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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Molecular Mechanisms of Synthesis of Noradrenaline as an Inducer of Development in the Adrenal Glands of Rats in Ontogenesis

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Abstract—The level of gene expression and the protein content of tyrosine hydroxylase and dopamine β-hydroxylase were determined. In the perinatal period of rats, when noradrenaline functions as a morphogenetic factor, the level of gene expression of these enzymes increased and the content of protein products of these genes was almost unchanged, indicating the difference in the regulatory mechanisms of their transcription and translation.

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Noradrenaline is one of the most important chemical signals that is involved as a morphogenetic factor in the regulation of development of many vitally important organs of mammals during the critical period of their ontogeny. In rats, this is the perinatal period, when noradrenaline is synthesized in the brain, adrenal glands, and paraganglia, including the organ of Zuckerkandl [1, 2].

One of the major sources of noradrenaline in the blood of animals is the adrenal medulla, which functions throughout their life. At the end of the prenatal period of rats, the medullar secretory cells contain all enzymes required for the synthesis of noradrenaline and adrenaline—tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine-n-methyltransferase (PNMT). This indicates their secretory activity, which is determined by the ability to synthesize, accumulate, and secrete noradrenaline [3]. In turn, the main indicators of the level of noradrenaline synthesis are the content and activity of the specified enzymes.

The purpose of this work was to study the molecular genetic mechanisms of noradrenaline synthesis in the adrenal gland of rats in ontogeny in the critical (perinatal) period of morphogenesis. The objectives of this work were to evaluate the expression level of the genes encoding the enzymes involved in the synthesis of noradrenaline (TH and DBH) and the content of enzymes involved in the synthesis of noradrenaline (TH and DBH) as well as to find a possible correlation between these indices.

The study was performed on male Wistar rats on day 21 of embryonic development (E21, 14 embryos) and on days 3 (P3, $n = 10$) and 30 (P30, $n = 7$) of postnatal development. The rats were kept under the standard vivarium conditions and had ad libitum access to food and water. The adrenal glands were isolated from rats that were anesthetized with chloral hydrate (Sigma-Aldrich, United States) at a dose of 400 mg/kg.

The content of TH and DBH mRNA was determined by real-time PCR as described by Kozina et al. [4]. The reaction was performed in a 7500 Real-Time PCR System automatic thermocycler (Applied Biosystems, United States) using the qPCRmix-HS SYBR + ROX mixture (Fermentas, United States) and specific primers (Evrogen, Russia and Litekh, Russia): forward primer 5'-AGTCTGGCCTTCCGCGTGTTTCAA-3' and reverse primer 5'-CCCCCAGAGATGCAAGTCCAATG-3' for TH and forward primer 5'-GCCGAAATCTGGAATCCGCATCTT-3' and 5'-TGGGGGCTGTAGTGGTTGTCTCTG-3' for DBH. The values obtained for each sample were normalized to the expression level of the reference housekeeping *GAPDH* gene and expressed in arbitrary units. The *GAPDH* gene expression level was determined using the forward primers 5'-CTGACATGCCGCCTGGAGAAA-3' and the reverse primer 5'-TGGAAGAATGGGAGTTGCTGTTGA-3'. PCR efficiency was determined by constructing the standard curves [5].

The content of TH and DBH in adrenal glands was determined by the Western blotting technique. The adrenal glands were homogenized in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, and

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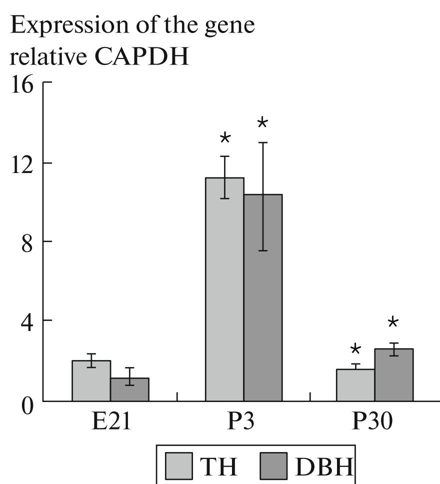

Fig. 1. The content of *TH* and *DBH* mRNA in the adrenal glands of rats in ontogeny. GAPDH is glyceraldehyde phosphate dehydrogenase. Data are represented as $M \pm m$. Measurements were performed on days E21 (n = 6), P3 (n = 6), P30 (n = 3). *$p < 0.05$ compared to the previous considered period of ontogeny.

1% NP-40) using a Labsonic M ultrasonic homogenizer (Sartorius AG, Germany) and centrifuged at 2000*g* for 20 min at 4°C. The protein content in the supernatants was determined using BSA. Then, the supernatant (100 μL) was supplemented with β-mercaptoethanol (5 μL) and boiled for 5 min. The resulting clarified homogenates were used for Western blotting analysis. Electrophoresis was performed according to Laemmli [6]. Each lane contained an equal amount of protein (20 μg of TH and 40 μg of DBH). The commercial kit Thermo Scientific PageRuler Plus Prestained Protein Ladder (Thermo Scientific, United Science) was used as a molecular weight marker. The proteins were transferred onto a nitrocellulose membrane for 1 h 15 min at 290 mA in a transfer buffer (25 mM Tris-HCl (pH 7.5), 192 mM glycine, and 20% ethanol). The transfer quality was assessed after staining with Ponceau S. The membranes were washed from the dye in TNT buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20). To prevent the nonspecific adsorption of antibodies, the membranes were incubated in a blocking solution (5% skim milk and 0.1% Tween 20) for 1 h at room temperature and then for 10 h with mouse monoclonal antibody to TH (1 : 1500, Sigma-Aldrich) and sheep polyclonal antibodies to DBH (1 : 1000, Abcam, United Kingdom) at 4°C. After incubation, the membranes were washed several times in TNT buffer and incubated with the second antibodies (conjugated to horseradish peroxidase; Jackson ImmunoResearch Labs, United States) to mouse IgG (1 : 50 000) and sheep IgG (1 : 50 000) at 20°C for 2 h. Then, the membranes were washed several times with TNT buffer and developed by the standard method using the enhanced chemiluminescence kit ECL Western Blotting Detection Reagent (Amersham, United States). X ray films were scanned, and the resulting images were processed using the ImageJ software, evaluating the integral absorption of protein bands. The content of DBH and TH was expressed as a percentage of the total protein content in the control sample (in this case, the brain tissue of rats on postnatal day 30).

The results were statistically processed using the GraphPad Prism6 program. The significance of differences was estimated using ANOVA and the Wilcoxon–Mann–Whitney test.

The *TH* mRNA content in the adrenal glands on day 21 of embryonic development (E21) was 2.07 ± 0.29 arb. units. By P3, the content of mRNA for this enzyme significantly (more than 5 times) increased and then decreased on day P30 to the level detected on day E21 (Fig. 1).

A similar picture was observed when measuring the level of DBH mRNA (Fig. 1). This means that the changes in the content of TH and DBH mRNA had a similar dynamics in the analyzed period of ontogeny. All these data are suggestive of the same mechanisms of transcription regulation of the genes for both enzymes.

The content of TH in the adrenal glands increased from day E21 to day P3 and did not change during further ontogeny (Fig. 2). The level of DBH increased by 42% from day E21 to day P3. A further increase in DBH (by 10%) on day P30 led to significant differences between the levels on days E21 and P30 (Fig. 2). In each studied period of ontogeny, the content of DBH exceeded the content of TH more than 3 times, and the ratio between them (TH : DBH) was 1 : 3.4 on day E21, 1 : 3.2 on day P3, and 1 : 3.3 on day P30. This may indicate the enhancement of upregulation of DBH translation in ontogeny. In addition, these data confirm the fact that TH is an rate-limiting enzyme of noradrenaline synthesis [7]. The high level of DBH may also be due to the fact that noradrenaline is the final and intermediate product synthesized by the adrenal medulla. However, the rate of its synthesis is ultimately determined not only by the content but also the activity of this enzyme.

Since the content of enzymes is the result of synthesis and degradation, it is determined by the rate of both processes. Tyrosine hydroxylase is located in the cytoplasm and is degraded by the ubiquitin–proteasome pathway [8]. Unlike TH, DBH, which is located in secretory granules [9], is protected from peptidases and is apparently degraded in the extracellular space after exocytosis of these granules. If the level of degradation of TH and DBH does not change in ontogeny, the increase in their content in the cell indicates an enhancement of synthesis. However, such information is almost absent in the literature. It is only known that the activation of the autonomic nervous system is accompanied by the stimulation of the synthesis of DBH in the adrenal glands, whereas glucocorticoids

inhibit its degradation [10]. Therefore, it can be assumed that the mechanisms of regulation of synthesis and degradation of DBH before and after establishment of functional adrenal innervation (approximately postnatal day 10 in rats) may differ.

Our findings should be considered in the context of the published data on the appearance of cells synthesizing adrenalin against the background of the population of cells that produce noradrenaline. This is indicated by the appearance of PNMT, which is first detected in the adrenal medulla of rats on day 18 of embryonic development. Then, up to day P3, all cells contain both noradrenaline and adrenaline. On days P2–P3, the separation of cells to those synthesizing predominantly noradrenaline or adrenaline begins, with increasing the number of the adrenalin-synthesizing cells [11, 12]. In young adult rats, as much as 80% of the adrenal medullar cells contain adrenaline [11–13]. We studied the period when the cells contain both noradrenaline and adrenaline, and only on day P30 the proportion of cells containing noradrenaline decreased [12]. Probably, the changes in the ratio of TH and DBH protein content, observed in this study, were largely determined by cell differentiation rather than by the changes in the mRNA content ratio, because the proportion of adrenaline in the total content of catecholamines in the adrenal medulla sharply increases in the period from day E18 until birth and then after birth reaches a level of 75% and then increases to ≥90% in adult animals [12].

Since the content of noradrenaline in the adrenal glands reflects their secretory activity and the involvement of noradrenaline in the regulation of morphogenesis of target organs, the assessment of its content in the adrenal glands in this period depending on the state of enzymes involved in different stages of its synthesis is particularly important. In our previous studies [14], we distinguished several stages at which the content of noradrenaline in rat adrenal glands increases in ontogeny. At the first stage (day E21 to P3), the content of noradrenaline sharply increases with increasing the content of TH and DBH mRNA. Then, in the period from day P7 to day P30, the content of noradrenaline continues to increase, despite the reduction in the content of mRNA and stabilization of TH and DBH protein synthesis, which is determined, apparently, by triggering other regulatory mechanisms, such as the maturation of secretory granules, reuptake mechanisms, and modification of enzyme activity.

In addition to the level of gene expression and the content of the enzymes, the noradrenaline synthesis rate is also determined by the activity of these enzymes. With this in mind, we also compared the activity of DBH in the adrenal glands in the studied period of ontogeny with the published data on the PNMT activity. Kvetňanský et al. [15] showed that the activity of DBH and PNMT in rats in the period from day P2 to day P30 changed insignificantly, similarly to the content of DBH in this study. However, the activity of PNMT was one order of magnitude higher than the activity of DBH [15], which may indicate that, in this case, the content of noradrenaline in the organ depends primarily on the content of DBH rather than on its activity.

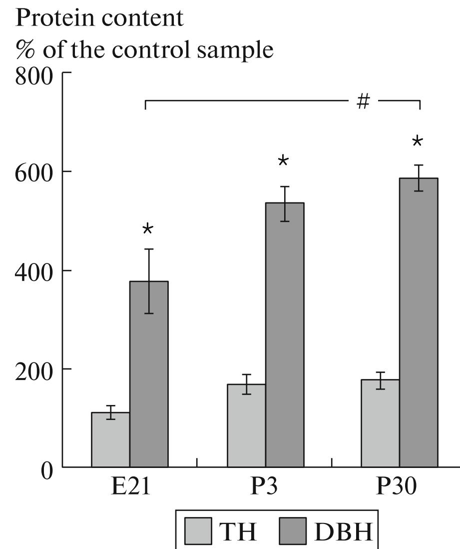

Fig. 2. The content of TH and DBH in the adrenal gland of rats in ontogeny. Data are represented as $M \pm m$. Measurements were performed on days E21 ($n = 8$), P3 ($n = 4$), P30 ($n = 4$). #$p < 0.05$ compared to E21, *$p < 0.05$ compared to TH.

Thus, in the ontogeny of rats, when noradrenaline functions as a morphogenetic factor, the expression level of TH and DBH genes in adrenal medullar cells increases, whereas the content of these enzymes remains virtually unchanged, suggesting different mechanisms of regulation of transcription and translation.

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