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Regulation of Gene Expression of Catecholamine Biosynthetic Enzymes in Dopamine- β -Hydroxylase- and CRH-Knockout Mice Exposed to Stress

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Abstract

Norepinephrine-deficient mice harbor a disruption of the gene for dopamine- β -hydroxylase (DBH-KO). Corticotropin-releasing hormone knockout mice (CRH-KO) have markedly reduced HPA activity. The aim of the present work was to study how deficiency of DBH and CRH would affect tyrosine hydroxylase (TH), DBH, and phenylethanolamine N-methyltransferase (PNMT) gene expression and protein levels in the adrenal medulla (AM) and stellate ganglia (SG) of control and stressed mice. Both in AM and SG, single immobilization significantly increased TH and DBH mRNA and protein levels both in wild-type (WT) and CRH-KO mice. On the other hand, the stress-triggered increase in the PNMT mRNA and protein levels seen in WT mice was absent in CRH KO mice. DBH-KO mice are more sensitive to stress but survive a single 2 h restraint stress in a tube (RES). The increase in TH mRNA levels induced by RES in WT was not observed in DBH-KO mice. PNMT mRNA and especially PNMT protein levels were significantly elevated in AM of DBH-KO mice. In SG of DBH-KO mice TH mRNA levels were not affected; however, PNMT gene expression was highly elevated. Thus, disruption of the DBH gene surprisingly blocks the stress-induced elevation of TH mRNA levels in AM but increases PNMT gene expression both in AM and SG. Our data indicate that adrenergic signaling is required for stress-induced increase in TH mRNA and that this signaling restrains stress-induced increase in PNMT mRNA. They also confirm that the HPA system plays a crucial role in the stress-induced regulation of PNMT gene expression.

Keywords

catecholamine biosynthetic enzymes; gene expression; DBH- and CRH- knockout mice; restraint stress; immobilization; glucocorticoid regulation

INTRODUCTION

Activation of the sympathoadrenal system (SAS) and elevated levels of released norepinephrine (NE) and epinephrine (EPI) represent a major and fast response of the mammalian organisms to stressors. Stress-induced activation of SAS is associated with increased catecholamine (CA) biosynthesis and elevated gene expression, proteosynthesis and activity of CA biosynthetic enzymes.^{1–3} These enzymes are: tyrosine hydroxylase (**TH** – conversion of tyrosine to DOPA), decarboxylase of aromatic amino acids (**AAAD** – decarboxylation of DOPA to dopamine-DA), dopamine- β -hydroxylase (**DBH** – hydroxylation of DA to NE), and phenylethanolamine N-methyltransferase (**PNMT** – N-methylation of NE to EPI).

Much data has been published on stress-induced changes of CA biosynthetic enzymes in the adrenal medulla and sympathetic ganglia. However, regulation of gene expression and proteosynthesis of these enzymes in DBH-knockout (DBH-KO) mice with targeted disruption of DBH gene has not been studied, especially under stress conditions. This mutant mouse model allows one to examine CA enzyme levels under control conditions as well as following their response to stressors in the complete absence of NE and EPI.^{4,5} The most clear phenotypical sign of these animals is ptosis; they are also cold intolerant^{6,7}, have increased basal levels of ACTH and corticosterone^{7,8}, are hypotensive with low heart rate⁹, have enhanced contractility and decreased β -adrenergic receptor kinase-1¹⁰, are deficient in social discrimination and lack isolation-induced aggression¹¹, and the density of β -adrenergic receptors in many brain areas is up-regulated.¹²

Corticotropin-releasing hormone knockout mice (CRH-KO) are unable to synthesize a functional CRH, the cortical fasciculate zone of their adrenal glands is markedly atrophic, they have profound impairment of corticosterone synthesis and secretion and elevated hypothalamic vasopressin mRNA levels.^{13–16} These animals have significantly lower plasma EPI levels and higher plasma NE levels mainly because their PNMT levels are lower.^{17,18} CRH KO mice represent an interesting model that can help to define mechanisms of HPA axis involvement in the regulation of the SAS including CA biosynthetic enzymes under normal conditions and during stress.^{14,15,17,18}

The aim of the present study was to investigate regulation of gene expression and translation of catecholamine biosynthetic enzymes (TH, AAAD, DBH and PNMT) in the adrenal medulla and stellate ganglia of dopamine- β -hydroxylase deficient and corticotropin-releasing hormone deficient mice under control conditions and after their exposure to stressors. The main aim of this work was to determine the significance and relative contributions of NE/EPI and CRH in the regulation of CA biosynthetic enzymes, or especially in stress conditions.

MATERIALS AND METHODS

Animals

The creation of DBH-KO mice was achieved by standard gene-targeting methods as described by Thomas et al. (1995)⁴ and Thomas (2002)⁷. Since these animals do not contain NE and EPI, they were prenatally treated with 1mg/ml L-threo-3,4-dihydroxyphenylserine (DOPS), a synthetic catecholamine precursor, in the maternal drinking water from embryonic day 9.5 until birth. No treatment was administered after birth. Administration of L-DOPS restores NE levels in many tissues of DBH-KO adult mice.¹⁹ Groups of both males and females (3–4 months old) were used for experiments.

Male CRH-KO mice (C57B1/129SV), approximately 3–4 months old were compared with wild-type mice (WT) in this experimental study. The CRH KO mouse line was originally donated by Dr. Joseph A. Majzoub (Harvard Medical School, Department of Endocrinology, Boston, USA) and currently the mice are bred in our facilities (Institute of Experimental Endocrinology). The presence of the CRH-KO or DBH-KO alleles was verified using DNA isolation from mouse tails with subsequent PCR.

Animals were housed three-four per cage under controlled environmental conditions (22±1°C, 12 h light/dark cycle, light on at 06:00 a.m.). Food and water were available *ad libitum*. The average weight of male mice was 25–30 g and female mice 20–24 g.

All animal experiments were approved by the Ethical Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences in Bratislava (protocol number 186-2-2003).

Stress procedure

Restraint stress (RES) was performed in 50 ml plastic tubes. Ventilation was provided by several holes on the upper side of the tube. Mice were restrained for 2 hours, transferred to home cages and sacrificed by decapitation 3 hours later.

Immobilization stress (IMO) was performed as described previously.²⁰ The size of immobilization boards was modified for mice. In the process of immobilization, mice were stressed for 2 hours and decapitated 3 hours after termination of the immobilization stimulus. Control mice were sacrificed immediately after removal from their home cages.

Organs collection

Adrenal medullae and stellate ganglia were rapidly extirpated, frozen in liquid nitrogen and stored at –70°C until assay.

Isolation of RNA and reverse transcription (RT-PCR)

Total RNA was prepared from frozen mice tissue using the RNeasyTM reagent (Tel-Test, USA) according to the manufacturer's instructions. Reverse transcription was performed from 1.5 µg of total RNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience) and pd(N)₆ primer according to the manufacturer's protocol. To determine mRNA levels in the adrenal medullae and stellate ganglia of mice the following primers

listed in Table 1 were used. After the initial denaturation at 94°C for 5 minutes, each cycle of amplification consisted of 60 seconds at 94°C, 30 or 60 seconds at the respective annealing temperature (Table 1), and 60 seconds at 72°C. Final polymerization was performed for 7 minutes at 72°C. All reactions were performed in the linear phase of amplification. PCR products were separated on 2% agarose/ethidium bromide gels. Density of individual bands was evaluated by system STS 6220I (Ultra-Lum, Inc.) and relative expression values were calculated by analysis of band intensities using PCBAS 2.08e software (Raytest, Inc.). Semi-quantitative values were expressed relatively to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot analysis

Adrenal medullae and stellate ganglia from individual animals were homogenized in 50–80 µl of 0.05M potassium phosphate, pH 6.65, 0.2% Triton X-100 and centrifuged at $10,000 \times g$ for 20 min at 4°C. Protein concentration was measured according to Bradford.²¹ Protein (10–15 µg) was loaded onto 10% polyacrylamide gels, separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA, USA) and transferred to the supported nitrocellulose membranes (Hybond CTM Extra, AP Biotech) using semidry electrophoretic blotting system (EBU-6000, C.B.S.Scientific Company Inc.). For immunoblot detection, membranes were first blocked in Tris-buffered saline Tween[®]20 (0.1%; TBST) containing 5% non-fat powdered milk for 1 hour at the room temperature on an orbital shaker. All subsequent washes and primary/secondary antibody incubations were also carried in TBST. Calibration curves were performed to select appropriate amount of protein loaded and film exposition time. Levels of the TH protein were determined using monoclonal anti-TH primary antibody (dilution 1:5000, Chemicon International), AAAD protein using polyclonal anti-AAAD antibody (1:1000, Calbiochem), DBH protein by polyclonal anti-DBH (1:1000, Sigma), and levels of the PNMT protein were determined using a polyclonal anti-PNMT primary antibody (dilution 1:1000, Protos Biotech Corporation). These incubations in primary antibodies were followed by incubations with horseradish peroxidase-conjugated secondary antibody appropriate to primary antibody (dilution 1:5000). Signal detection was facilitated with enhanced chemiluminescence (ECLTM, Amersham Life Science) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Buckinghamshire, UK). Optical density of immunoreactive bands was detected by PCBAS 2.08e software (Raytest, Inc.). Densitometric values were normalized for protein loading using glyceraldehyde-3-phosphate dehydrogenase (dilution 1:2000, Chemicon) as a control for loading and for local background.

Statistical evaluation

Results are presented as mean $\pm$ S.E.M. and each value represents an average of 3–7 mice. Statistical differences among groups were evaluated by one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant. For multiple comparisons, an adjusted t -test with p values corrected by Bonferroni method was used (Jandel SigmaStat Software, Jandel Corporation).

RESULTS

DBH-KO Mice at Control Conditions: mRNA and Protein Levels in Adrenal Medulla

TH and AAAD mRNA and protein levels did not differ in AM of male (left) or female (right) DBH-KO mice when compared to the WT mice (Fig. 1A, B).

Neither DBH gene expression, nor DBH protein levels were detected in AM of DBH-KO mice (Fig. 1C). Both, PNMT mRNA and protein levels were significantly increased in AM of DBH-KO male and female mice (Fig. 1D).

DBH-KO Mice with Restraint Stress: mRNA and Protein Levels in Adrenal Medulla

DBH-KO mice, compared to WT, are more sensitive to stress situations and survive only a single restraint exposure but not forced immobilization exposure. The restraint-induced significant increase in TH mRNA levels in WT mice is not observed in DBH-KO mice (Fig. 2). TH protein levels are not changed by a single restraint stress exposure either in WT or DBH-KO mice. AAAD gene expression and protein levels do not show any changes with restraint either in WT or DBH-KO mice (Fig. 2).

DBH mRNA levels are significantly elevated in WT mice after restraint stress exposure but in DBH-KO mice no DBH mRNA or protein levels were found (Fig. 3).

The biggest changes were found in PNMT levels (Fig. 3). Both PNMT mRNA and protein levels were elevated in control DBH-KO mice. A restraint-induced increase in PNMT mRNA levels was found in WT mice; however, no significant change was seen in DBH-KO mice (Fig. 3). PNMT protein levels were not influenced by a single restraint exposure.

DBH-KO Mice at Control Conditions: mRNA Levels in Stellate Ganglia

TH and AAAD mRNA levels were unchanged in stellate ganglia (SG) of DBH-KO mice (Fig. 4). However, PNMT mRNA levels in SG of these mice were increased 2.5 fold (Fig. 4).

DBH-KO Mice with Restraint Stress: mRNA Levels in Stellate Ganglia

Acute restraint stress does not significantly affect gene expression of enzymes TH, AAAD, and PNMT in SG of DBH-KO mice (Fig. 5).

CRH-KO Mice at Control and Stress Conditions: Adrenal Medulla and Stellate Ganglia

TH mRNA levels (Fig. 6A) and DBH mRNA levels (Fig. 6C) were significantly increased in AM and SG of WT mice exposed to a single immobilization stress. A similar increase was seen also in CRH-KO mice.

AAAD mRNA levels were not affected by immobilization stress either in WT or CRH-KO mice (Fig. 6B).

However the immobilization-induced increase in PNMT mRNA levels found both in AM and SG of WT mice was blocked in CRH-KO mice (Fig. 6D).

DISCUSSION

The aim of the present paper was to determine the effect of disruption of the DBH gene and thus elimination of NE/EPI levels or the CRH gene, on gene expression and protein levels of CA biosynthetic enzymes in the AM and sympathetic ganglia of mice under control conditions and during stress.

Dopamine- β -hydroxylase is one of the key enzymes catalysing the biosynthesis of catecholamines. Disruption of the DBH gene blocks the synthesis of NE and EPI.^{4,7} CA biosynthesis is also regulated by HPA axis, especially by glucocorticoids.²² In the promoter area of CA biosynthetic enzymes genes are elements regulated by glucocorticoids.^{2,23–27} In activation of HPA axis, CRH plays a key role. Knocking out the CRH gene greatly reduces the activity of HPA axis, especially during exposure to stressors. There are large reductions in corticosterone and EPI levels, together with reductions in PNMT gene expression and protein levels in CRH-KO animals.^{14,17,18,28}

To our knowledge this is the first study to examine changes in gene expression of CA enzymes in DBH-KO mice under stress. Compared to wild-type control mice, in AM and SG of DBH-KO control mice TH and AAAD mRNA and protein levels were unchanged, while DBH mRNA levels were absent, and PNMT mRNA and protein levels were significantly elevated.

Since TH is the rate-limiting enzyme for CA biosynthesis and DBH-KO animals do not have NE and EPI, we anticipated a potential increase in TH gene expression. Unchanged levels of TH mRNA found in catecholaminergic neurons of DBH-KO mice (Fig. 1A) might be explained by inhibitory feed-back activity of elevated dopamine levels in the DBH-KO mice.^{4,7,19}

Unchanged mRNA levels of AAAD (Fig. 1B) are not surprising because this enzyme is present in tissues in large excess and it does not play a role in limiting CA biosynthesis. No changes in AAAD gene expression or activity were described in the AM of stressed rats, in which TH, DBH, and PNMT activities²⁹ and mRNA levels were manyfold elevated.³⁰ The only reported change in AAAD mRNA level (increase) was found in stellate ganglia of repeatedly immobilized rats.³¹

In all DBH-KO mice (males and females) elevated levels of PNMT mRNA were found in the AM and SG. This could be due to loss of feed-back activity since DBH-KO mice do not synthesize EPI. Further, PNMT activity and gene expression are significantly regulated by the HPA axis.^{2,23–27} Interestingly, DBH-KO mice have increased basal activity of the HPA axis and contain higher ACTH and corticosterone levels.⁷ This could be responsible for the elevated PNMT mRNA levels in DBH-KO mice.

In addition, in sympathetic ganglia there is also a direct effect of ACTH on PNMT expression^{3,32,33}, as expression of MC-2 (ACTH) receptors is expressed there. Therefore elevated PNMT mRNA in stellate ganglia of DBH-KO mice might also be affected via a direct stimulation of these receptors by the elevated levels of ACTH found in DBH-KO mice.⁷

Both males and females DBH-KO mice showed almost identical levels of TH, AAAD, DBH, and PNMT mRNA and protein levels versus WT mice, supporting the uniformity of regulation of these processes in DBH-KO mice independently on sex.

Acute restraint stress produced elevated levels of TH, DBH, and PNMT mRNA but no changes in their protein levels in the AM of WT mice. However, in DBH-KO mice these restraint-induced increases in mRNA levels were absent. A potential explanation of this finding is that elevated extracellular levels of NE/EPI activate adrenergic receptors, which contribute to this increase in gene expression. The unchanged levels of TH mRNA might also be explained by feed-back effect of elevated DA levels especially in the adrenal medulla of DBH-KO mice.^{4,7,19} In addition, because PNMT expression is already elevated in control DBH-KO mice, further elevation may have been limited. In support of this, levels of PNMT mRNA in the AM were equivalent between samples from stressed WT and DBH-KO mice.

Protein levels of these enzymes are not changed by a single restraint stress either in WT or DBH-KO mice. This is not surprising because elevation of proteins require a longer or repeated stress exposure.^{2,34} Discrepancies between TH gene transcription, TH mRNA, and TH protein levels have suggested that post-transcriptional mechanisms could play an important role in TH^{35,36} and PNMT expression.^{27,36} Post-transcriptional mechanisms that control TH mRNA translation are not appropriately regulated. During a single stress exposure TH protein is connected with less TH mRNA molecules associated with polysomes while long-term stress regulates mechanisms that enhance TH mRNA translation. Trans-acting factors binding to the polypyrimidine-rich region of the 3'UTR of TH mRNA are proposed to play a role in these post-transcriptional mechanisms.³⁵ Thus, unchanged levels of TH protein, because of an inefficient translation, may play a vital role in fine-tuning the capacity of the adrenal medulla to synthesize CA during a single stress exposure.

Restraint stress did not affect mRNA levels of CA enzymes in the stellate ganglia. The explanation of this finding is most probably in the kinetics of stress triggered changes in gene transcription in sympathetic ganglia. The mRNA levels in the AM of stressed animals reach peak levels about 3 hours after the stress exposure is stopped,³⁰ and 24 hours later the levels were back at baseline. In sympathetic ganglia, however, mRNA levels peak about 24 hours after stress exposure and even 48 hours later the levels remain significantly elevated.^{30,37}

In CRH-KO mice, no differences were observed in TH mRNA and DBH mRNA levels in AM and SG when compared to WT mice both at control and stress conditions. These data indicate that CRH and HPA axis deficiency in CRH-KO mice do not affect the gene expression of TH and DBH. Similar changes in mRNA and also protein levels were found in CRH-KO mice exposed to repeated immobilization.^{17,28} AAAD mRNA levels did not show any changes in AM and SG of CRH-KO and WT mice at rest or during stress.

Stress-induced increases in PNMT mRNA levels both in AM and SG of WT mice were absent in the CRH-KO mice. Indeed, CRH via the HPA axis contributes to the regulation of PNMT activity, protein and gene expression^{13–15,17,18,28} and CRH-KO mice exhibit

impaired glucocorticoid synthesis and secretion.^{14,16,17} PNMT is regulated by glucocorticoids via two mechanisms: post-transcriptionally via regulation of the PNMT cosubstrate S-adenosylmethionine and transcriptionally through stimulation of GRE elements in the PNMT gene promoter.^{14,24–27}

Our results together with previous reports indicate that CRH deficiency in CRH-KO mice does not affect TH and DBH gene expression but leads to impaired PNMT gene expression during stress exposure, both in AM and SG.

Thus, disruption of the DBH gene elevates glucocorticoids and PNMT gene expression and proteosynthesis, suggesting that adrenergic signaling has an inhibitory role on these processes. The mechanism of these findings needs further study. On the other side, disruption of the CRH gene substantially reduces stress-induced increases in PNMT mRNA and protein levels both in AM and SG of mice. In both situations glucocorticoids and ACTH are very important factors responsible for the regulation of the sympatho-adrenomedullary and sympatho-neural systems.

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Figure 1.

DBH-knockout male and female mice at control conditions. The mRNA and protein levels of catecholamine biosynthetic enzymes, TH, AAAD, DBH, and PNMT, in the adrenal medulla of wild type (WT) and DBH-knockout (DBH-KO) mice are shown. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (6–7 males and 3–4 females/group). Statistical significance between DBH-KO and WT groups, * $p < 0.05$.

Figure 2.

DBH-knockout mice after exposure to restraint stress. The TH and AAAD mRNA and protein levels in the adrenal medulla of wild type (WT) and DBH-knockout (DBH-KO) mice are shown. Animals were sacrificed 3 hours after the end of the 2-hour restraint. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (3–4 female mice/group). Statistical significance between control and restraint groups, ^x $p < 0.05$.

Figure 3.

DBH-knockout mice after exposure to restraint stress. The DBH and PNMT mRNA and protein levels in the adrenal medulla of wild type (WT) and DBH-knockout (DBH-KO) mice are shown. Animals were sacrificed 3 hours after the end of the 2-hour restraint. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (3–4 female mice/group). Statistical significance between control and restraint groups, ^{xx} $p < 0.01$; and between DBH KO and WT groups, * $p < 0.05$.

Figure 4.

DBH-knockout mice at control conditions – stellate ganglia. The mRNA levels of catecholamine biosynthetic enzymes TH, AAAD, DBH, and PNMT in stellate ganglia of wild type (WT) and DBH-knockout (DBH-KO) mice are shown. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (3–4 female mice/group). Statistical significance between DBH KO and WT group, ** $p < 0.01$.

Figure 5.

DBH-knockout mice after exposure to restraint stress – stellate ganglia. The TH, AAAD, DBH, and PNMT mRNA levels in the stellate ganglia of wild type (WT) and DBH-knockout (DBH-KO) mice are shown. Animals were sacrificed 3 hours after the end of the 2-hour restraint. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (3–4 female mice/group). There are no statistically significant changes induced by restraint stress exposure. Statistical significance between DBH-KO and WT groups ** $p < 0.01$.

Figure 6.

CRH-knockout mice. Effect of a single immobilization stress exposure for two hours on TH, AAAD, DBH, and PNMT mRNA levels in the adrenal medulla (left side) and stellate ganglia (right side) of corticotropin-releasing hormone knockout (CRH-KO) and wild type mice (WT). Animals were sacrificed 3 hours after the end of the immobilization. Results are normalized relative to expression of the housekeeping gene GAPDH and are expressed in arbitrary units. Values are displayed as mean $\pm$ S.E.M. (4–6 animals/group). Statistical significance between control and immobilized groups, ^x $p < 0.05$; ^{xx} $p < 0.01$; ^{xxx} $p < 0.001$.

Table 1

Sequences of used primers and conditions of PCR reaction.

Gene	Primer sequence	Annealing temperature [°C]/time[s]	Number of cycles
<i>TH</i>	For: 5'-GAAGGGCCTCTATGCTACCCA-3' Rev: 5'-TGGGCGCTGGATACGAGA-3'	63/30	35
<i>AAAD</i>	For: 5'-AGCATGCACAGAGCTGGAGAC-3' Rev: 5'-AAGAAAGGAATCAGGCCAGC-3'	62/60	35
<i>DBH</i>	For: 5'-GACTCAACTACTGCCGGCACGT-3' Rev: 5'-CTGGGTGCACTTGTCTGTGCAGT-3'	60/30	35
<i>PNMT</i>	For: 5'-TACCTCCGCAACAACACTACGC-3' Rev: 5'-AAGGCTCCTGGTTCCTCTCG-3'	60/30	35
<i>GAPDH</i>	For: 5'-AGATCCACAACGGATACATT-3' Rev: 5'-TCCCTCAAGATTGTCAGCAA-3'	60/60	30