Correlation of Hepatic Thyroxine 5'-Monodeiodination with Hexose Monophosphate Shunt in Young Rats

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Summary

The role of NADPH and glutathione (GSH) in hepatic thyroxine (T4) 5'-deiodination and possible metabolic linkage between T4 converting system and hexose monophosphate shunt were studied in young rats during maturation. Low activity of T4 5'-deiodinase in young rats was enhanced 2-4-fold with the addition of 1 mM NADPH and GSH *in vitro*, the effect of which was more prominent with NADPH than with GSH. The highest enhancement was observed at 2-3 wk of age, whereas basal T4 5'-deiodinase activity was gradually increased until 5-6 wk of age, decreasing to adult level thereafter. This change was associated with a rise in GSH and glycogen content in the liver and significantly correlated to the changes in glutathione reductase activity ($r = 0.622$, $P < 0.001$). In contrast, glucose-6-phosphate dehydrogenase (G6PD) activity remained depressed until 5 wk of age and rose sharply thereafter. Between T4 5'-deiodinase and G6PD activities after 6 wk of age, an inverse correlation was noted ($r = -0.749$, $P < 0.01$). A dose-response relationship between triiodothyronine (T3) production and NADPH *in vitro* showed similar age-related changes, whereas dose-dependency of T3 formation on GSH was decreasing with age, especially under the presence of 1 mM NADPH.

These results indicate that: (1) NADPH and GSH are important cofactors of T4 conversion to T3; (2) NADPH appears to be more rate-limiting in the maturational process of the system; and (3) hexose monophosphate shunt plays a significant role in the regulation of T4 5'-monodeiodination through NADPH and GSH formation.

Speculation

The physiologic hyperactivity of the thyroid gland during growth appears to be associated with an increased peripheral conversion of T4 to T3, which is closely correlated to the carbohydrate metabolism through hexose monophosphate shunt.

The extrathyroidal conversion of thyroxine (T4) to 3,5,3'-triiodothyronine (T3) has been established as the major pathway of T3 production (7, 27, 31). In the fetal and neonatal period, hepatic T4 5'-monodeiodination is extremely low, but rises promptly after birth (13, 15). Little is known, however, about the regulatory mechanism(s) of this reaction during the maturational process. It was suggested that reduced activity of T4 5'-deiodination in the neonate was not due to the deficiency of the enzyme protein *per se*, but rather to inactivation of existing enzymes or to a deficiency of cofactors (11). Importance of sulphydryl (SH) groups in the reaction has been repeatedly stressed (2-4, 11, 12, 15, 19, 21, 26, 34, 35), and nonprotein SH or glutathione (GSH) in tissue is regarded as an essential cofactor (4, 15, 35). On the other hand, the close association of carbohydrate metabolism with T4 converting system has also been documented (29, 30, 33). Taken together, it is postulated that there may be a metabolic linkage between hexose monophosphate shunt (HMS) and T4 mono-

deiodinating system through NADPH-GSH cycle. In the present study, we attempted to elucidate the changes in the two pathways during maturation, and the role of NADPH and GSH in the hepatic conversion of T4 to T3, by sequential determination of the activity of glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49), glutathione reductase (GSSG-R) (EC 1.6.4.2), T4 5'-deiodinase and the content of GSH and glycogen in liver.

MATERIALS AND METHODS

Pregnant Wistar rats were provided with commercial rat chow and water *ad libitum*. On the 21-22nd day of pregnancy, 11-13 pups were delivered from each rats. Five to eight rats from different litters were used as the source of liver at weekly intervals up to 7 wk of age. Maternal rats served as control. Under ether anesthesia, the liver was removed, weighed and homogenized in 5 volume 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. NADP, NADPH, GSH, GSSG, glycogen, G6P, T4 and T3 were purchased from Sigma Chemical Co.

T4 5'-deiodinase assay. The activity was determined by the method of Chopra (10) under the presence or the absence of 1 mM NADPH, 1 mM GSH and a combination of both in each sample. On each assay, duplicated samples of liver homogenate from an adult female rat or those from maternal rat after weaning (3 wk postpartum) were settled as control. One ml of assay mixture contained 400 μ l of 20% liver homogenate, 100 μ l of T4 (10 μ g/ml in buffer) and 500 μ l of phosphate-EDTA buffer. When requested, 100 μ l of cofactor (10 mM in the buffer) was substituted. The mixture was usually incubated at 37°C for 60 min. The reaction was stopped by the addition of 2 ml of 95% ethanol. Generated T3 was extracted into ethanol (extraction rate; $85 \pm 5\%$). After centrifugation, 10-50 μ l of the supernatant was used for T3 double-antibody radioimmunoassay with commercially available kit (Eiken T3 RIA kit). To eliminate the effect of ethanol on the assay system, the equal amount of 63.3% ethanol was added to the standard curve. Endogenous T3 concentration was also measured in each sample and subtracted from estimated T3 value. Generated T3 was expressed as ng-T3/h/mg-cytosol protein.

G6PD assay (6). Two and one-half ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.2 ml of 0.1 M $MgCl_2$, 0.1 ml of NADP Na-salt (10 mg/ml) and 0.1 ml of G6P Na-salt (10 mg/ml) were mixed and preincubated at 25°C. The reaction was started with the addition of 20 μ l of supernatant of liver homogenate. Increase in absorbance at 340 nm was recorded with Hitachi spectrophotometer Model 124. For calculation of NADPH formed, extinction coefficient of $E_{340}^{mM} = 6.22$ was used and enzyme activity was reported as μ M-NADPH/min/mg-protein.

GSSG-R assay (6). 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 25°C. The reaction was started with the addition of 20 μ l of supernatant of liver homogenate. Decrease in adsorbance at 340 nm was recorded and formed NADP was calculated in the same way as G6PD assay.

GSH content. Determination of GSH was performed by a modification of the method of Hissin and Hilf (16). One-half ml of 20% homogenate, 0.5 ml of 25% HPO_3 and 1.0 ml of 0.1 M phosphate buffer (pH 8.0) containing 5 mM EDTA were mixed and centrifuged. To 100 μ l of the supernatant, 100 μ l 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 the activation at 350 nm. Standard GSH (2.5–10 nmoles/tube) was dissolved in phosphate-EDTA buffer just before use.

Liver glycogen was precipitated with 2 volume of ethanol, standing at 4°C overnight after 30% KOH digestion. The precipitate was dissolved in 1.0 ml of water and glycogen concentration was estimated by the method of Montgomery (24), using phenol-sulfuric acid. Protein was estimated by the method of Lowry *et al.* (22), with bovine serum albumin as the standard. Statistical analysis was carried out by Student's *t* test and the correlation coefficient was calculated by general formulas.

Fig. 1. Age-related changes in T4 5'-deiodinase activity with (open column) or without (shaded column) supplement of 1 mM NADPH and GSH. The activities of mother rats serve as controls. Number in parenthesis; number of rats. 2/7; 2 days of age. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

Fig. 2. Comparison of T4 5'-deiodinase activation with 1 mM NADPH (solid column) or 1 mM GSH (shaded column) to the combined activation (open column), which are expressed in percent activity of basal level (without supplement). Number in parenthesis; number of rats. 2/7; 2 days of age. * $P < 0.01$ vs those of mother.

Fig. 3. Changes in hepatic glycogen (open circle) and GSH (solid circle) contents during maturation. Bar indicates mean $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs those of mother rats.

RESULTS

Age-related changes in T4 5'-deiodinase activity and effects of NADPH and/or GSH enrichment (Fig. 1 and 2). The basal T4 5'-deiodinase activity (without supplementation of NADPH or GSH) was low in the neonatal period. It rose gradually with age and reached a peak at 5–6 wk of age, decreasing to adult level thereafter. The addition of 1 mM NADPH and GSH *in vitro*, however, markedly enhanced the activity. The relative enhancing rate was the greatest on 2–3 wk of age, but was diminished after 6 wk of age. The individual contribution of NADPH or GSH to the enhancement was shown in Figure 2. The additive effect of NADPH and GSH was apparent only in the neonate. Most of the activating effect was due to NADPH, although a small but definite effect was noted with 1 mM GSH. These data suggest that NADPH exerts a significant effect on T3 generating system, and reduced production of T3 during the neonatal period may be due partially to the deficiency of NADPH and GSH.

GSH and glycogen contents in liver (Fig. 3). Changes in GSH and glycogen concentration were almost parallel to each other. Both were extremely depressed in the neonatal period and rose progressively between 2–4 wk of age. The cause of transient decrease at 4–5 wk of age was not apparent, but it may be due to the effect of weaning.

Changes in the activity of GSSG-R and G6PD (Fig. 4 and 5). The GSSG-R activities were significantly reduced from 2 days to 3 wk of age ($P < 0.001$ versus adult), which increased rapidly above adult level during 4–6 wk of age. These changes were approximately parallel to the mean value of T3 production *in vitro*. In contrast, G6PD activities remained depressed until 5 wk of age, rising sharply thereafter. It is noteworthy that T4 deiodinating activity was reciprocally decreasing in the period.

Figure 5 shows the relation between generated T3 and GSSG-R or G6PD activities. As mentioned above, there was a significant correlation between T3 formation and GSSG-R activities ($r = 0.622$, $P < 0.001$). G6PD activities before 5 wk of age were low and relatively stable, whereas those after 6 wk of age were inversely correlated with generated T3 ($r = -0.749$, $P < 0.01$). This suggests that response of T4 5'-deiodinase to NADPH may be different with age. This possibility was tested *in vitro*.

Dose-response relation of T3 formation to NADPH and GSH (Fig. 6). Dose-response curve of T3 production to NADPH showed a plateau at the concentration of 0.25–0.5 mM NADPH. The level of plateau was low in 1 wk of age; the highest in 3 wk of age; and decreased again with age. The addition of 1 mM GSH to NADPH doses did not affect the pattern of this age-related change. In contrast, a linear dose-response relationship was noted in the range of 0.5–5 mM GSH, but the degree of activation was not so great as that of NADPH. The supplement of 1 mM NADPH to graded doses of GSH again markedly enhanced T3 formation,

which eliminated the dose-dependency of T3 production on GSH in older and adult rats. These results indicate that T4 5'-monodeiodination in young rats is dependent on both NADPH and GSH, whereas in older rats NADPH is more rate-limiting than GSH. Furthermore, NADPH exerts its effect by direct stimulation of T4 5'-deiodinase rather than by increase in GSH formation.

DISCUSSION

Peripheral conversion of T4 to T3 is modulated by several conditions, such as thyroid hormone excess and deficiency (2, 5, 15, 17, 18), stress (8, 9), age (13, 15, 36), sex (14) and dietary effects (3, 4, 12, 15, 19, 29, 30, 33). In each situation, however, the mechanism by which conversion of T4 to T3 is regulated may be different. Carbohydrate deprivation reduced T3 formation by a reduction in the hepatic content of endogenous cytosolic thiol compounds (principally GSH) (20, 23), restoration of which can improve the rate of conversion *in vitro* (4, 15). During starvation, lack of glucose substrate and decreased activity of G6PD and malate dehydrogenase result in a decrease in cytosol NADPH (1, 25, 28, 32). NADPH is an essential cofactor of GSH formation, which is primarily generated by HMS. Considering these data, it is suggested that there may be a metabolic linkage between HMS

Fig. 4. Maturation changes in GSSG-R (open circle) and G6PD (solid circle) activities in liver. Mean values of generated T3 (triangle) are also shown, which are approximately parallel to GSSG-R activities. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs those of mother rats.

Fig. 5. (A) Correlation between T4 5'-deiodinase and GSSG-R activities. A statistically significant correlation is noted ($r = 0.622$, $P < 0.001$). (B) Correlation between T4 5'-deiodinase and G6PD activities. (Solid circle) 0-5 wk of age; (open circle) 6-7 wk of age; (double circle) mother rats. An inverse correlation is obtained after 6 wk of age ($r = -0.749$, $P < 0.01$).

Fig. 6. Dose-response curves of T3 production to NADPH with (B) or without (A) 1 mM GSH. Lower panel; those to GSH with (D) or without (C) 1 mM NADPH. (Solid circle) 1 wk of age; (open circle) 3 wk of age; (double circle) 6 wk of age; (triangle) mother rats.

and hepatic T4 5'-deiodination (15). In the fetus and neonate, the reduced conversion of T4 to T3 is reported to be due to the diminished content of cofactors rather than the amount of T4 5'-deiodinase *per se* (11, 15). The results of the present study demonstrate that the maturational changes in HMS are closely associated with those in T4 converting system. The addition of NADPH and/or GSH to liver homogenates of young rats enhanced T3 generation 2-5-fold. Age-related increase in hepatic GSH content and in GSSG-R activity is also parallel to the change in T4 5'-deiodinating activity. Lower activity of G6PD in young rat liver was correlated with greater enhancement of T3 production by exogenous NADPH enrichment *in vitro*. These indicate that NADPH and GSH are important cofactors of T4 conversion to T3.

There is considerable difference, however, between the effect of NADPH and that of GSH. The magnitude of activation was always greater with NADPH than with GSH. The additive effect of GSH to NADPH on T3 production was apparent only in young rats. A dose-response curve of T3 generation to increasing doses of GSH was also blunted with advance of age. Probably, this is due to the endogenous GSH level, which seems to be present in enough amounts in older rat liver. These findings suggest that NADPH is more rate-limiting than GSH in T4 converting system during maturation. Furthermore, NADPH is supposed to affect directly the thiol radicals in the enzyme protein rather than via formation of GSH. In this regard, it is of interest that a reciprocal change was found between generated T3 and G6PD activities after 6 wk of age. This is a rather unexpected result. The increase in G6PD activity in this period may be the reflection of an accelerated turnover of NADPH in liver, which can result in the reduction of T3 production. Another possible explanation is that hormonal influence related to sexual maturation may be involved in the fall of T3 production during this period, because the sexual difference in T4 monodeiodination has been apparent after puberty (14). However, the exact cause of decrease in T3 formation after puberty remains to be elucidated in future.

In summary, the present study demonstrates that there is a close relationship between HMS and the converting system of T4 to T3

through the formation of NADPH and GSH during the maturational process.

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