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Hypothyroidism modulates renal antioxidant gene expression during postnatal development and maturation in rat

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ABSTRACT

In the present study effects of 6-*n*-propyl thiouracil (PTU)-induced hypothyroidism on renal antioxidant defence system during postnatal development (from birth to 7, 15 and 30 days old) and on adult rats were reported. Hypothyroidism in rats was induced by feeding the lactating mothers (from the day of parturition till weaning, 25 days old) or directly to the pups with 0.05% PTU in drinking water. The activities of Cu/Zn-superoxide dismutase (SOD1) and glutathione peroxidase (GPx) were increased in 30 days old hypothyroid rats with respect to their respective controls, on the other hand, levels of translated products and activities of Mn-superoxide dismutase (SOD2) and catalase (CAT) were decreased in hypothyroid rats of all age groups as compared to their respective control rats. SOD1 activity remained unchanged in persistent (PTU-treatment from birth to 90 days old) hypothyroid rats as compared to euthyroid. However, a decreased activity of SOD1 was recorded in transient (PTU-treatment from birth to 30 days then withdrawal till 90 days old) hypothyroid rats with respect to control rats. The mRNA level, protein expression and activity of SOD2 and CAT were significantly decreased in persistent hypothyroid rats as compared to euthyroid rats. The activity of GPx was significantly increased in both persistent and transient hypothyroid rats with respect to euthyroid rats. The present study indicates modulation of antioxidant defence status of rat kidney during postnatal development and maturation by hypothyroidism.

1. Introduction

Oxidative stress (OS) is a pathophysiological condition of aerobes when the rate of generation of reactive oxygen species (ROS) such as $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 surpasses their rate of neutralization [17]. Mitochondria are considered as one of the main sites for the production of ROS in cells due to incomplete reduction of molecular oxygen in electron transport chain [8,32]. The primary ROS in this event is superoxide radical ($O_2^{\cdot-}$) which is produced in mitochondria. Superoxide dismutase (SOD) enzyme dismutates $O_2^{\cdot-}$ to H_2O_2. Both H_2O_2 and $O_2^{\cdot-}$ can react with transition elements to form highly reactive $^{\cdot}OH$ which can potentially damage the biomolecules present in their vicinity. Cellular system is equipped with two important enzymes which protect cells from toxic effects of H_2O_2. The enzyme catalase (CAT) converts H_2O_2 to molecular oxygen and H_2O, whereas glutathione peroxidase (GPx) catalyses H_2O_2 with an expense of reduced glutathione (GSH) to oxidized glutathione (GSSG) and H_2O. Oxidized glutathione recycled to GSH in this cascade by the enzyme glutathione reductase (GR) by oxidation of NADPH to $NADP^+$. Non-enzymatic antioxidants like GSH and ascorbic acid also play important role in scavenging ROS [19].

Thyroid hormones are critical for normal development and functioning of kidney in vertebrates [44,49]. Hypothyroidism is frequently associated with impairment of several renal functions such as escalation of serum creatinine level, reduction in glomerular filtration rate (GFR), renal plasma flow (RPF) rate, disruption of the capacity to excrete free water and hyponatremia [30]. Also hypothyroidism is reported to be coupled with hypometabolic state, nevertheless, generation of ROS in hypothyroid animals is not less in comparison to normal individuals as revealed by flow cytometric analyses [38]. Whilst several reports have shown that hypothyroidism modulates antioxidant defences in various tissues of adult rats [7,29,37,46–48] including kidney [20,28,29,50], the effect of hypothyroidism in modulating antioxidant defences of kidney during early postnatal development remains ambiguous. Available literature is also limited to provide conclusive evidence on the regulatory mechanism of oxidative stress and antioxidant protection associated with hypothyroidism particularly during postnatal development. Therefore, further experiments are necessary to understand the effect of hypothyroidism on antioxidant defence strategy in rat kidney during development. Hence, two experiments were designed to elucidate the mechanism of

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hypothyroidism-induced oxidative stress response and regulation of antioxidant genes expression and associated kidney dysfunctions during postnatal development. In the first experiment, hypothyroidism was induced in the neonates by 6-*n*-propyl thiouracil (PTU)-treatment and parameters of antioxidant defences were analysed to ascertain the oxidative stress response in renal tissues during postnatal development and maturation. Second experiment was designed to find out whether induction of neonatal hypothyroidism and subsequent withdrawal of PTU (on 30 days) restore altered antioxidant defences, if any, in adulthood.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: Effect of PTU-induced hypothyroidism during postnatal development

As soon as the pups were born, dams (6 month old, weight about 225–250 g) were divided into two groups: control mothers ($n = 15$), given drinking water, and hypothyroid mothers ($n = 15$), given 0.05% PTU in drinking water [7,22,37]. After weaning (25 days) pups were supplied with 0.05% PTU directly in drinking water for the remaining period of the experimentation. All pups used in this experiment were male Wistar rats (*Rattus norvegicus*). Effect of neonatal hypothyroidism on kidney antioxidant defence system was assessed by comparing various antioxidant and oxidative stress parameters between 7, 15 and 30 days old euthyroid and hypothyroid pups.

2.1.2. Experiment 2: Effect of neonatal PTU-treatment on adult rats

Male pups of Wistar rats obtained after breeding were made hypothyroid from birth till 30 or 90 days of postnatal age as explained in experiment 1. Animals were divided into three groups each containing five animals. Group I: control rats (90 days old). Group II: rats treated with PTU from birth to 90 days old (persistent hypothyroidism). Group III: rats treated with PTU for 30 days after birth followed by withdrawal of treatment till 90 days old (transient hypothyroidism). All control and experimental rats were sacrificed on 91st day of age.

Animal care, maintenance and experiments were done under the supervision of the Institutional Animal Ethics Committee (IAEC) regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2. Sample preparation for biochemical assays

Whole kidney was dissected out and a 20% (w/v) homogenate was prepared in 50 mM phosphate buffer, pH 7.4 with a Potter-Elvejhem type motor-driven homogenizer. The crude homogenate was centrifuged at 600*g* for 10 min at 4 °C to pellet down nuclei and other cell debris. In the resultant supernatant (S1), oxidative stress and non-enzymatic antioxidant defence parameters were estimated. A part of the S1 was treated with Triton X-100 (final concentration 0.1%, v/v) and centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant (S2) so obtained was used for enzymatic assays.

2.3. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from tissues of kidney using Trizol reagent (Molecular Research Center Inc., USA). RNA samples were subjected to DNase1 treatment to remove genomic DNA contamination in the presence of RNase inhibitor. Primer sequences (Table 1) of antioxidant genes were custom made by Integrated DNA technology, USA. G3PDH served as the internal standard. Reverse transcription of 5 μg total RNA was performed using 200 ng random hexamer, 20 U RNase inhibitor, 1.0 mM dNTPs, and 40 U of M-MuLV reverse transcriptase (Fermentas), to make ss-cDNA at 37 °C for 1 h. The reverse transcribed products (cDNA) were then subjected to PCR at 94 °C for 3 min, 3-step cycling, each cycle consist of denaturation at 94 °C for 30 s, annealing for 30 s, extension at 72 °C for 1 min (30 cycle for G3PDH, SOD1, SOD2, CAT and GPx1, and 33 cycle for GR) followed by final extension at 72 °C for 5 min. The PCR mixture of 25 μl included 1 μl RT product as template, 2.5 μl 10× buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Fermentas) and 25 pmol of each primer. The PCR products were electrophoresed on ethidium bromide stained 1.2% agarose gels. The expression levels were measured by densitometry.

2.4. Preparation of tissue homogenate and Western blotting

A 10% homogenate of kidney sample was prepared in 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 104 mM PMSF, 100 μM E-64, 80 μM aprotinin, 100 μM leupeptin, 1% Triton X-100 and 0.1% SDS to avoid protein degradation. Homogenates were centrifuged at 1000*g* for 20 min at 4 °C. Fifty micrograms of total cellular protein was resolved in 12% SDS–PAGE and transferred to PVDF membrane (0.4 μm, PALL Life Sciences) at 23 mA current for 1 h. The membrane was blocked in 5% blocking solution for 1 h at room temperature. The blot was then incubated with rabbit polyclonal anti-G3PDH (1:1000), anti-CAT (1:5000), anti-SOD1 (1:5000) or anti-SOD2 (1:2500) for 1 h at room temperature. Anti-G3PDH, anti-CAT and SOD1 primary antibodies were obtained from Imgenex India Pvt. Ltd., Orissa, while SOD2 was obtained from Santa Cruz Biotechnology, Inc., USA. The membrane was washed (three times, 5 min each) with washing solution and subsequently incubated with HRP-conjugated anti-rabbit goat IgG (1:7500, Santa Cruz Biotechnology, Inc., USA) for 1 h at room temperature. After washing, specific immunoreactive proteins were detected with ECL kit (Santa Cruz Biotechnology, Inc., USA) in X-ray film and their expression level was measured by densitometry.

2.5. Determination of levels of oxidative stress parameters (lipid peroxidation and protein carbonylation)

Lipid peroxidation (LPx) and protein carbonyl (PC) are the two major indices of oxidative stress parameters. Lipid peroxidation and protein carbonyl content were determined in S1-supernatant samples. Lipid peroxidation was estimated by monitoring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. Ohkawa et al. [34]. Concentration of TBARS in samples was calculated from its extinction co-efficient, $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and was expressed as nmoles TBARS formed per mg protein. Protein carbonyl content in samples was estimated by the method of Levine et al. Levine et al. [23] and was expressed as nmoles carbonyl/mg protein.

2.6. Estimation of small antioxidant molecules (reduced glutathione and ascorbic acid)

Reduced glutathione (GSH) and ascorbic acid (ASA) are the non-enzymatic small antioxidant molecules which help in detoxification of cellular oxidants. Supernatant (S1)-fraction were precipitated in ice-cold TCA (final concentration 5%) containing 0.01 N HCl at 4 °C for 30 min followed by centrifugation at 2000 rpm for 15 min at room temperature. The supernatant so obtained was used to measure GSH and ASA contents according to the methods of Sedlak and Lindsay [41] and Mitsui and Ohta [27], respectively and expressed as μg/g tissue.