Effect of thiocyanate induced hypothyroidism on 5'deiodinase activity and T₃ receptors in developing rat brain

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Experiments were performed in weaning rats to understand the influence of thiocyanate, an hydrolytic product of glucosinolates present in foods, on the generation of T₃ in situ by type II 5'deiodinase and the binding of [¹²⁵I]T₃ to specific nuclear receptors in developing brain. Feeding of thiocyanate through gestation and lactation resulted in an increase in type II 5'deiodinase activity in cerebrum, cerebellum and brainstem of the 21 day old pups compared to controls. Hypothyroidism induced by thiocyanate further resulted in augmentation of the maximum binding capacity of receptors in the cerebrum of the weaning pups. Affinity constants for binding of [¹²⁵I]T₃ were however, unaltered. Increase in type II 5'deiodinase activity and the number of binding sites point to an adaptive increase in response to thiocyanate induced hypothyroidism to maintain the cellular T₃ levels within a narrow limit.

Thyroid hormone plays an important role in the developing central nervous system. Its most striking effects are seen during maturation of brain. Hypothyroidism during birth leads to severe physiological, biochemical and behavioral defects which can be corrected only if an adequate replacement therapy is provided as early as possible. Experimentally induced hypothyroidism in rats has been shown to result in defective myelination and hypoplastic neuropile. Naturally occurring substances with the potential to cause goiter and cretinism have been identified in the staple foods of many developing countries. However, studies specifically directed to understand their influence on brain development are scanty. Impairment in postnatal cell acquisition and other biochemical events unique to developing brain in response to thiocyanate treatment in rats suggest hypothyroidism. In the brain most of the physiologically active hormone, T₃, is derived from thyroxin by the action of type II 5'deiodinase. The type II deiodinase is located predominantly in brain, pituitary and adipose tissue. High enzyme activity is reported in developing brain. The T₃ so formed triggers cascade of thyroid hormone dependent reactions by binding to specific receptors. T₃ receptors are members of a family of hormone responsive transcription factors that are similar in structure and mechanism of action. There are two types of receptor genes, α gene coding for α₁ and α₂ receptors and β gene coding for β₁ and β₂ receptors. The T₃ receptors are expressed in developmentally specific pattern especially in brain. In the neonatal rat brain, β receptor increases 40 folds during thyroid hormone sensitive developmental period. Thyroidal state has been shown to influence the T₃ concentration in brain during development. It is therefore proposed to study influence of thiocyanate feeding to rats through gestation and lactation on the activity of type II 5'deiodinase and the [¹²⁵I]T₃ binding capacity in brain of neonates. Further the effect of iodine supplementation on thiocyanate induced changes, if any, have also been studied.

Materials and Methods

Wistar/NIN strain weaning female rats (30) were used. They were divided into 3 groups of 10 each. Group I received a casein based diet adequate in all respects (control). Group II received a casein based diet devoid of potassium iodide and in addition 25 mg of thiocyanate (mixed in the diet) (+KI + SCN group). Group III received casein based diet containing thiocyanate and in addition potassium iodide (31.6μg/g of diet) (+KI + SCN group). The rats were maintained on the respective diets for 2 months. At the end of 2 months the circulating levels of T₃ and T₄ were estimated in serum by RIA using kits from BARC, Bombay. The rats were allowed to mate and were continued on the same dietary regimen throughout the gestation and lactation period. Pups.
born (8 in each case) were sacrificed at 7, 14, and 21 days (spanning the brain growth spurt period) by decapitation. Brains were dissected out immediately into major anatomical regions, viz. cerebrum, cerebellum and brainstem and transferred to ice and processed for the assay of type II 5' deiodinase and cerebrum for T₃ receptor binding capacity (whole brain from 7 day old pups).

For assay of type II 5' deiodinase, separately pooled samples of cerebrum, cerebellum and brainstem (3 in each case) were homogenised in 4 volumes of ice cold buffer containing 0.32 M sucrose, 10 mM DTT and 10 mM HEPES (pH 7) using a Potter Elvehjem homogeniser fitted with a teflon pestle. Microsomal membranes were prepared by the conventional ultracentrifugation procedure. Briefly, tissues were homogenised in ice cold buffer containing 0.32 M sucrose, 10 mM HEPES (pH 7) and 10 mM DTT. The homogenates were centrifuged at 4°C at 100,000 g for 10 min followed by centrifugation of the supernatant at 12,000 g for 15 min. The resulting supernatant was subjected to high speed centrifugation at 1,000,000 g for 1 hr at 4°C in a Beckmann ultracentrifuge. The pellet obtained after high speed centrifugation was suspended in buffer containing 100 mM potassium phosphate (pH 7), 1 mM EDTA and 20 mM DTT. Aliquots of the suspension were rapidly frozen and stored at -20°C until use. The protein content of microsomal preparations was measured as per Lowry et al.

The Type II 5' deiodinase activity was estimated by the method of Visser et al. Briefly, the reaction mixture for the enzyme assay contained in the final volume, 100 mM potassium phosphate (pH 7), 1 mM EDTA, 20 mM DTT and 1,000,000 cpm (35S 125I) T₄ (sp.ac.1500 μCi/μg) from Amersham plus unlabelled T₄ to make up the concentration of substrate to 2.5 nM. The reaction was started by addition of microsomal suspension and continued for 1 hr. Reaction was terminated by adding 50 μl of ice cold normal human serum. After TCA precipitation radioiodine was separated by passing through Dowex 50W×2 (100-200 mesh) resin and counted in a gamma counter. The activity was expressed as T₄ moles/iodine/mg protein/hr.

To estimate the receptor binding capacity pooled cerebrows (3) were homogenised in ice cold buffer containing 0.32 M sucrose, 1 mM MgCl₂, and 20 mM Tris HCl (pH 7.5)(STM buffer). The nuclei obtained after low speed (800 g) centrifugation were suspended in buffer containing triton-X-100 to yield purified preparations. The T₃ binding capacity of the nuclear preparations was assayed by the method of Ishiguro et al. Briefly, nuclei equivalent to 50-100 μg of DNA were incubated with STM buffer containing 5 mM DTT containing 0.09 pmol of [125I] L-T₃ and various amounts of unlabelled L-T₃ (0.2 to 5 pmol). After incubation at 25°C for 3 hr, samples were chilled in an ice bath and centrifuged at 1800 g for 10 min. The supernatants were saved for determination of free T₃. The nuclear pellets were washed once with STM-Triton buffer and radioactivity was determined in a well type gamma counter. Non specific binding was determined by adding 100 fold excess of cold T₃. All data were corrected for non specific binding. The receptor bound T₃ and free T₃ were expressed as p moles/mg DNA. DNA was estimated by the method of Dische. The maximal binding capacity (MBC) in p moles/mg DNA and the dissociation constant, Kd, in nM, were calculated from the Eadie Hofstee plot. Statistical analysis of the data was done by ANOVA.

Results

Feeding of thiocyanate for 2 months significantly (P<0.001) reduced T₃ levels when compared to the control group (Table 1). The T₃ levels were however unaltered. Rats receiving diet supplemented with both iodine and thiocyanate (group III) had near normal T₄ and T³ levels.

The type II 5' deiodinase activity was higher in the 21 day old brain region as compared to 14 day old brain regions (Table 2). The increase was 45% in cerebrum, 220% in cerebellum and 173% in brainstem in controls and 81% in cerebrum, 213% in cerebellum and 129% in brainstem of treated rats (group II). Exposure to thiocyanate resulted in an increase in the type II 5' deiodinase activity in both cerebrum (36%; P<0.001) and brainstem (90%, P<0.001) of 14 day old pups as compared to controls (group II vs. group I). In the potassium iodide supplemented group (group III) the activity was

<table>
<thead>
<tr>
<th>Group (Treatment)</th>
<th>T₄ (μg/dl)</th>
<th>T₃ (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (control)</td>
<td>6.56 ± 0.346a</td>
<td>0.118 ± 0.008</td>
</tr>
<tr>
<td>II (+KI)</td>
<td>3.81 ± 0.370b</td>
<td>0.108 ± 0.005</td>
</tr>
<tr>
<td>III (+KI+SCN)</td>
<td>5.61 ± 0.488b</td>
<td>0.110 ± 0.006</td>
</tr>
</tbody>
</table>

*P values : Values bearing different superscripts are significantly different by ANOVA.

* is different from † at P<0.001
similar to controls. The effect of thiocyanate was also evident at the end of third postnatal week with significant (P<0.001) augmentation of type II 5'deiodinase activity in cerebrum (70%) and brainstem (60%). Supplementation of potassium iodide failed to reverse the effects completely.

The maximum binding capacity (MBC) and dissociation constant (Kd) which were arrived at from Eadie Hofstee plots are depicted in Table 3. No change in receptor numbers was apparent with age in controls. Thiocyanate treatment significantly (P<0.05) increased the receptor density in the cerebrum of 14 (45%) as well as 21 (49%) day old pups as against controls. The Kd was however unaltered with age or with thiocyanate treatment. MBC in the potassium iodide supplemented group was not reversed completely.

Discussion

Feeding of thiocyanate (25 mg/day in the diet) to rats for 2 months resulted in hypothyroidism as evidenced by decrease in circulating T₄, T₃ levels were however unaltered. Congenital hypothyroidism in humans, like dysgenesis or ectopically located or poorly functioning thyroid tissue also present a similar situation with alteration in only T₄. Thus the experimental condition presented by feeding this level of thiocyanate parallels certain human situations.

The developing brain is under the stress of maternal hypothyroidism due to thiocyanate feeding. Several studies have shown that optimal maternal thyroxine levels are crucial to the development of the fetal brain. Hypothyroidism could result from thiocyanate exposure through placenta during fetal development and through milk in the neonates. The thiocyanate induced hypothyroidism is of significance to the developing brain in view of the fact that the cellular supply of T₃ in brain depends to a great extent on conversion of T₄ to T₃ in situ by type II 5'deiodinase, the exchange of plasma T₃ being relatively lower in the brain.

High activity of type II 5'deiodinase in cerebrum, cerebellum and brainstem at 14 and 21 days coincide with the time of rapid development and maturation of the nervous system. Kaplan and Yaskoski have reported a peak in the activity of type II 5'deiodinase activity between 14 and 21 days in different regions of the brain. Many important biochemical processes unique to the developing brain are associated with this period of high 5'deiodinase activity.

Thiocyanate induced hypothyroidism enhanced the type II 5'deiodinase activity in cerebrum and brainstem of three weeks old rats compared to control 21 day old pups. An increase in type II 5'deiodinase activity has been reported in rats made hypothyroid by potent antithyroid drugs like propyl thiouracil and methimazole or by surgical thyroidectomy. It is suggested that the augmentation in the microsomal type II 5'deiodinase activity could be the result of an adaptive mechanism operating in the organism in

Table 2—Type II 5'deiodinase activity (1 moles I released/mg protein/hr) in different regions of the brain in response to thiocyanate feeding

<table>
<thead>
<tr>
<th>Group (Treatment)</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WB</td>
<td>CB</td>
<td>CL</td>
</tr>
<tr>
<td>I (Control)</td>
<td></td>
<td>148.35±15.63</td>
<td>140.32±10.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>II (-Kl+SCN)</td>
<td></td>
<td>144.45±15.60</td>
<td>191.40±11.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III (+Kl+SCN)</td>
<td></td>
<td>140.53±25.75</td>
<td>147.19±5.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

WB-Whole Brain, CB-Cerebrum, CL-Cerebellum and BS-Brainstem
Those bearing different superscripts are significantly different by ANOVA.
<sup>a</sup>"a" is different from "b" at P<0.05, "a" is different from "c" at P<0.01, "a" is different from "d" at P<0.001.

Table 3—Maximum binding capacity and Kd of nuclear preparation from cerebrum of 14 and 21 day old rat pups in response to thiocyanate feeding

<table>
<thead>
<tr>
<th>Group (Treatment)</th>
<th>MBC (p moles/mg DNA)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14d</td>
<td>21d</td>
</tr>
<tr>
<td>I (Control)</td>
<td>1.56±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>II (-Kl+SCN)</td>
<td>2.27±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.20±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III (+Kl+SCN)</td>
<td>1.67±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values bearing different superscripts are significantly different by ANOVA.
"a" is different from "b" at P<0.05.
response to hypothyroidism to maintain optimal $T_3$ levels in the brain which is critical during development. However several studies have shown that despite three to five fold increase in the enzyme activity there is a decrease in thyroid hormone responsive enzymes like aspartate transaminase, succinic dehydrogenase and Na$^+$/K$^+$-ATPase pointing to tissue hypothyroidism.$^{20,21}$ Significant alterations in thyroid hormone dependent parameters were also observed in the developing brain (mostly evident at 21 days) in response to thiocyanate feeding.$^{7,8}$ This implies that the success of the adaptive mechanism in maintaining brain euthyroidism would be under the influence of more than one factor. Other factors which may influence $T_3$ economy include a) uptake of $T_4$ by the brain; b) the metabolism of $T_3$ (catalysed by type III deiodinase) and c) $T_3$ disposal rate. All these factors have been shown to alter in response to hypothyroidism induced by antithyroid drugs$^{21,25}$.

However, to what extent thiocyanate induced hypothyroidism affects uptake of $T_4$ or rate of $T_3$ disposal, remains to be studied.

Binding of the $T_3$ (generated by in situ conversion of $T_4$ to $T_3$ by the type II 5-deiodinase) to specific nuclear receptors would act as a trigger in regulating the thyroid hormone responsive genes.$^{26}$ The values for MBC and Kd for $T_3$ receptors observed agrees with that reported in nuclear extract of neonatal rat brain by Valcana and Timiras.$^{12}$ There was no difference in density of binding sites between second and third postnatal week. Schwartz and Oppenheimer$^{27}$ have reported high concentrations of nuclear $T_3$ receptors in neonatal rat brain which decline to adult levels by second postnatal week and remain at that level until 6 months of age. An increase in receptor population as evident by augmented maximum binding capacity in response to thiocyanate induced hypothyroidism points to protective mechanism aimed at providing the maximum hormone response in a situation of reduced availability. However, in absence of sufficient hormone, the unoccupied $T_3$ receptors could exert a repressive effect on gene expression.$^{28}$ In view of reduced availability of $T_3$, major source of $T_3$ generation in brain, this may assume importance. No change in the affinity of the receptors was however evident as reflected by the dissociation constant. A similar increase in MBC has been reported in radioiodide-decomised rats by Valcana and Timiras$^{12}$ and Bellabarba et al$^{29}$, and in propyl thiouracil treated rats by Ishiguro et al$^{31}$. Iodine supplementation in the diet afforded protection against the effects of thiocyanate.

The study indicates that partial suppression of thyroid hormone function as a consequence of exposure to thiocyanate during pregnancy and lactation resulted in an adaptive increase in brain type II 5-deiodinase activity and the $T_3$ nuclear receptor population. Iodine supplementation was however able to correct to a large extent the changes induced by thiocyanate, which acts mainly by inhibiting the uptake of iodine.

Acknowledgement

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References