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Akt1 protects against germ cell apoptosis in the postnatal mouse testis following lactational exposure to 6-*N*-propylthiouracil☆

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ABSTRACT

Exposure to 6-propyl-2-thio-uracil (PTU), a neonatal goitrogen, leads to increased testis size and sperm production in rodents. Akt1, a gene involved in cell survival and proliferation is also phosphorylated by thyroxine (T_4). Therefore, we examined the requirement for Akt1 in germ cell survival following PTU-induced hypothyroidism. Experiments were performed using Akt1+/+, Akt1+/−, and Akt1−/− mice. PTU was administered (0.01% w/v) via the drinking water of dams from birth to PND21. At PND15, T_4 serum levels were similar in all control groups, and significantly lower in all exposed groups with a dramatic decrease in Akt1−/− mice. PTU-exposed Akt1−/− testes displayed smaller tubules, increased apoptosis, delayed lumen formation, and increased inhibin B and AMH mRNA. Relative adult testis weights were similar in all exposure groups; however, no increase in daily sperm production was observed in PTU-exposed Akt1−/− mice. In conclusion, Akt1 contributes to the effects of thyroid hormone on postnatal testis development.

1. Introduction

An extensive body of literature demonstrates that thyroid hormone inhibits proliferation of Sertoli cells and stimulates their functional maturation in the postnatal rodent testis [1,2]. In contrast, hypothyroidism extends the Sertoli cell proliferative window resulting in a larger number of Sertoli cells with a concomitant increase in germ cells, Leydig cells, and consequently a larger testis [3–7]. Thyroid hormone receptors (TRs) are present in human and rodent testes from birth to adult life [8,9] which further emphasizes a role for thyroid hormone in postnatal testicular development [10,11]. The increase in testis weight has recently been demonstrated to primarily occur through activation of the thyroid receptor alpha 1 isoform (TRα1) [12]. Although the mechanism(s) whereby T_4 (thyroxine) and T_3 (triiodothyronine) regulate Sertoli cell proliferation remains unclear, recent studies have suggested that the suppressive effects of T_3 on Sertoli cell proliferation might be mediated by increased levels of expression of the cyclin-dependent kinase inhibitors (CDKIs) [13] and/or connexin43 (Cx43) [14]. PKBα/Akt1 dependent regulation of both cell number and cell size contributes to the establishment of organ size [15,16]. Hormones and metabolites are known to play a role in the signaling networks which control these developmental processes. PI3K catalyzes the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which then binds to other signaling molecules via a canonical pleckstrin homology domain. PDK1, phosphoinositide-dependent kinase 1, and PDK2, phosphoinositide-dependent kinase 2, then cooperate to activate Akt1/protein kinase B (Akt1/PKB) by phosphorylation at two critical residues [17]. Despite their significant homology, Akt1 and Akt2 have distinct functions in the control of growth and metabolism, respectively [18]. Akt1−/− mice display impaired overall growth, whereas Akt2 null mice are insulin intolerant, demonstrating a diabetes-like syndrome [19]. The distribution of Akt3 mRNA is more limited than that of either Akt1 or Akt2. Akt3 mRNA is most highly expressed in the brain and testes, but it is also detected in fat, lungs, and mammary glands [18]. Akt3 deficiency in mice results in a smaller brain size in adult mice [20]. Importantly, T_4 (thyroxine) has been shown to phosphorylate Akt in neonatal cardiomyocytes preventing their apoptotic cell death [21].

In the testis, the PI3K/Akt pathway is reported to play a role in Sertoli cell proliferation and differentiation *in vitro*. In immature rat Sertoli cells, follicle-stimulating hormone has been shown to amplify the insulin-like growth factor I-mediated activation of AKT/protein kinase B signaling [22,23]. This may be mediated, in

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part by Akt1-dependent aromatase transcription in immature Sertoli cells [24]. We have previously shown that Akt1 plays a role in the response to testicular injury following exposure to radiation and phthalates; therefore, we wanted to determine if lactational exposure to PTU, a neonatal goitrogen would result in increased germ cell apoptosis and/or influence the overall growth of the testis. Our results demonstrate that Akt1 prevents germ cell apoptosis following lactational PTU exposure. Interestingly, we found that Akt1-deficiency alone did not result in decreased T_4 levels relative to Akt1+/+ or Akt1+/− animals; however, Akt1-deficiency in PTU-exposed animals led to a dramatic decrease in T_4 levels following PTU-exposure relative to PTU-exposed Akt1+/+ and Akt1+/− animals. Expression of inhibin B and antimullerian hormone (AMH), two markers previously associated with Sertoli cell immaturity [25], was found to be increased in both control and PTU-exposed Akt1-deficient seminiferous tubules indicating a less mature Sertoli cell phenotype. In addition, we found no redundancy of Akt2 or Akt3 for Akt1-deficiency in our model system. Interestingly, Akt3 mRNA was induced 2-fold following transient PTU-exposure in Akt1+/+ testes suggesting that Akt3 may play a functional role in the postnatal testis. In summary, we propose that Akt1 promotes the maturation of the Sertoli cell. Loss of Akt1 leads to the expression of genes associated with delayed maturation of the Sertoli cell. Therefore, following exposure to PTU, this phenotype is not able to support germ cells consequently resulting in increased germ cell death.

2. Materials and methods

2.1. Mice

Akt1+/− mice were obtained from the laboratory of Dr. Morris Birnbaum (University of Pennsylvania) and have been backcrossed a minimum of five times into a C57BL/6 background. Mice were given water and standard lab chow *ad libitum*. The animal room climate was kept at a constant temperature (23.3 ± 2 °C) at 30–70% humidity with a 12 h alternating light–dark cycle. All procedures involving animals were performed in accordance with the guidelines of Brown University's institutional animal care and use committee in compliance with the guidelines established by the NIH.

2.2. Induction of neonatal hypothyroidism

Neonatal hypothyroidism was induced as described previously [3]. The pups in the experimental groups (transient neonatal hypothyroid) received PTU treatment administered by adding 0.01% (w/v) to the dams water flavored with Raspberry Crystal Light® an artificial sweetener (30 mg/L) to offset the bitter taste of PTU from day 1 of birth through day 21 postpartum. Control dams received tap water flavored with Raspberry Crystal Light® only and food *ad libitum* throughout the entire study period.

2.3. Primers

For genotyping by PCR, the following primers were used in a single PCR: 853,5′-GTGGATGTGGAATGTGTGCGAG-3′; 854, 5′-GCTCAGTCAGTGAGGCCAGACC-3′; 855, 5′-CACCCCACAAGCTCTTCTTCCA-3′. The PCRs were run with an initial denaturing step of 94 °C for 5 min, 39 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. PCR genotyping of progeny, the wild-type and targeted bands are 310 and 194 bp, respectively.

2.4. Serum collection and T_4 analyses

Animals were decapitated on PND15 and trunk blood collected in serum separator tubes. Serum was separated via centrifugation of clotted samples and stored at −80 °C for later analyses. Serum concentrations of total thyroxine (T_4) were analyzed by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). All samples were run in duplicate except for five samples in which only single replicates could be performed due to sample volume constraints. The inter-assay variations were less than 5%. The lowest calibrator was 5 ng/ml for the detection of T_4. In those cases where the sample result fell below the level of the lowest calibrator, the result was set by default to 5 ng/ml for statistical purposes.

2.5. Body and testis weights and spermatid head counts

The body and testis weights of control and PTU-exposed mice were weighed and recorded at 15, 25, and 90 days of age denoted PND15, PND25 and PND90, respectively. Testes obtained 90 days after transient neonatal PTU exposure were homogenized separately, and sperm heads were counted on a hemacytometer using previously described methods [26]. The counts from both testes of at least three animals were averaged for statistical analysis.

2.6. TUNEL staining and quantitation

Paraffin fixed testis sections were cut to 7 μm thickness and mounted on poly-L-lysine-coated glass slides (VWR Scientific, West Chester, PA). Germ cell apoptosis was detected in sections of paraffin-fixed testis by the TUNEL labeling method using the ApopTag kit (Chemicon, Temecula, CA). Tissue was counterstained with methyl green. Testis sections were viewed using a Nikon E800 microscope (Melville, NY) using differential interference contrast microscopy. The images were captured with a Kodak DC120 digital camera equipped with a MDS120 adapter (Eastman Kodak Co., Rochester, NY) and processed using Adobe Photoshop 6.0 software (Adobe, San Jose, CA). TUNEL-positive germ cells were quantitated in each tissue section by counting the number of TUNEL-positive cells in each essentially round seminiferous tubule. The incidence of apoptosis was then categorized into one of the three groups, defined as none, one to three, or more than three TUNEL-positive germ cells per seminiferous tubule cross-section. In the control mouse testis, the percentage of seminiferous tubules with more than three TUNEL-positive cells is less than 10%, so that an increase in apoptosis is easily determined using this data presentation. A minimum of 100 tubules per mouse were counted. The data, calculated as a percentage of the total, are expressed as the mean ± SEM.

2.7. RNA isolation and quantitative RT-PCR

Total RNA was isolated from testes of control and PTU-exposed Akt1+/+ and Akt1−/− mice. PND15 and PND25 testes were detunicated, weighed, and homogenized in TriReagent (Sigma Aldrich, St. Louis, MO) and RNA isolation was performed according to the TriReagent manufacturer's instructions. Total RNA (1 μg) was DNase-I (Invitrogen, Carlsbad, CA) treated and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocols, and the cDNA templates were amplified with each of the primer pairs in independent sets of PCR using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad). Mouse-specific primers were designed using Molecular Beacon Design 4.0 Software (Bio-Rad). The concentration of Mg^{2+} and the linear range of amplification of cDNAs with each primer pair first were optimized, and cDNAs were subsequently tested. Each sample was run in triplicate, and mRNA levels were analyzed relative to hypoxanthine