Propylthiouracil-induced congenital hypothyroidism upregulates vimentin phosphorylation and depletes antioxidant defenses in immature rat testis

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Abstract

Congenital hypothyroidism was induced in rats by adding 0.05% 6-propyl-2-thiouracil in the drinking water from day 9 of gestation, and continually up to postnatal day 15. Structural alterations observed by light microscopy of seminiferous tubules and by transmission electron microscopy of Sertoli cells of treated animals were consistent with hypothyroid condition. Hypothyroidism was also associated with high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 levels. Furthermore, the phosphorylation and the immunoreactivity of cytoskeletal-associated vimentin were increased without altering vimentin expression, suggesting an accumulation of insoluble and phosphorylated vimentin. These alterations in intermediate filament dynamics could result in loss of Sertoli cell cytoskeletal integrity and be somewhat related to the deleterious effects of hypothyroidism in testis. In addition, the mitochondrial alterations observed could also be related to defective cytoskeletal dynamics implying in cell damage. Moreover, we observed decreased oxygen consumption and unaltered lipid peroxidation in hypothyroid testis. However, we demonstrated decreased enzymatic and non-enzymatic antioxidant defenses, supporting an increased mitochondrial reactive oxygen species (ROS) generation, contributing to biochemical changes in hypothyroid testis. In addition, the changes in the testis histoarchitecture could be ascribed to cytoskeletal alterations, decreased antioxidant defenses, and increased ROS generation, leading to oxidative stress in the organ.

Journal of Molecular Endocrinology (2008) **40**, 125–135

Introduction

The Sertoli cells are the principal structural elements of the seminiferous epithelium, providing physical support and an environment conducive to germ cell development and maturation (Holsberger & Cooke 2005). These cells play a pivotal role in the regulation and maintenance of spermatogenesis and are the site of action of all hormonal influences modulating testis development. They also provide physical support to germ cells, form the blood–testis barrier, and secrete protein products thought to be essential to the maintenance and control of spermatogenesis (Maran *et al.* 1999). The hormonal factors controlling the duration of Sertoli cell proliferation are critical determinants of fertility (Jannini *et al.* 1995, Holsberger & Cooke 2005).

Thyroid hormones (THs) are essential for normal postnatal growth and development, and are known to

play a fundamental role in the regulation of the energy metabolism of almost all mammalian tissues. TH receptors are highly expressed in neonatal Sertoli cells, indicating that the developing Sertoli cells and testis may be important TH targets (Jannini *et al.* 1999, Rao *et al.* 2003). In this context, Jannini *et al.* (1995) and Holsberger *et al.* (2005) provided strong evidence that THs regulate Sertoli cell proliferation and differentiation in the neonatal testis. Accordingly, alterations in thyroid activity are frequently associated with changes in male reproductive functions, since hypothyroidism is associated with a marked delay in sexual maturation and development (Holsberger & Cooke 2005). However, the complete mechanisms by which THs are able to control Sertoli cell and testicular development is still uncertain.

Intermediate filaments (IFs) are cytoskeletal polymers that provide crucial structural support in the nucleus and cytoplasm of eukaryotic cells. Perturbation of their function and dynamics accounts for fragile cells

that cannot sustain mechanical and non-mechanical stress. In addition, the IFs are able to modulate cellular responses to metabolic stress, programmed cell death, cell migration, and even tissue growth (Coulombe & Wong 2004). Vimentin is an IF protein often expressed transiently during development (Menet *et al.* 2001) that has been described in Sertoli cells during fetal and postnatal periods (Romeo *et al.* 1995, Show *et al.* 2003, Franke *et al.* 2004), where it plays important roles in the modifications of Sertoli cell morphology, junctional processes, structural integrity, and cytoplasmic organization that occur during spermatogenesis (Russell & Peterson 1985, Tanemura *et al.* 1994, Show *et al.* 2003, He *et al.* 2007).

Phosphorylation modulates both reciprocal interactions of IF proteins with other cytoskeletal components and the continuous exchange of IF subunits between a soluble pool and the polymerized IF (Inagaki *et al.* 1987, Chou *et al.* 1996, Inada *et al.* 1999, Zamoner *et al.* 2005, 2006, 2007). In this context, the mitogen-activated protein kinase (MAPK) family have been described to phosphorylate IF proteins (Brownlees *et al.* 2000) and participate in cell cycle arrest and inhibition of cell proliferation, a hallmark of hypothyroidism (Moro *et al.* 2004, Franco *et al.* 2006). We have recently described that THs are able to modulate the *in vivo* and *in vitro* IF phosphorylation and expression in rat testis and cerebral cortex through genomic and non-genomic mechanisms (Zamoner *et al.* 2005, 2006, 2007). Furthermore, we have also demonstrated that vimentin hyperphosphorylation was associated with MAPK activation and oxidative stress in testis from hyperthyroid rats (Zamoner *et al.* 2007).

The THs are largely described as important modulators of cellular metabolism. Several studies demonstrated that hyperthyroid tissues exhibit increased reactive oxygen species (ROS) generation (Venditti & Di Meo 2006). The generation of ROS *in vivo* is a constant phenomenon due to either physiological metabolism or pathological alterations, and therefore oxidative stress consists one of the major threat to cell homeostasis in aerobic organisms (Gaté *et al.* 1999). In the male reproductive system, the testis, epididymis, sperm, and seminal plasma contain high activities of antioxidant enzymes that protect sperm against ROS deleterious effects (Zini & Schlegel 1996, 1997a,b, Potts *et al.* 2000). However, the effect of congenital hypothyroidism in oxidative stress and antioxidant defenses is still unclear.

In an attempt to better understand some mechanisms underlying the testicular alterations observed in hypothyroidism, we investigated the effects of 6-propyl-2-thiouracil (PTU)-induced congenital hypothyroidism on testicular morphology, somatic indices, involvement of MAPK cascade, vimentin expression and phosphorylation, and some aspects of oxidative stress in young rats in the present study.

Materials and methods

Chemicals

[³²P] Na₂HPO₄ was purchased from CNEN (São Paulo, -Brazil). Benzamidine, leupeptin, antipain, pepstatin, chymostatin, anti-vimentin antibody (clone vim 13.2), peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG), monoclonal anti-β-actin antibody, acrylamide, and bis-acrylamide were obtained from Sigma Chemical Co. Anti-p44/42 MAP kinase (anti-extracellular signal-regulated kinases 1/2 (ERK1/2)), anti-phospho-p44/42 MAP kinase (anti-phospho ERK1/2), anti-p38 MAPK, anti-phospho p38 MAPK, anti-stress-activated protein kinase (SAPK)/Jun N-terminal kinases (JNK), or anti-phospho SAPK/JNK, antibodies were from Cell Signaling Technology Inc. (Danver, MA, USA). The chemiluminescence ECL kit and rat thyroid-stimulating hormone (rTSH) kit were obtained from Amersham Bioscience. TRIzol reagent and SuperScript II RT are from Invitrogen and Taq DNA Polymerase from CENBIOT (Porto Alegre, RS, Brazil).

Animals

Fifteen-day-old male Wistar rats used in this study were originated from litters in our rat colony. The rats were maintained under a 12 h light:12 h darkness cycle in constant temperature (22 °C) room. On the day of birth, the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and 20% (w/w) protein commercial chow were available *ad libitum*. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

Induction of hypothyroidism

Wistar rats were mated and the day of appearance of the vaginal plug was considered day 0 of fetal age. Congenital hypothyroidism was induced by adding 0.05% PTU in the drinking water from day 9 of gestation, and continually up to postnatal day 15. Euthyroid rats, receiving only water during the same period, were used as controls. The dosage of PTU used is in agreement with previous reports (Choudhury *et al.* 2003, Dong *et al.* 2005, Gravina *et al.* 2007). Control and hypothyroid animals were used when 15 days old, considering that we have previously demonstrated the *in vivo* (Zamoner *et al.* 2007) and *in vitro* (Zamoner *et al.* 2005) cytoskeleton susceptibility to 3,5,3'-triiodo-L-thyronine (T₃) in 15-day-old rat testis. In all animal groups, the testes weight was measured on the day of the experiment.

Determination of hormone levels

Blood was collected, separated by centrifugation (500 g, 10 min) and the serum was stored at -20°C . Twelve to fifteen samples of control and treated groups were used to measure the serum hormone levels. Serum levels of T_3 and thyroxine (T_4), total and free, and TSH (ultrasensitivity method) were determined by RIA. The hTSH assay sensitivity was $0.003\ \mu\text{U}/\text{ml}$ (CRIESP, São Paulo, Brazil). In order to better characterize the hypothyroidism, the TSH levels were also measured by rTSH kit – Biotrak Assay System. The rTSH assay sensitivity was $0.05\ \text{ng}/\text{tube}$. The cross-reactivity with rat TSH is 100% and the non-specific binding was determined to be $<5\%$. The assay procedures were run according to the manufacturer's specifications.

Light and transmission electron microscopy

For light microscopy, the testes were removed, fixed in Bouin's solution overnight, and subsequently transferred to 70% ethanol for storage. The specimens were then dehydrated in a graded series of ethanol and embedded in Paraplast Plus. They were sectioned in $5\ \mu\text{m}$ slices, stained with hematoxylin and eosin, and examined by light microscopy.

For transmission electron microscopy, the contralateral testes were fixed with 3% glutaraldehyde, buffered with $0.2\ \text{M}$ phosphate buffer (pH 7.4), and post-fixed with 1% osmium tetroxide in the same buffer. They were dehydrated in a crescent concentration series of ethanol and passage through propylene oxide. The specimens were embedded with Spurr. Thin sections were obtained with glass knives in a Porter Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate (Watson 1958, Reynolds 1963), and observed with a JEM-1200Ex transmission electron microscope.

In vitro ^{32}P incorporation into vimentin

The *in vitro* phosphorylation of vimentin was carried out as described previously by Funchal *et al.* (2003) and Zamoner *et al.* (2005). In brief, testes of 15-day-old rats were pre-incubated at 30°C for 20 min in a Krebs–HEPES medium containing $124\ \text{mM}$ NaCl, $4\ \text{mM}$ KCl, $1.2\ \text{mM}$ MgSO_4 , $25\ \text{mM}$ Na-HEPES (pH 7.4), $12\ \text{mM}$ glucose, $1\ \text{mM}$ CaCl_2 , and the following protease inhibitors: $1\ \text{mM}$ benzamidine, $0.1\ \mu\text{M}$ leupeptin, $0.7\ \mu\text{M}$ antipain, $0.7\ \mu\text{M}$ pepstatin, and $0.7\ \mu\text{M}$ chymostatin. Incubation was carried out with $100\ \mu\text{l}$ of the basic medium described above, containing $80\ \mu\text{Ci}$ [^{32}P] orthophosphate. The labeling reaction was allowed to proceed for 30 min at 30°C and then stopped with $1\ \text{ml}$ of cold stop buffer containing $150\ \text{mM}$ NaF, $5\ \text{mM}$

EDTA, $5\ \text{mM}$ EGTA, $50\ \text{mM}$ Tris–HCl (pH 6.5), and the protease inhibitors described above. Testes were then washed twice by decanting with the stop buffer to remove excess radioactivity. After the *in vitro* phosphorylation procedures, the IF-enriched cytoskeletal fraction was extracted as described below.

Extraction of IF-enriched cytoskeletal fraction

The extraction of IF-enriched cytoskeletal fraction was proceeded as described by Funchal *et al.* (2003). Briefly, testes were homogenized in $600\ \mu\text{l}$ ice-cold buffer containing $5\ \text{mM}$ KH_2PO_4 , $600\ \text{mM}$ KCl, $10\ \text{mM}$ MgCl_2 , $2\ \text{mM}$ EGTA, $1\ \text{mM}$ EDTA, 1% Triton X-100, and the protease inhibitors described above. The homogenate was centrifuged at $15\ 800\ \text{g}$ for 10 min at 4°C in an Eppendorf centrifuge. The insoluble material was resuspended in $600\ \mu\text{l}$ of the same buffer and centrifuged as described. The pellet constituted the high-salt Triton-insoluble IF-enriched cytoskeletal fraction. This pellet was then dissolved in 1% SDS. Some experiments used total protein homogenate of the testis. For this, the tissue was homogenized in $300\ \mu\text{l}$ of a lysis solution ($2\ \text{mM}$ EDTA, $50\ \text{mM}$ Tris–HCl (pH 6.8), 4% SDS). The protein content was measured by the method of Lowry *et al.* (1951). For SDS–PAGE analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, $50\ \text{mM}$ Tris–HCl (pH 6.8), and boiled for 3 min. Equal protein concentrations of the total protein homogenate or the IF-enriched cytoskeletal fraction were analyzed by 10% SDS–PAGE (Laemmli 1970) and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer ($48\ \text{mM}$ Trizma, $39\ \text{mM}$ glycine, 20% methanol, and 0.25% SDS). In the experiments on *in vitro* ^{32}P incorporation, the nitrocellulose membranes containing the IF-enriched cytoskeletal fraction were exposed to X-ray films (T-mat G/RA) at -70°C with intensifying screens, and finally the autoradiograms were obtained and quantified as described below.

Western blot analysis

The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; $0.5\ \text{M}$ NaCl, $20\ \text{mM}$ Trizma (pH 7.5)), followed by 2-h incubation in blocking solution (TBS plus 5% defatted dried milk (MTBS)). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween 20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the monoclonal anti-vimentin antibody (clone vim 13.2), and diluted 1:400. In experiments designated to study the MAPK cascade anti-ERK1/2, anti-phospho ERK1/2, anti-p38 MAPK, anti-phospho p38

MAPK, anti-SAPK/JNK, or anti-phospho SAPK/JNK, antibodies were diluted 1:2000. The monoclonal anti-β-actin was diluted 1:1000 in blocking solution. The blots were then washed twice for 5 min with T-TBS and incubated for 2 h in MTBS containing peroxidase-conjugated rabbit anti-mouse IgG diluted 1:4000 (for anti-vimentin and anti-actin) or anti-rabbit IgG 1:1000 (for MAPK cascades). The blots were washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blots were then developed using a chemiluminescence ECL kit. Autoradiograms and immunoblots were quantified by scanning the films with a Hewlett-Packard ScanJet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company, Canberra, Australia).

Reverse transcription PCR (RT-PCR)

Total RNA from testes cells was isolated using TRIzol reagent. CDNA was synthesized from 1 μg total RNA. RNA was primed with 0.5 μg oligo (dT)_{12–18} primer (reaction volume 20 μl). After the RNA was denatured (10 min at 70 °C) and cooled on ice, the following reagents were mixed and then added: 10 mM of each deoxynucleoside triphosphate (dNTP), 6 μl 5× reverse transcription (RT) buffer, 0.1 mM dithiothreitol, and 200 U SuperScript RT (reaction volume 30 μl). Reaction was performed by incubation for 1 h at 42 °C. Amplification reactions consisted of 0.2 μM primer (described in Table 1), 10 μM of each dNTP, PCR buffer, 5 μl of the cDNA reaction, 0.5 U *Taq* DNA polymerase. Amplification was carried out during 30 PCR cycles, each cycle consisting of a denaturation step at 94 °C for 1 min, an annealing step at 60 °C for 2 min, and an extension step at 74 °C for 3 min after the last cycle, and finally, incubation for another 7 min at 74 °C was performed. The PCR products (5 μl) were analyzed on agarose gel containing 0.5 μg/ml ethidium bromide (Guma *et al.* 2001).

Oligonucleotides of 5' primers and 3' primers of target genes.

	5' primer sequence (5'–3')	3' primer sequence (5'–3')	Size of PCR product (bp)
mRNA species			
Vimentin	GCCTATGT- GACCCGGT- CCTCGGCAGT- GCGCCT	AGACGTGCCA- GAGAAG- CATTGTCAA	478
β-Actin	GTGGGCCGC- TCTAGGCAC- CAA	CTCTTTGATG- TCAACGCAC- GATTTC	540

Table 1 Effect of congenital hypothyroidism on serum levels of thyroid hormones and thyroid-stimulating hormone (TSH) in rats

	Control	Hypothyroid
FT ₃ (ng/ml)	3.25 ± 0.01	0.35 ± 0.003 [†]
FT ₄ (ng/dl)	2.21 ± 0.003	0.30 ± 0.002 [†]
T ₃ (ng/ml)	9.39 ± 0.01	0.72 ± 0.003 [†]
T ₄ (μg/dl)	6.11 ± 0.001	0.80 ± 0.001 [†]
TSH (μU/ml) – hTSH kit	0.03 ± 0.001	0.46 ± 0.002 [†]
TSH (ng/ml) – rTSH kit	0.93 ± 0.03	4.13 ± 0.2*

Serum levels of free and total T₃ (FT₃ and T₃ respectively), free and total T₄ (FT₄ and T₄ respectively), and TSH. Data are reported as means ± s.e.m.; n=9 from controls and n=9 from hypothyroid group. hTSH, human TSH; rTSH, rat TSH. Statistically significant differences from controls, as determined by Student's *t*-test, are indicated. **P*<0.0001, [†]*P*<0.001.

Oxygen consumption measurements

Testis from controls and hypothyroid pups were carefully excised, surface dried with filter paper, weighted, and exhaustively washed in ice-cold isotonic Krebs–Ringer bicarbonate (KRb) solution containing 15 mM glucose (pH 7.4). Small slices (50–80 mg) from these tissues were resuspended in 2 ml of the KRb buffer settled in a Tucker chamber containing a Clark electrode, and rates of oxygen consumption were recorded on an oxygraph for 2 min at a controlled temperature (25 ± 1 °C). The oxygen consumption was expressed as μmol O₂/min per g and measured in triplicate (Estabrook 1984).

Antioxidant enzyme assays

Tissue was homogenized (~500 mg tissue in nine volumes of buffer) in a buffer containing 0.1% Triton X-100, 0.12 M NaCl, 30 mM NaH₂PO₄ (pH 7.4), and also containing freshly prepared protease inhibitors (0.3 mM phenylmethylsulphonyl fluoride and 0.05 mM trypsin inhibitor). The use of Triton is essential for the measurement of total antioxidant enzymatic capacity of the tissue. Homogenization was carried out at 4 °C, using 15 strokes in a Potter-Elvehjem homogenizer, followed by centrifugation at 1000 *g* for 5 min at 4 °C. The supernatants were used for enzymatic evaluations and TBARS contents. Aliquots of the extracts were stored in liquid nitrogen (–170 °C) and examined separately for each enzyme. Superoxide dismutase (SOD) activity was measured according to the method of cytochrome *c* reduction (Flohé & Gunzler 1984). Catalase (CAT) activity was determined by the decrease in hydrogen peroxide (10 mM solution) concentration at 240 nm (Aebi 1984). Glutathione peroxidase (GPx) was measured through the system glutathione/

NADPH/glutathione reductase by the dismutation of *tert*-butylhydroperoxide (Flohé & Gunzler 1984). Glutathione reductase (GR) was measured through the oxidation rate of NADPH, in a reaction medium containing 0.1 M NaH₂PO₄ buffer (pH 7.0) containing 0.1% DPTA and 1.0 mM oxidized glutathione (GSSG) (Carlberg & Mannervik 1985). The enzyme glutathione S-transferase (GST) was evaluated according to Habig *et al.* (1974), using CDNB as a substrate.

Glutathione assay

GSH was measured according to Beutler (1975), using Elmann's reagent (DTNB). Tissue acid extracts were obtained immediately after tissue excision by the addition of 12% trichloroacetic acid (1:4 v/v), and then centrifuged. Fresh supernatants from the acid extracts were added to 0.25 mM DTNB in 0.1 M NaH₂PO₄ (pH 8.0), and the formation of thiolate anion was determined at 412 nm. TG was measured according to the method of Tietze (1969), and GSSG was calculated in equivalents of GSH (1 GSSG = 2 GSH).

Lipid peroxidation (TBARS)

Determination of thiobarbituric acid-reactive substances (TBARS) was used to assay endogenous lipid oxidation according to Ohkawa *et al.* (1979) and Bird & Draper (1984). Fresh homogenates were added to 0.2 mM butylhydroxytoluene (BHT) to avoid further artifactual lipid oxidation. Tissue acid extracts were obtained by the addition of the homogenate to trichloroacetic acid 12% (1:4 v/v), and then centrifuged at 5000 g for 3 min. Supernatants were added to 0.67% (w/v) 2-thiobarbituric acid, maintained in boiling water for 60 min, cooled at 5 °C for 30 min, and then measured spectrophotometrically at 535 nm in triplicate. Absorbance was expressed as nmol TBARS/g tissue ($E_{535} = 153 \text{ mM/cm}$). Frozen samples were not used because even when reacted with BHT, they showed artifactual lipid autoxidation and, therefore, enhanced TBARS levels (Wilhelm Filho *et al.* 2001).

Statistical analysis

Data were statistically analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison tests when the *F*-test was significant or by Student's *t*-test. All analyses were performed using the GraphPad InStat Software (San Diego, CA, USA) version 1.12a.

Results

Hypothyroidism was induced by adding 0.05% PTU in the drinking water from day 9 of gestation, and

continually up to postnatal day 15, resulting in decreased serum concentrations of free and total FT₃, T₃, and FT₄, T₄, associated with a significant increase in the TSH serum levels (Table 1). Furthermore, we observed a decrease in body and testicular weights of hypothyroid rats (Table 2).

Light micrographs of testes sections from control and hypothyroid rats are shown in Fig. 1. The seminiferous tubules from control testes had the typical organization and the lumen was already formed (Fig. 1A), while in the hypothyroid rats the seminiferous tubules were disorganized, showing no lumen formation, when compared with control animals (Fig. 1B). Transmission electron microscopy of Sertoli cells from hypothyroid rats showed atypical Golgi apparatus presenting swollen and disorganized vesicles, as well as abundant secretion vesicles. Moreover, hypothyroid Sertoli cells showed swollen mitochondria with markedly dilated cristae compared with control cells (Fig. 1C and D).

In addition, the effect of hypothyroidism on the *in vitro* phosphorylation of the Triton-insoluble cytoskeletal-associated vimentin was evaluated. The results demonstrated that congenital hypothyroidism increased the *in vitro* phosphorylation of this protein (Fig. 2).

We also analyzed the immunocontent of vimentin both in the total tissue homogenate and in the cytoskeletal fraction. The results showed that the vimentin immunocontent in tissue homogenate was unaltered, whereas it was increased in the cytoskeletal fraction (insoluble pool) from hypothyroid rat testis (Fig. 3). Furthermore, RT-PCR analysis showed that vimentin expression was not altered in the testes of hypothyroid animals (Fig. 4).

We next investigated the effect of hypothyroidism on MAPK signaling pathways (ERK1/2, – SAPK/JNK and p38 MAPK). Results showed that in congenital hypothyroidism the expression of total and phospho-ERK1/2 were diminished while total and phospho-SAPK/JNK were unaltered, nonetheless, despite the decrease in total p38 MAPK, phospho-p38 was increased (Fig. 5A–C).

Taking into account that THs are associated with hypermetabolic activity in several cell types (Venditti & Di

Table 2 Effect of congenital hypothyroidism on body and testicular weight

	Body weight (g)	Testis weight (mg)
Treatment		
Control	23.0 ± 1.5	51.0 ± 0.9
Hypothyroid	15.3 ± 0.33*	29.1 ± 0.3*

Data are reported as means ± S.E.M.; *n* = 12 for control and hypothyroid groups. Statistically significant differences from controls, as determined by Student's *t*-test, are indicated. **P* < 0.001.

Figure 1 Light and transmission electron micrographs of control and hypothyroid rat testis. (A and B) Hematoxylin–eosin-stained sections of seminiferous tubules from control and hypothyroid rat testis. (A) Seminiferous tubules showing lumen formation (Lu) and tubular integrity in control cells. (B) Disorganized seminiferous tubules from hypothyroid rat testis without appearance of lumen. (C and D) Transmission electron micrograph of Sertoli cells. (D) shows disorganized Golgi apparatus (Go) with secretion vesicles and swollen mitochondria (Mi) with dilated cristae in Sertoli cell from hypothyroid rat testis compared with controls (C). Nu, nucleus.

Meo 2006), we investigated some parameters of oxidative metabolism in congenital hypothyroid testis, such as oxygen consumption, lipid peroxidation, as well as the enzymatic and non-enzymatic antioxidant defenses. The results showed that oxygen consumption was diminished in hypothyroid testis, compared with the control group, a metabolic state compatible with the hypothyroid status

Figure 2 Effect of congenital hypothyroidism on vimentin phosphorylation in immature rat testis. Fifteen-day-old rat testes were incubated in the presence of ^{32}P orthophosphate for 30 min. The high-salt Triton-insoluble cytoskeletal fraction 0.0001. was extracted and the radioactivity incorporated into vimentin was measured. Data are reported as means $\pm$ S.E.M. of 17 animals and expressed as percentage of control. Statistically significant difference from controls, as determined by Student's *t*-test, is indicated. * $P < 0.0001$

(Fig. 6). In addition, TBARS levels were not altered, suggesting no modifications in lipid peroxidation associated with hypothyroidism in rat testes (Fig. 7). Moreover, we observed a decrease in total (TG) and reduced (GSH) glutathione contents, while the levels of oxidized glutathione (GSSG) were unaltered. Furthermore, the activities of glutathione reductase (GR) and GST were significantly inhibited, without altering the GPx activity in hypothyroid group (Fig. 8). In addition, hypothyroid rat testis presented increased catalase (CAT; Fig. 9A) and decreased SOD activities (Fig. 9B), when compared with normal animals.

Discussion

In the present study, the effectiveness of PTU in inducing a hypothyroid condition was confirmed by the reduction of T_3 and T_4 serum levels with a marked increase in TSH secretion, as well as by the impairment of testicular growth and decreased body weight.

The light microscopy results showed a disorganization of the seminiferous tubules, indicating that PTU treatment provoked significant structural alterations in the germinative epithelium. In addition, the

Figure 3 Effect of congenital hypothyroidism on total and cytoskeletal vimentin immunoccontent of immature rat testis. The immunoccontent of total (tissue homogenate) and high-salt Triton-insoluble vimentin was measured. Representative immunoblots: C, control; H, hypothyroid. Scans from 15 different animals from each group were quantified. Results are expressed as mean $\pm$ S.E.M. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. * $P < 0.0001$.

appearance of lumen, which marks the maturation of the Sertoli cells (Tindall *et al.* 1975, Russel *et al.* 1989), was observed only in the seminiferous tubules from control animals. Our results are in line with previous reports describing that hypothyroidism in the neonatal rat impairs testicular growth, germ cell maturation, formation of the lumen in seminiferous tubules, and other developmental events (Palmero *et al.* 1989, Francavilla *et al.* 1991, De França *et al.* 1995, Maran *et al.* 2001, Holsberger & Cooke 2005).

The results of transmission electron microscopy demonstrated an atypical and disorganized Golgi apparatus, supporting alterations in protein processing. We also observed swollen mitochondria containing markedly dilated cristae in hypothyroid rat testis. These alterations in organelle morphology suggest a cellular response to the hypothyroid status.

We have recently demonstrated that both *in vivo* and *in vitro* treatment with T_3 increased cytoskeletal vimentin phosphorylation in immature rat testis (Zamoner *et al.* 2005, 2007). In this context, hyperthyroidism could be associated with vimentin hyperphosphorylation and ERK pathway activation (Zamoner *et al.* 2007).

Figure 4 RT-PCR analysis of vimentin mRNA expression in control and hypothyroid rat testis. The larger band of DNA ladder correspond to 600 bp. C, control; H, hypothyroid.

Figure 5 Effect of congenital hypothyroidism on MAPK pathways ERK1/2, JNK, and p38 kinase of immature rat testis. The immunoccontent of total and phospho-ERK1/2, JNK, and p38 was measured in testis homogenate. Densitometry of (A) total and phospho-ERK1/2, (B) total and phospho-JNK, (C) total and phospho-p38 kinase, (D) actin immunoblot showing equal protein loading. Representative immunoblots: C, control; H, hypothyroid. Scans from ten different animals from each group were quantified. Results are expressed as mean $\pm$ S.E.M. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. * $P < 0.01$, ** $P < 0.001$.

Figure 6 Effect of congenital hypothyroidism on oxygen consumption in immature rat testis. Data are reported as means $\pm$ S.E.M. of ten animals from each group. Statistically significant differences from controls, as determined by Student's *t*-test, are indicated. * $P < 0.01$.

Interestingly, in hypothyroidism we also observed cytoskeletal-associated vimentin hyperphosphorylation; however, this effect could not be associated with ERK signaling, considering that we demonstrated decreased total and phospho-ERK levels. In addition, no changes were observed both in total and in phosphorylated JNK levels. On the other hand, the phospho-p38 MAPK levels were increased, despite decreased total p38 MAPK contents, suggesting that vimentin hyperphosphorylation might be, at least in part, related to alterations in p38 MAPK activity in hypothyroid rat testis. Our present findings concerning MAPK signaling pathways are in agreement with Franco *et al.* (2006) who have demonstrated that hypothyroidism was associated with high phospho-p38 MAPK and decreased phospho-ERK levels in liver. Our results emphasize that both hyperthyroidism (Zamoner *et al.* 2007) and hypothyroidism were associated with vimentin hyperphosphorylation, implying that alterations in the phosphorylation/dephosphorylation equilibrium of cytoskeletal proteins are a target for different physiological stimuli through different signaling mechanisms. The relevance of phosphorylation as a regulatory mechanism of the cytoskeletal dynamics lies on the fact

Figure 7 Effect of congenital hypothyroidism on lipid peroxidation in immature rat testis. Thiobarbituric acid-reactive substances (TBARS) measurement of lipid peroxidation. Data are reported as means $\pm$ S.E.M. of 15 animals from each group as determined by Student's *t*-test.

that IF proteins have multiple phosphate acceptor sites that could be sequentially and synergistically phosphorylated by several protein kinases leading to a multiplicity of cell responses (Roach 1991, Inagaki *et al.* 1997). Consequently, the sites of vimentin, which are phosphorylated in the hypothyroid status, could be different from the ones phosphorylated in the hyperthyroid status, which would explain the different polymerization/depolymerization equilibrium observed in these treatments. In hypothyroid rats, vimentin hyperphosphorylation was accompanied by increased vimentin immunoreactivity in the cytoskeletal fraction (vimentin in insoluble pool), despite the unaltered vimentin immunocontent in tissue homogenate that reflects the total content of the protein in the tissue (soluble and insoluble pool). These findings are in agreement with RT-PCR results showing no alterations in vimentin mRNA, indicating unaffected vimentin expression in hypothyroid rat testis. Nonetheless, the increased cytoskeletal-associated immunocontent of vimentin (insoluble) suggests that hypothyroidism might stimulate the ability of the phosphorylated form of this protein to polymerize/aggregate into cell testis. The modified vimentin phosphorylation/dephosphorylation equilibrium we are evidencing could support the altered structural organization observed in the hypothyroid testis cells, since it is known that the

Figure 8 Effect of congenital hypothyroidism on glutathione levels and glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST) activities in immature rat testis. (A) Total (TG), reduced (GSH), and oxidized (GSSG) glutathione levels. (B) Enzymatic activities of GR, GST, and GPx. Data are reported as means $\pm$ S.E.M. of 12 animals from each group. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. * $P < 0.001$, ** $P < 0.0001$.

Figure 9 Effect of congenital hypothyroidism on catalase (CAT) and superoxide dismutase (SOD) activities in immature rat testis. (A) CAT and (B) SOD activities. Data are reported as means $\pm$ S.E.M. of ten animals from each group. Statistically significant differences from controls, as determined by Student's *t*-test, are indicated. * $P < 0.01$, ** $P < 0.0001$.

cytoarchitecture is dependent upon the dynamic properties of the cytoskeleton, which, in turn, are regulated by the phosphorylation/dephosphorylation equilibrium of their constituent proteins (Inagaki *et al.* 1996). Moreover, our findings concerning vimentin hyperphosphorylation indicate that the mechanisms underlying vimentin aggregation into the cytoskeletal fraction in hypothyroid rat testis does not involve increased vimentin expression, which is in contrast with the previously reported increased expression of phosphorylated vimentin in hyperthyroid rat testis (Zamoner *et al.* 2007).

In this context, an implication of the present findings is that the deleterious effects of hypothyroidism may be elicited, at least in part, by altering the physiological equilibrium of cytoskeletal protein polymerization/depolymerization leading to loss of Sertoli cell IF integrity. Moreover, the morphological alterations observed in Sertoli cell mitochondria and Golgi apparatus could be related to defective cytoskeletal dynamics implying in cell damage. The loss or disruption of vimentin filament structure and dynamics is a cause of germ cell apoptosis after testicular insult (Romeo *et al.* 1995), occurs in concert with the failure of spermatogenesis after hormonal withdrawal (Show *et al.* 2003), and may be one of the mechanisms of reproductive toxicity (He *et al.* 2007).

We have previously described vimentin hyperphosphorylation and overexpression associated with increased oxygen consumption and oxidative stress in hyperthyroid rat testis (Zamoner *et al.* 2007). On the contrary, the present results of decreased oxygen consumption in TH-deficient testis are supported by the classical TH modulation of the metabolism of several cell types (Norman & Litwack 1997). Although lipid peroxidation, here estimated by TBARS levels, has been frequently used as an index of oxidative stress (Fernández *et al.* 1985, Venditti *et al.* 1997, Tapia *et al.* 1999, Zamoner *et al.* 2007), no alterations in this parameter were detected in hypothyroid rat testis.

The lower oxygen consumption in TH-deficient testis might be responsible for a lower ROS generation and therefore for the maintenance of lipoperoxidation levels. However, the induction of oxidative stress in the hypothyroid rat testis was detected in some enzymatic and non-enzymatic parameters of antioxidant defenses.

Total and reduced glutathione levels were decreased in TH-deficient rat testis, although GSSG was unaltered. These findings are consistent with the inhibition observed in GR and GST activities, since the inter-conversion GSH/GSSG/GSH is maintained by GR (Halliwell & Gutteridge 1999, Dröge 2002, 2005, Venditti & Di Meo 2006). However, the GPx activity was unaltered while the CAT activity was paradoxically increased and the SOD activity was also decreased in hypothyroid rat testis. In this context, the decline observed in TG and GSH levels, as well as the inhibition in SOD, GST, and GR activities highlight a relationship between hypothyroidism and oxidative stress in rat testis. GST uses GSH for its activity and depletion of reduced glutathione might be reflected in the lower GST activity. These enzymatic and non-enzymatic antioxidant defense dysregulation could be either the cause or the consequence of the oxidative stress due to poor ROS scavenging and, consequently, resulting in damage to testes cells. Irrespective of the levels of ROS generation, oxidative stress occurs if the antioxidants are lowered (Halliwell & Gutteridge 1999).

In conclusion, the above results support the hypothesis that p38 MAPK activation, the cytoskeletal alterations, and the overall decreased antioxidant defenses leading to oxidative stress could represent some of the mechanisms underlying cell dysfunction induced by hypothyroidism in rat testis. Since the cytoskeleton participates in critical cell functions, including the physiological regulation of ROS release from mitochondria (Gourlay & Ayscough 2005), cytoskeletal alterations induced by hypothyroidism might have important association with oxidative stress, as well as with morphological and biochemical changes in this tissue.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and PROPEsq-UFRGS. The authors are indebted to the Universidade Federal do Paraná (UFPR, Curitiba-PR, Brazil) for the electron microscope facilities. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 5 December 2007

Accepted 2 January 2008

Made available online as an Accepted Preprint

2 January 2008