Existence of cardiac PNMT mRNA in adult rats: elevation by stress in a glucocorticoid-dependent manner

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Received 5 December 2000; accepted in final form 30 April 2001

Križanová, O., L. Mičutková, J. Jeloková, M. Filipenko, E. Sabbán, and R. Kvetňanský. Existence of cardiac PNMT mRNA in adult rats: elevation by stress in a glucocorticoid-dependent manner. Am J Physiol Heart Circ Physiol 281: H1372–H1379, 2001.—Phenylethanolamine N-methyltransferase (PNMT) is the enzyme that synthesizes epinephrine from norepinephrine. The aim of this study was to determine potential PNMT gene expression in the cardiac atria and ventricles of adult rats and to examine whether the gene expression of this enzyme is affected by immobilization stress. PNMT mRNA levels were detected in all four parts of the heart, with the highest level in the left atrium. Both Southern blot and sequencing verified the specificity of PNMT detected by RT-PCR. Single immobilization for 2 h increased gene expression of PNMT in both atria and ventricles. In atria, this effect was clearly modulated by glucocorticoids, because either adrenalectomy or hypophysectomy prevented the increase in PNMT mRNA levels in response to immobilization stimulus. This study establishes, for the first time, that PNMT gene expression occurs in cardiac atria and also, to a small extent, in ventricles of adult rats. Immobilization stress increases gene expression in atria and ventricles. This increase requires an intact hypothalamus-pituitary-adrenocortical axis, indicating the involvement of glucocorticoids.

Catecholamines, especially epinephrine (Epi), are known to be involved in augmenting cardiac function. Epi is a potent agonist of cardiac α- and especially β2-receptors, and it has an ~70-fold higher affinity for β2-receptors than its precursor norepinephrine (NE) (30). Stimulation of β2-receptors in cardiac atria increases both the rate and force of cardiac contractions (6). Physiological concentrations of plasma Epi affect stroke volume and cardiac output (12). Chronic elevations of plasma Epi result in cardiac hypertrophy (16), and very high concentrations can induce cardiomyopathy (17).

Epi is synthesized from NE by the enzyme phenylethanolamine N-methyltransferase (PNMT), which is most densely localized in the adrenal medulla. In small concentrations, Epi is found in almost all the organs of the body (5). Epi in the heart is believed to be taken up mainly from the circulation by sympathetic nerve endings (1, 13). However, some investigators (3, 37) have predicted that a portion of Epi in the heart might be synthesized directly in the cardiac tissue. Kvetňanský and co-workers (29) found a slight but significant elevation of plasma Epi levels in adrenalectomized rats exposed to stress, suggesting an extraadrenal source of Epi. The extraadrenal source of Epi was also proposed in humans, because after adrenalectomy patients maintain nearly normal levels of urinary Epi (40). Patients with heart transplants maintain adequate cardiac function even in the absence of sympathetic reinnervation (32, 34). Abnormal cardiac Epi production in patients with heart failure may be derived in part from sources other than the uptake from plasma or sympathetic nerves, because enhanced cardiac Epi spillover into the coronary circulation is unrelated to stress-induced cardiac sympathoadrenal activation (18).

Extraneuronal Epi synthesis in the heart has been repeatedly reported by Ziegler et al. (11, 23, 24). Huang et al. (14) demonstrated the existence of intrinsic cardiac adrenergic (ICA) cells in rodent and human hearts. ICA cells contain mRNA levels for enzymes involved in catecholamine synthesis. They concluded that ICA cells are different from sympathetic neurons. These findings suggest the existence of an intrinsic cardiac adrenergic signaling system capable of participating in cardiac regulation that appears to be independent of sympathetic innervation. Recently, it has been shown that enzymes involved in the synthesis of dopamine, NE, Epi, serotonin, and histamine exist within neurons of adult human cardiac ganglia (36). Immunoreactivity toward tyrosine hydroxylase, L-dopa decarboxylase, dopamine β-hydroxylase,

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PNMT, etc. was found in neurons of adult human cardiac ganglia. PNMT serves as a marker for tissues and cells producing Epi. Evidence that PNMT activity is present in the heart has been reported by several authors (3, 9, 11, 19, 23, 37, 39, 43). PNMT activity in the heart of adult rats is much lower compared with values found in newborn animals (7) or in rat fetuses (24). This is the most likely reason that PNMT gene expression was only found in embryonic rat hearts (10) and the presence of PNMT gene expression in the adult rat hearts has not been previously reported.

The present study focused on identifying the gene expression of the Epi biosynthetic enzyme PNMT in cardiac atria and ventricles of adult rats under basal conditions and during stress.

MATERIALS AND METHODS

Animals and immobilization. Male Sprague-Dawley rats (280–320 g) ~3 mo old from Charles River Farm (Suzfeld, Germany) were used in the majority of the experiments. Before the experiments, animals were housed for 1 wk with four animals per cage in a controlled environment (22 ± 2°C, 12:12-h light-dark cycle, light on at 6:00 AM). Food and water were available ad libitum. The Ethics Committee of the Institute of Experimental Endocrinology approved all presented experiments.

Immobilization stress was performed as described previously (27). Animals were immobilized for 2 h, transferred to home cages, and decapitated 3 h after the end of the immobilization. In specified experiments, bilaterally adrenalectomized or hypophysectomized male Sprague-Dawley rats were used. Adrenalectomized rats (~232.0 ± 3.3 g) and sham-operated rats (304.0 ± 3.4 g) were obtained from IFFA Credo Laboratories and were used for the experiment 10 days after the surgery. The hypophysectomy (hypophysectomized rats, 237.0 ± 5.0 g) and shamp operation (sham-operated rats, 362.0 ± 4.0 g) were performed by IFFA Credo Laboratories, and animals were used for the experiment 20 days after surgery. All adrenalectomized and hypophysectomized rats received isotonic saline instead of drinking water. Appropriately sham-operated rats were used as a control group. The success of both the adrenalectomy and hypophysectomy was checked by visual control of adrenal or pituitary removal and by levels of plasma corticosterone. Both procedures clearly documented the complete extirpation of adrenals or pituitaries.

RNA isolation and relative quantification of mRNA levels by RT-PCR. RNA was isolated by RNAzol. RT was performed using Ready-To-Go You-Prime First-Strand beads (AP Bio-tech) and pd(N)6 primer. PCR specific for PNMT was carried out afterward using the primers PT1 5'-TAC CTC CGC AAC AAC TAC GC-3' (position 1,171–1,190 in exon 1) and PT2 5'-AGG GCT CCT GGT TCT CG-3' (position 1,904–1,923 in exon 2), yielding a 260-bp fragment. For genomic DNA, these primers were intron spanning [in position 1,271–1,763, sequence according to Shuh et al. (35)]. The PCR program included 35 cycles of denaturing at 94°C for 1 min, annealing at 56°C for 1 min, and polymerizing at 72°C for 1 min. The number of cycles was determined by testing 15, 20, 25, 30, 35, 37, and 40 cycles (data not shown) to be within the linear range of amplification.

As a control for quantitative evaluation of PCR, primers for the housekeeper glyceraldehyde-3-phosphate dehydrogenase (GADPH1: 5'-AGA TCC ACA ACG GAT ACA TT-3'; GADPH2: 5'-TCC CTC AAG ATT GTC AGC AGC AA-3') were used to amplify a 309-bp fragment from each first strand sample. After denaturation at 94°C for 5 min, 30 cycles of PCR at 94°C, 60°C, and 72°C for 1 min each were performed (31).

For the seminested PCR, primers were designed as described in Comer et al. (8): PNMT3–01: 5'-AGG TCT CGG ACC TCA TAA CC-3'; PNMT3–02: 5'-CCG ATG AGA AGG AGA TGA CGG CC-3'; and PNMT5–01: 5'-CTA CCT CCG CAA CAA CTA CG-3'. As a negative control, amplification was performed on mRNA omitting the RT.

PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. Intensity of the individual bands was evaluated by Image software.

Sequencing of PCR fragment. The PCR product was purified using a Qiagen purification kit (Qiagen) and sequenced using the 32P end-labeled PT1 or PT2 primer as described by Wang et al. (41).

Southern blot analysis. Southern blot was performed as described by Sambrook et al. (33). The reaction product (10 μl) was fractionated on a supported nitrocellulose membrane (Hybond-ECL, AP Bio-tech) using semidy blotting. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline-Tween and incubated with the rabbit polyclonal antibody against bovine PNMT (Protos Biotech; dilution 1:1,000). This antisera is known to cross-react with PNMT protein in humans, monkeys, rodents, and cats. After the membrane was washed three times, it was incubated in the secondary anti-rabbit antibody conjugated to horseradish peroxidase (dilution 1:5,000). Secondary antibody was visualized by enhanced chemiluminiscence (AP Biotech).

Activity of PNMT. The activity of PNMT was determined by a radiometric assay as described by Culman et al. (9). PNMT was determined in tissue homogenates using S-[methyl-3H]adenosyl-L-methionine as the methyl donor and phenylethanolamine as the substrate. The radioactive product of the enzymatic reaction (N-methylphenylethanolamine) was extracted and then separated by thin layer chromatography.

Statistical analysis. Each value represents the average of at least 5 (mostly 8–15) animals. Results are presented as mean ± SE. Statistical differences among groups were determined by one-way ANOVA. Values of P < 0.05 were considered to be significant. For multiple comparisons, an adjusted t-test with P values corrected by the Bonferroni method was used (Instat, GraphPad Software).

RESULTS

Identification of PNMT gene expression in the rat heart. PNMT mRNA levels were determined by RT-PCR. The level of PNMT mRNA was quantified relatively to the housekeeper GAPDH. The 260-bp fragment of PNMT was detected in both cardiac atria and ventricles (Fig. 1). The highest levels of PNMT mRNA were found in cardiac atria compared with ventricles and control tissues.
were observed in the left atrium. In the left and right cardiac ventricles of control rats, the level of PNMT mRNA was very low, just at the detection limit. Therefore, we used another set of primers, which gave a larger band (656 bp). The RT-PCR yielded similar results as the previous set. Afterward, a third primer with a sequence within the fragment sequence was used, and seminested PCR was performed (Fig. 2A) to confirm the presence of PNMT mRNA in the cardiac ventricles. With the seminested PCR, a clear signal also appeared in the left and right ventricles of control animals. Thus PNMT mRNA is present in both cardiac atria and ventricles, although atria contain much higher levels.

Identity of PNMT fragment was verified by both Southern blot analysis and sequencing of the PCR fragment. The radiolabeled probe for PNMT hybridized to the amplified fragment (Fig. 2B). Additional verification was obtained by direct sequencing of this fragment. The sequence of the fragment was 100% identical to the published PNMT sequence (35) (GeneBank Accession No. GI 414186; data not shown), which proved that the PCR fragment was of PNMT origin.

The presence of immunoreactive PNMT in the rat cardiac atria, ventricles, and adrenal medulla (20 μg of protein as a positive control) was examined by Western blot and hybridization with specific PNMT antibody. The signal was visualized by enhanced chemiluminescence detection. Although we used as much as 50 μg of protein from cardiac atria and ventricles, we failed to detect a signal for PNMT, most probably because of its very low abundance (Fig. 3A).

PNMT activity was also measured in cardiac atria and ventricles (Fig. 3B). Although the PNMT activity represents both endogenous and exogenous enzyme production, the highest activity was also observed in the left atrium, the region that revealed the highest PNMT mRNA levels by RT-PCR.

Effect of immobilization stress on cardiac PNMT gene expression. The effect of immobilization stress on cardiac PNMT mRNA levels was examined. Single immobilization stress for 2 h significantly increased

![Fig. 1. Phenylethanolamine N-methyltransferase (PNMT) mRNA levels in cardiac left (LA) and right atria (RA) and left (LV) and right ventricles (RV). Top: representative result of gel electrophoresis is shown for both PNMT mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Bottom: columns represent the average of 15 animals. Results are normalized relatively to the housekeeper GAPDH. Each column is displayed as the mean ± SE.](http://ajpheart.physiology.org/)

![Fig. 2. A: RT-PCR and seminested PCR for the cardiac LV and RV as well as for the RA and LA. Lane 1, the 656-bp fragment after normal RT-PCR. Lane 2, 1 μl of the product was used for seminested PCR. This approach resulted in obtaining the 543-bp fragments in all four parts of the rat heart. B: Southern blot analysis with a hybridized cDNA probe for PNMT. The strong signal was obtained in both the LA and RA. A weak signal was visible in the RV, whereas practically no signal was observed in the LV, probably because the very low amount of PNMT mRNA was below the detection limit of this method.](http://ajpheart.physiology.org/)

![Fig. 3. Protein amount (A) and activity of PNMT (B) in cardiac atria and ventricles. Protein was determined by Western blot with a specific antibody against PNMT. From each part of the heart (LA, RA, LV, and RV), 50 μg of total protein was loaded. As a positive control, 20 μg of the adrenal medulla (AM) was loaded. PNMT activity (B) was determined as described in MATERIALS AND METHODS. Results are displayed as means ± SE, and each value represents the average of 8 animals.](http://ajpheart.physiology.org/)
levels of PNMT mRNA in both atria and ventricles (Fig. 4). The highest elevations compared with corresponding controls occurred in the left atrium [from 6,091 ± 612 to 22,079 ± 2,743 optical density (od) per mm²] and the right ventricle (from 168 ± 93 to 1,767 ± 352 od/mm²). However, PNMT activity did not show such a large change (Fig. 5). Although there is a tendency for an immobilization-induced increase in PNMT activity, especially in atria, the only significant increase (P < 0.05) in PNMT activity due to immobilization was observed in the left ventricle.

Effect of adrenalectomy and hypophysectomy on cardiac PNMT gene expression. Because the mechanism of the increase in cardiac PNMT mRNA levels induced by immobilization is not known, we examined the possible involvement of glucocorticoids in the observed upregulation. Glucocorticoids have been shown to be rapidly elevated by immobilization stress (25, 29). Therefore, we used adrenalectomized (removal of adrenals) or hypophysectomized (removal of pituitaries) rats and compared them with sham-operated control groups of rats. Both adrenalectomy and hypophysectomy failed to significantly affect PNMT mRNA levels in cardiac atria and ventricles in control unstressed rats. After a single immobilization, there were significant increases of PNMT mRNA levels in hearts of sham-operated rats. However, neither adrenalectomized (Fig. 6) nor hypophysectomized (Fig. 7) rats revealed any significant increase in cardiac PNMT mRNA levels compared with adrenalectomized and/or hypophysectomized control groups.

DISCUSSION

Presence and localization of PNMT mRNA in the rat heart. We show here, for the first time, PNMT gene expression in the heart of the adult rat under resting conditions and its increase during stress conditions.
Preliminary data on these findings have already been reported (26).

Previously, PNMT gene expression (determined by RNase protection assay) was only found in embryonic rat hearts and was not detected in adult rat hearts (10). Different methodological approaches as well as different parts of the heart used in the experiments may account for the inability to detect PNMT mRNA previously in the heart of the adult rat. The RT-PCR method with the specific primers used was sensitive enough to detect PNMT mRNA in the atria but was almost at the limit for its measurement in the ventricles. Therefore, seminested PCR was used to prove the presence of PNMT mRNA in the cardiac ventricles. With the use of this technique, a sufficient signal was also attained in the ventricles. The identity of the PNMT fragment was verified by Southern blot assay and by sequencing the PCR fragment, which confirmed 100% identity with the corresponding cDNA sequence. This methodological approach clearly proved that the fragment amplified by RT-PCR is of PNMT origin and that we specifically detected PNMT mRNA in heart tissues of adult rats.

Fig. 6. The effect of adrenalectomy (adrex) on mRNA levels of PNMT in the LA (A), RA (B), LV (C), and RV (D). Values are displayed as means ± SE, and each value represents the average of at least five animals. Statistical significance was calculated as described in MATERIALS AND METHODS and shows the difference between the control and immobilized animals in all parts of the heart. **P < 0.01; ***P < 0.001. ns, No statistical significance was observed between sham-operated controls and adrenalectomized control animals. #No difference between adrenalectomized controls and adrenalectomized immobilized animals.

Fig. 7. The effect of hypophysectomy (hypox) on mRNA levels of PNMT in the LA (A) and RA (B) of adult rat hearts. Values are displayed as means ± SE. Statistical significance was calculated as described in MATERIALS AND METHODS and shows the difference between the control and immobilized animals in both parts of the heart. ***P < 0.001.
Our data show that the distribution of PNMT mRNA levels within the heart is not uniform. The left cardiac atrium has a much higher abundance of PNMT mRNA than the right atrium, whereas the levels in ventricles are ~10 times lower. These findings are in very good agreement with our data on the distribution of PNMT activity in the areas of the heart (see Fig. 3) as well as with published reports (9, 11, 19, 37) on the localization of cardiac PNMT activity. PNMT activity in the adult rat heart was found to be relatively low and higher in the left atrium than in the right atrium and many times lower in the ventricles (9, 37). Although in our experiments the gene expression of PNMT was significantly increased in both cardiac atria and ventricles, PNMT activity was significantly increased only in the left ventricle. Two possible explanations may clarify this difference. First, a single immobilization with a subsequent 3-h rest period is a relatively short time, and cells probably do not have enough time to translate the information into functional protein. Second, besides endogenous PNMT (which was expressed and synthesized directly in the heart), exogenous PNMT protein could be transported through the sympathetic innervation, probably from stellate ganglia, and might affect the final PNMT activity in the cardiac tissue. Nevertheless, further experiments are needed to clarify this question. It has also been shown that the cardiac atria and ventricles contain two different inducible adrenaline-forming enzymes: PNMT and non-specific N-methyltransferase (NMT) (11). Cardiac atria contain primarily PNMT with a high affinity for NE, which is inhibited by specific PNMT inhibitor. Cardiac ventricles contain both PNMT and NMT. Ventricular NMT can synthesize Epi from NE and also N-methyldopamine from dopamine (43).

Although PNMT mRNA and activity were measured, we failed to detect PNMT immunoreactive protein in any of the investigated heart areas. Evidently, the Western blot analysis was not sensitive enough to detect small amounts of PNMT protein even when we used as much as 50 μg of total protein for the assay. To our knowledge, there are only a few studies (14, 23) showing a PNMT signal determined by Western blot analysis in the heart. Kennedy and Ziegler (23) reported that increased immunoreactive amounts of cardiac PNMT protein after dexamethasone treatment were detected by administration of anti-PNMT antibody. However, Huang and co-workers (14) got a positive PNMT signal by the Western blot technique in ICA cells and sympathetic neurons innervating the heart but not in myocytes. We assume that because in our samples the amount of cardiomyocytes was much more abundant compared with ICA cells and neurons, dilution of PNMT protein was so high that it was under the detection limit of Western blot analysis.

An immunohistochemical approach can overcome this problem. In human heart tissue, it has been shown that PNMT protein was localized within neurons of cardiac ganglia (36).

**Stress-induced changes in cardiac PNMT mRNA levels.** Immobilization stress is known to produce significant changes in the plasma and tissue levels of many hormones and neurotransmitters. In 1970, our laboratory (28) reported increased stress-elicited PNMT activity in the adrenal medulla, especially in repeatedly immobilized rats (29). Later on, we (38) reported that immobilization stress also highly increased PNMT mRNA levels in the rat adrenal medulla and that the increase was abolished by hypophysectomy.

In the present study, immobilization was found to significantly elevate PNMT mRNA levels in left and right cardiac atria and ventricles. The largest increases were seen in the left atrium and right ventricle. These data suggest that the gene expression of PNMT, which is localized mainly in heart nonneuronal cells (14, 23, 43), is regulated by very fast and robust processes. Cardiac PNMT activity did not follow the PNMT mRNA level increases induced by immobilization. This is not surprising because PNMT protein synthesis and its activation need more time than we used in the present experiments (5 h) for mRNA determination.

The cardiac Epi concentration was significantly elevated in all four parts of the heart of rats exposed to immobilization. Even if changes in cardiac PNMT mRNA and Epi levels during immobilization correlate very well, we believe that the increased cardiac Epi level in the given time interval is predominantly a consequence of Epi uptake from the circulation, where very high Epi levels were seen during immobilization (25, 29). Cardiac Epi production may be a reserve security mechanism that is activated mainly in emergency situations to supply the heart with the Epi levels necessary for physiological functions.

Because immobilization stress activates many cardiovascular, metabolic, neuroendocrine, and other processes, the highly stress-induced increase in cardiac PNMT mRNA levels suggests a participation of the cardiac adrenergic system in some physiological and/or pathophysiological processes. Cardiac adrenergic system has been suggested to play an important role in hypertension (21), arrhythmias and cardiomyopathies, and diabetes and insulin resistance (22).

**Regulation of cardiac PNMT mRNA levels.** Because adrenal medullary PNMT activity and gene expression are known to be regulated mainly by glucocorticoids via glucocorticoid receptors, the PNMT gene response element (GRE), and the transcription factor Egr-1 (42), and because plasma corticosterone levels are increased severalfold during immobilization stress (25, 29), we expect a similar mechanism also in regulation of the immobilization-induced increase in cardiac PNMT gene expression. Our data obtained in adrenalectomized or hypophysectomized rats only partially confirmed this prediction. Although adrenalectomy shows a tendency for elevation of PNMT mRNA compared with control rats, no statistically significant change was observed. When adrenalectomized rats were immobilized, no significant changes in PNMT mRNA were observed compared with adrenalectomized con-
control rats, in contrast with sham-operated immobilized rats. Hypophysectomy did not produce any change in PNMT mRNA levels in all studied heart areas of control animals. In hypophysectomized rats, no increase in PNMT mRNA was observed after immobilization. These data suggest that the PNMT gene expression in cardiac ventricles might be regulated by other factors besides glucocorticoids. At present, there are no available data to explain the regulatory mechanism of cardiac ventricular PNMT gene expression. This problem might be associated with a very low PNMT gene expression in cardiac ventricles.

The mRNA encoding for PNMT was also detected in the rat lung (20), spleen, and thymus (2, 15). Glucocorticoids increased lung PNMT activity by increasing levels of mRNA coding for this enzyme (20). Hybridization between adrenal PNMT cDNA and lung mRNA provided evidence that the lung Epi-forming enzyme is very similar in structure to the adrenal PNMT. Our sequencing of the PCR product clearly demonstrates that PNMT mRNA is identical with that in the adrenal medulla.

Adrenalectomy failed to reduce PNMT activity in cardiac atria and ventricles, but chronic dexamethasone administration significantly increased PNMT activity in these cardiac tissues (23). In our experiments, PNMT mRNA levels in atria and ventricles followed that trend and did not show any significant changes in adrenalectomized rats. However, an immobilization-induced increase in atrial PNMT mRNA levels (produced most probably by highly elevated corticosterone levels) was completely blocked by adrenalectomy. Neither dexamethasone nor adrenalectomy altered NMT activity in cardiac atria and ventricles. Our data demonstrate changes in PNMT gene expression exclusively because we proved that the measured mRNA coded specifically for the PNMT gene.

Recently, two forms of regulating PNMT activity in the hearts of embryonic rats have been described (24). One form is glucocorticoid dependent, and the other form is glucocorticoid independent. How the glucocorticoid-independent PNMT form is regulated is not known. Similar distinct mechanisms in regulation of PNMT gene expression might exist in cardiac atria and ventricles of adult rats at control and stress conditions.

Chemical sympathectomy with 6-hydroxydopamine increased heart PNMT activity (23, 37), most probably as a compensatory mechanism of activation of nonneuronal intrinsic cardiac adrenergic cells instead of destroyed heart sympathetic innervation. Our preliminary observations suggest that sympathetic ganglionic cells, e.g., of the stellate ganglia of adult rats, contain also PNMT mRNA (J. Jeloková, O. Krížanová, and R. Kvetnánský, unpublished observations). Thus the PNMT activity measured in the heart might be a mixture of PNMT present in ICA cells and in sympathetic innervation coming from ganglia. The level of the found PNMT mRNA is an indicator of the presence of intracardiac nonneuronal adrenergic cells.

Thus our data have shown that at least two mechanisms exist in the regulation of cardiac PNMT gene expression. The regulatory mechanisms depend on the homeostatic state of the organism (control condition, stress situation) and also on the considered cardiac area (atria, ventricles). On the basis of our data obtained in adrenalectomized and hypophysectomized rats, it appears that, under control conditions, PNMT gene expression in atria and ventricles is not regulated by a glucocorticoid mechanism. The stress-induced increase in atrial PNMT mRNA levels is fully dependent on the presence of glucocorticoids. However, the stress-induced increase in ventricular PNMT mRNA levels is mainly regulated by a mechanism other than one associated with glucocorticoids.

This work was supported by Slovak Grant Agency Grants VEGA 26109 and 27158 and by Fogarty Collaborative Research Award 1R03TW00894.

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