 3rd day after the last injection. IG group was injected with 8 U/rat of semilente insulin (Novo) subcutaneously once a day for 6 days and given 20% glucose instead of water ad libitum. FF group was fasted for 48 h followed by 3 days of refeeding. After these treatments, blood samples were obtained by heart puncture under ether anesthesia and the liver was removed immediately. One gram of liver was weighed, homogenized in 5 ml of ice-cold 0.1 M phosphate buffer (pH 7.0) containing 5 mM EDTA with Teflon homogenizer. After centrifugation at 3000 rpm for 10 min at 4°C, the supernatant was immediately used for the following assays except for protein and glycogen analysis. The remaining liver was stocked in a refrigerator at -20°C until use. NAD, NADH, NADP, NADPH, FMN, FDN, GSH, GSSG, G6P, glycogen, PTU and T₄ were purchased from Sigma Chemical Co.

T₄ 5’deiodinase assay: The activity was determined by the method of Chopra (1977). Each 1 ml of assay contained 400 µl of 20% liver homogenate, 1.29 µM of T₄ and 500 µl of phosphate-EDTA buffer. When requested, 100 µl of cofactor (10 mM in phosphate buffer) was substituted for the buffer. The mixture was usually incubated for 60 min at 37°C. The reaction was stopped by the addition of 2 ml of 95% ethanol. The T₃ generated was extracted into ethanol and 10-50 µl of the supernatant was used for T₃ radioimmunoassay with a commercially available kit (Eiken T3 RIA kit). The limit of sensitivity for T₃ measurement was 1.25 ng/dl. Endogenous T₃ concentration together with the cross reactivity of added T₄ was measured in each sample without incubation and subtracted from the estimated T₃ value.

G6PD assay (Bergmeyer, 1974)
2.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.2 ml of 0.1 M MgCl₂, 0.1 ml of 10 mM NADP and 0.1 ml of G6P Na-salt (10 mg/ml) were mixed and preincubated at 30°C. The reaction was started with the addition of 20 µl of liver homogenate. The increase in absorbance at 340 nm was recorded and enzyme activity was calculated as µmole-NADPH/min/mg-protein.

GSSG-R assay (Bergmeyer, 1974)
The incubation mixture consisted of 2.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.1 ml of EDTA (10 mg/ml), 0.2 ml of GSSG (50 mg/ml) and 0.05 ml of NADPH Na-salt (10 mg/ml), which was preincubated at 30°C. The reaction was started with the addition of 20 µl of liver homogenate. The decrease in absorbance at 340 nm was recorded with an Hitachi Spectrophotometer, Model 124. To calculate the amount of NADPH formed, the extinction coefficient of ε₃₄₀=6.22 was used and enzyme activity was reported as µmole-NADPH/min/mg-protein.

GSH content
Determination of GSH was performed by a modification of the method of Hissin and Hilf (1976). A mixture of 0.5 ml of 20% homogenate and 1.0 ml of 0.1 M phosphate-5 mM EDTA buffer
(pH 8.0) was deproteinized with 0.5 ml of 25% HPO₃ and centrifuged. To 100 µl of the supernatant, 100 µl of o-phthalaldehyde (1 mg/ml in absolute methanol) and 3.0 ml of phosphate-EDTA buffer were added. After standing for 15 min at room temperature, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with activation at 350 nm. Standard GSH (2.5-10 nmole/tube) was dissolved just prior to use.

Non-protein SH (NPSH) content
Thiol groups were determined by a modification of the method of Sedlak and Lindsay (1968). A mixture of 100 µl of 20% homogenate and 0.9 ml of 5% sulfosalicylic acid was centrifuged. Thiol content of the supernatant was estimated by the addition of 2.5 ml of 0.4 M Tris-HCl buffer (pH 8.9) and 0.1 ml of Ellman's reagent (150 mg of 5, 5'-dithiobis-2-nitrobenzoic acid in 100 ml Tris-HCl buffer). After incubation for 5 min at room temperature, the resulting color was read at 412 nm against a blank. As a reference, 2.5-25 mg/100 ml of GSH solution was used.

Liver glycogen was precipitated with 2 volumes of ethanol at 4°C overnight after 30% KOH digestion and the concentration was determined by the method of Montgomery (1957). Protein was estimated by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Serum T4 and T3 concentration was measured by RIA with a commercially available kit (Spac T4 kit; Daiichi RI Lab. and Eiken T3 RIA kit). Statistical analysis was carried out by Student's t test and the correlation coefficient was calculated by the general formula.

Table 1. Effect of nicotinamide or flavin adenine nucleotides and glutathione on T4 5'-deiodinase activity in vitro.

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>T3 generated (ng/h/mg-protein)</th>
<th>Percent activity for control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>4</td>
<td>1.9 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td>+FDN (1 mM)</td>
<td>4</td>
<td>2.0 ± 0.2</td>
<td>124 ± 5</td>
</tr>
<tr>
<td>+FMN (1 mM)</td>
<td>4</td>
<td>2.1 ± 0.2</td>
<td>132 ± 11</td>
</tr>
<tr>
<td>+NAD (1 mM)</td>
<td>4</td>
<td>2.3 ± 0.3</td>
<td>138 ± 13</td>
</tr>
<tr>
<td>+NADH (1 mM)</td>
<td>4</td>
<td>3.3 ± 1.1</td>
<td>201 ± 44</td>
</tr>
<tr>
<td>+NADP (1 mM)</td>
<td>4</td>
<td>6.7 ± 1.6</td>
<td>409 ± 73</td>
</tr>
<tr>
<td>+NADPH (1 mM)</td>
<td>4</td>
<td>7.6 ± 0.8</td>
<td>463 ± 55</td>
</tr>
<tr>
<td>+GSH (1 mM)</td>
<td>4</td>
<td>2.5 ± 0.5</td>
<td>155 ± 16</td>
</tr>
<tr>
<td>+GSSG (1 mM)</td>
<td>4</td>
<td>1.5 ± 0.2</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>Preheated control**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+NADPH (1 mM)</td>
<td>4</td>
<td>0.4 ± 0.06</td>
<td>28 ± 4</td>
</tr>
</tbody>
</table>

*Livers stocked for 2W at -20°C were used.
**Liver homogenates were preheated at 60°C for 30 min before assay.

Preheated control vs control.

Values represent mean ± SD.

Results

1) Studies in vitro
Effect of the addition of NAD, NADH, NADPH, FDN, FMN, GSH and GSSG on hepatic T4 5'-deiodinase activity is listed in Table 1. Since the effect of these compounds added to fresh liver was rather small in the previous reports (Visser et al., 1975, 1976. Chirasevenuprapund et al., 1978), the homogenates stocked for 2 w at -20°C were used. Marked enhancement of T3 generation from T4 was observed with the supplement of 1 mM NADPH or NADP. A small but significant rise in activity was also noted with 1 mM NAD, NADH, FDN, FMN or GSH, while GSSG showed an inhibitory effect. Preheated homogenates at 60°C for 30 min lost most of the activity.

The dose-response relation between T3 formation and NADPH or GSH is shown in Fig. 1. An exponential curve was obtained with NADPH doses, exhibiting a plateau at 0.25 mM concentration. With 1–5 mM GSH doses, definite activation was also noted, but this effect was masked in the presence of 1 mM NADPH. From these results, however, it is not clear whether NADPH exerts its activating effect via GSH formation or not. Therefore, the time course of T3 generation was examined (Fig. 2). The definite activating effect of NADPH was observed at 15 min of incubation and was 2 fold higher than that of 5 mM GSH throughout the period. The effect of adding GSH to NADPH was not conspicuous. In the next experiment, inhibition of the action of NADPH by graded doses of methylene blue was tested in the presence or the absence of GSH (Fig. 3). Again, GSH did not show any remarkable effect on T3 generation in the system. These results are
Fig. 1. Dose-response relations between generated T3 and NADPH (panel A) or GSH (panel B) with or without 1 mM GSH or 1 mM NADPH supplement, respectively. Liver homogenate stocked at −20°C for 3 w was used. Basal activity of sample A and B was 1.2 and 2.0 ng-T3/h/mg-protein respectively. Each point represents mean of duplicated estimates. The activating effect is more prominent in NADPH than in GSH, which reaches a plateau at 0.25 mM of NADPH. In panel B, dose-related enhancement of T3 formation to GSH is eliminated in the presence of 1 mM NADPH.

Fig. 2. Time courses of T3 generation in the presence of 1 mM NADPH (solid circle), 5 mM GSH (open circle), combination of both (double circle) and control. T3 generation is 2 fold greater with the addition of NADPH than that of GSH, and additive effect is not apparent.

Fig. 3. Comparison of the blocking effect of graded doses of methylene blue on T3 generation with or without 5 mM GSH. Each sample was supplemented with 1 mM NADPH. Endogenous concentration of GSH is 1.1 μmole/g-liver. No significant difference is noted.
against the possibility that NADPH activates the T4 converting system via the GSH generating pathway.

II) Studies in vivo

Table 2 lists changes in body and liver weight, protein and glycogen content, and serum T4 and T3 concentration in the experimental groups and controls. Fasting-refeeding markedly depleted glycogen content, while insulin administration reduced the relative weight of the liver. PTU treatment depressed serum T4 and T3 levels and a prominent increase in glycogen content was noteworthy in this group. T4 group showed a significant decrease in serum T3 and a slight fall in T4 levels, which may be attributable to the feedback inhibition of thyroid hormone secretion after the cessation of exogenous T4.

In Table 3, T4 5'-deiodinase, G6PD and GSSG-R activity, and NPSH and GSH content are shown. T3 generation was significantly decreased in all experimental groups, whereas G6PD and GSSG-R activity was increased. A concomitant rise in GSH and NPSH was noted in PTU group, while these were reduced in IG group. However, no consistent relation was observed between GSH or NPSH and T4 deiodinating activity (Fig. 4) By contrast, a significant inverse correlation was obtained between generated T3 and G6PD or GSSG-R activity (r = -0.802 and -0.933 respectively, P < 0.001) (Fig. 5). G6PD activity in the FF group was markedly increased apart from the regression line.

III) Recovery of T4 5'-deiodinase activity with the supplementation of NADPH and GSH (Fig. 6)

After storing these homogenates for 4 w at -20°C, restoration of T4 5'-deiodinase activity with the addition of 1 mM NADPH and GSH was examined. The homogenates of FF and T4 group recovered a level of activity comparable to that of controls which corresponded to approximately 60%
Fig. 4. Relation between T4 5'-deiodinase activity and GSH or NPSH content. Asterisk; PTU group, square; T4 group, triangle; IG group, open circle; FF group and solid circle; control. No consistent relation is observed.

of the initial activity. In contrast, IG and PTU groups exhibited only a small increase with the addition of NADPH and GSH (37% and 19% respectively). These suggest that the major cause of reduced T3 production in FF and T4 is depression of cofactors, especially NADPH content, whereas that in IG and PTU group is due to a decrease in enzyme concentration per se or blocking of thiol radicals in enzyme protein by PTU.

Discussion

The involvement of thiol groups in the system converting T4 to T3 has been repeatedly demonstrated in vitro (Visser et al., 1976. Chopra, 1978) and in vivo (Harris et al., 1979). Dithiothreitol enhanced the reaction while sulphydryl blocking agents inhibit the conversion. In hypothyroid, starved and neonatal rodents, T4 5'-deiodinase activity well correlated with NPSH content in liver, indicating the importance of NPSH as a cofactor in liver T4 5-deiodinase (Harris et al., 1979). The essential role of GSH in the reaction has been also established (Balsam et al., 1979). Recently, however, Gavin et al. (1980) reported that the effect of diet on T4 monodeiodination in liver was not mediated by changes in the tissue level of SH compounds, but rather involved a change in the concentration of the deiodinase. The results of the present study were also in accordance with their findings. NPSH and GSH content was markedly increased in PTU group. PTU acts as a substrate for GSH transferase, sparing GSH consumption in tissues (Yamada et al., 1978). In contrast, these were significantly decreased in IG group. GSH is known to be a cofactor of insulinase and a large dose of exogenous insulin consumes GSH in tissues (Ammon et al., 1980). Despite these changes, no consistent relation was found between NPSH or GSH content and T4 deiodinase activity (Fig. 4). This suggests the presence of another regulating mechanism in T4 conversion.

NADPH is known to be a cofactor in the GSH generating system. As for the effect of NADPH on the conversion of T4 to T3, Balsam et al. (1979) proposed that NADPH exerted a promoting effect within cytosol, by increasing the concentration of GSH through the action of GSSG-R, because NADPH did not show any effect on microsomal fraction. The results of the present study, however, are rather against this possibility, because (1) the activating effect of NADPH was always greater than that of GSH despite the low endogenous GSH level (1.0 μmole/g-liver) (Table 1, Fig. 1), (2) at the early stage of incubation, NADPH already showed more prominent
enhancement than GSH (Fig. 2), and (3) the presence of GSH did not affect the inhibition of T3 generation by methylene blue, the blocking agent of NADPH (Fig. 3). These results suggest that NADPH exerts a direct effect on the activation of T4 5'-deiodinase rather than that of GSSG-R.

As for the discrepancy on the role of NADPH, a possible explanation is that Balsam et al. used cytosol or microsome fraction of fresh liver, whereas we used liver homogenate stocked for several weeks, by which time it was supposed to be completely depleted of NADPH. As indicated in the dose-response relation of NADPH (Fig. 1), T3 formation reached a plateau at the concentration of 0.25 mM NADPH. Therefore, a fresh sample containing endogenous NADPH might respond less sensitively to an additional dose of NADPH (Visser et al., 1975, 1976).

It is well known that hexose-monophosphate shunt is the major pathway of NADPH generation. Changes in the enzyme activity of this pathway have been reported in several situations. During starvation, there
is an early fall in the activity of G6PD, which returns to normal on refeeding (Anderson and Hollifield, 1966. Szepesi et al., 1971). The induction of G6PD is also observed in high carbohydrate diet or insulin administration (Rudack et al., 1971), and there is a negative feedback regulation between G6PD activity and NADPH content (Nakamura et al., 1979). On the other hand, the effect of diet or starvation on the peripheral T4 monodeiodination has been well documented (Vagenakis et al., 1975, Balsam et al., 1978, 1979, Harris et al., 1979). Fasting decreases the hepatic T3 generating activity, which is restored to normal by enrichment with NADPH (Balsam et al., 1978). Similar results were obtained in this study also. Although direct measurement of NADPH was not performed, T4 5'-deiodinase activity was significantly correlated with G6PD and GSSG-R activity (Fig. 5). Probably the elevated activity of these enzymes reflect an increased turnover or depletion of tissue level of NADPH, which may result in reduced conversion of T4 to T3 by the possible equation: T4 + NADPH → T3 + NADP'* + I'.

In summary, the results of the present study indicate that although both GSH and NADPH play an important role as cofactors in the hepatic conversion of T4 to T3, NADPH appears to be more rate-limiting in the reaction in vitro and in vivo. It remains to be elucidated in future whether T4 5-deiodinase is one of the NADPH-dependent enzymes.

Acknowledgements

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