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PTU-induced hypothyroidism modulates antioxidant defence status in the developing cerebellum

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ABSTRACT

The objective of the present study was to evaluate the effect of 6-*n*-propylthiouracil (PTU)-induced hypothyroidism on oxidative stress parameters, expression of antioxidant defence enzymes, cell proliferation and apoptosis in the developing cerebellum. PTU challenged neonates showed significant decrease in serum T_3 and T_4 levels and marked increase in TSH levels. Significantly elevated levels of cerebellar H_2O_2 and lipid peroxidation were observed in 7 days old hypothyroid rats, along with increased activities of superoxide dismutase and glutathione peroxidase and decline in catalase activity. In 30 days old hypothyroid rats, a significant decline in cerebellar lipid peroxidation, superoxide dismutase and glutathione peroxidase activity and expression was observed along with an up-regulation in catalase activity and expression. Expression of antioxidant enzymes was studied by Western blot and semi-quantitative rt-PCR. A distinct increase in cell proliferation as indicated by proliferating cell nuclear antigen (PCNA) immunoreactivity was observed in the internal granular layer of cerebellum of 7 days old hypothyroid rats and significant drop in PCNA positive cells in the cerebellar molecular layer and internal granular layer of 30 days old PTU treated rats as compared to controls. *In situ* end labeling by TUNEL assay showed increased apoptosis in cerebellum of hypothyroid rats in comparison to controls. These results suggest that the antioxidant defence system of the developing cerebellum is sensitive to thyroid hormone deficiency and consequent alterations in oxidative stress status may play a role in regulation of cell proliferation of the cerebellum during neonatal brain development.

1. Introduction

Brain development is a consequence of highly synchronized changes in gene expression coordinated by transcription and suppression of transcription of specific genes regulated by important growth factors including GH, EGF, BDNF, NGF and thyroid hormones (Samuels and Shapiro, 1976; Barlow et al., 1986; Fujieda et al., 1993). Several elegant reports illustrate significant functions of thyroid hormones with regard to neonatal brain development through modulation of genomic and non-genomic actions (Porterfield and Hendrich, 1993; Denver, 1997; Koibuchi et al., 2003; Boelaert and Franklyn, 2005; Farwell et al., 2006). Neonatal thyroid hormone deficiency is associated with profound neurological and morphogenetic deficits including defects in interneuronal connectivity and synaptogenesis, decreased myelination, defective cell migration, Purkinje cell development, cell proliferation and apoptosis (Oppenheimer and Schwartz, 1997; Thompson and Potter, 2000; Zoeller et al., 2002; Bernal et al., 2003; Koibuchi, 2008).

Furthermore, thyroid hormones are known to set the cellular basal metabolic rate and are considered as major regulators of energy metabolism; mitochondrial activity and biogenesis; oxygen consumption and active oxygen metabolism (Katyare et al., 1994; Vega-Nunez et al., 1997; Wrutniak-Cabello et al., 2001; Martinez et al., 2001). Hypothyroidism has been associated with a submetabolic state and lowered energy and oxygen metabolism (Weitzel et al., 2003). Brain development is an exceptionally active process capable of generating enormous number of cells and should it coincide with a compromised energy state, perturbations in brain maturation and neurological dysfunction may occur (Lust et al., 2003). The brain processes a great amount of oxygen per unit tissue mass as it consumes 20% of the entire oxygen consumed by the

Abbreviations: BDNF, Brain derived neurotrophic factor; CAT, catalase; EGF, Epidermal growth factor; EGL, External granular layer; GH, Growth hormone; GPx, Glutathione peroxidase; GR, Glutathione reductase; GSH, Reduced glutathione; HIF, Hypoxia inducing factor; IGL, Internal granular layer; LPx, Lipid peroxidation; ML, Molecular layer; NGF, Nerve growth factor; PCNA, Proliferating cell nuclear antigen; PTU, 6-n-propylthiouracil; ROS, Reactive oxygen species; SOD, superoxide dismutase; TBARS, Thiobarbituric acid reactive substances; TH, Thyroid hormone; TSH, thyroid stimulating hormone.

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body though its weight is only 2% of the total body weight (Halliwell, 2006); more so in the postnatal period due to higher brain to body ratio. Most of the oxygen consumed by cells is tetravalently reduced to water during mitochondrial respiration. Nevertheless, incomplete reduction of small quantities of oxygen forms superoxide radicals, which, though highly reactive, cannot diffuse through cellular membranes. They are converted by superoxide dismutase enzymes (SOD; EC 1.15.1.1), SOD1 (CuZn SOD) and SOD2 (Mn SOD) to hydrogen peroxide (H_2O_2), which is stable and can unreservedly move to different cellular compartments (Halliwell, 2006). While both H_2O_2 and $O_2^{\bullet -}$, themselves can set off reactions with cellular macromolecules, they can also react with transition elements to form highly reactive hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 2001) which can damage biomolecules in its vicinity. Two major antioxidant enzymes, catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GPx; EC 1.11.1.9) play a crucial role in the cellular defence by scavenging H_2O_2 in addition to preventing $^{\bullet}OH$ formation (Halliwell and Gutteridge, 2001). It has been suggested that higher peroxide degrading enzymes (CAT and GPx) coupled to a lag in expression of the peroxide generating enzyme (SOD) in the neonatal brain may be a crucial factor in protection of neurons from oxidative injury (Folkerth et al., 2004). However, regardless of the prevailing low oxygen tensions in the brain microenvironment, it remains at risk due to abundance of highly unsaturated fatty in addition to transition elements, dopamine metabolism, hydrogen peroxide formation and high iron contents.

Of great interest is the aspect that rate of mammalian growth and gene expression are strongly modulated by only slight variations in metabolic gradients, ambient oxygen tension and ROS (Allen and Tresini, 2000). Reactive oxygen species are known to play important roles in regulating proliferation and differentiation by modulating various growth factors as well as signaling molecules like HIF, AP-1, NFκB, MAP kinases, cell cycle and apoptotic proteins (Hancock et al., 2001). Several authors have reported that altered thyroid states modulate generation of reactive oxygen species (ROS) (Rahaman et al., 2001; Yilmaz et al., 2003; Venditti et al., 1997; Venditti and Di Meo, 2006) in adult rats. We have earlier shown that altered thyroid states may induce changes in antioxidant defence system in various tissues including brain of adult rats (Das and Chainy, 2001, 2004; Chattopadhyay et al., 2007; Sahoo et al., 2005, 2006). However, despite enormous progress in our insight vis-à-vis TH action, studies regarding the role of antioxidant defence enzymes during neonatal hypothyroidism in the cerebellum are inadequate. In this regard, the rodent cerebellum is one of the finest illustrations of temporal changes in the sensitivity to TH during brain development. It is an especially good model to study developmental changes owing to its significant period of postnatal terminal differentiation and distinct changes in cell number and migration within the first three to four postnatal weeks. Against this background, the present investigation aims at creating a temporal profile of changes in oxidative stress status, expression of antioxidant enzymes, cell proliferation and cell death in the developing cerebellum during neonatal hypothyroidism.

2. Materials and methods

2.1. Materials

6-*n*-Propylthiouracil (PTU), diaminobenzidine tetrahydrochloride, horseradish peroxidase (HRP), thiobarbituric acid, bovine serum albumin, Coomassie Brilliant Blue G250, sodium dodecyl sulphate, agarose and glutathione reductase were obtained from Sigma Chemical Co (St. Louis, MO, USA). T_3, T_4 and TSH were assayed using ELISA kits from Monobind Inc. (Costa Mesa, CA, USA). Ribonuclease inhibitor, DNase I, RevertAid H-minus first strand c-DNA synthesis kit, M-MuLV Reverse Transcriptase, Taq DNA polymerase and 10x Taq buffer were purchased from Fermentas Inc, USA. Trizol was obtained from Invitrogen, CA, USA. G3PDH, SOD2, PCNA and HRP-conjugated anti-rabbit goat IgG antibodies were procured from Santa Cruz Biotechnology Inc., CA, USA. Rabbit polyclonal antibodies for SOD1 and CAT were developed in collaboration with Imgenex Biotech Pvt. Ltd., Orissa, India.

2.2. Animals

Thirty adult pregnant female Wistar strain rats (*Rattus norvegicus*) weighing approximately 300 g were procured from National Institute Nutrition, Hyderabad, India and maintained under standard conditions in the animal room at 25 ± 2 °C with 12-h light:12-h dark cycles and supplied food and water ad libitum (Samanta and Chainy, 1997). Protocols for animal care, maintenance and experiments were approved by the Institutional Animal Ethics Committee (IAEC).

As soon as the pups were born, dams were divided into 2 groups: control mothers (n = 15), given water and hypothyroid mothers (n = 15), given 0.05% PTU in drinking water for 7, 15 or 30 days (Ladenson et al., 1986). The numbers of pups per litter were kept constant and were weaned at 25 days of age after which the pups were given 0.05% PTU in drinking water. Each pup for each group was taken from separate litters. Pups were grouped as: 7 days old euthyroid, 7 days old hypothyroid, 15 days old euthyroid, 15 days old hypothyroid, 30 days old euthyroid and 30 days old hypothyroid.

2.3. Tissue preparation

Animals were sacrificed after 7, 15 and 30 days of treatment. Blood samples were collected and serum levels of T_3, T_4 and TSH were analyzed using commercially available ELISA kits in a microplate reader (BIO-RAD model 550). Immediately after sacrifice, tissue samples of different brain regions were collected along with other organs and washed in cold 0.9% (w/v) normal saline solution, pat dried, weighed, frozen in liquid nitrogen and in liquid nitrogen and stored at −80 °C for further analysis. Crude homogenates (10% w/v) of cerebella were prepared homogenate was prepared in phosphate buffer (50 mM, pH 7.4) with the help of a Potter-Elvehjem type motor driven glass Teflon homogenizer in ice. Hydrogen peroxide and TBARS contents were estimated in the crude homogenate. The crude homogenate was then centrifuged at 10,000 × g for 20 min at 4 °C in the presence of 0.1% Triton X-100. The supernatant so obtained was used to assay the activities of antioxidant enzymes. Estimation of protein in samples was done by Bradford's method (1976), using bovine serum albumin as the standard.

2.4. Estimation of H_2O_2 content and lipid peroxidation

Hydrogen peroxide content was measured in the cerebellum according to the method of Pick and Keisari (1981) based on the H_2O_2-mediated HRP dependent oxidation of phenol red and expressed as H_2O_2 nmoles per mg protein. Lipid peroxidation level in each sample was estimated by monitoring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Okhawa et al. (1979) in the presence of 0.02% (w/v) butylated hydroxytoluene to suppress peroxidation during the subsequent boiling. Concentration of TBARS was calculated in terms of nmol TBARS formed per mg protein from its extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.