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ADRENERGIC RESPONSES TO STRESS: TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL CHANGES

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Abstract

Stress effects on adrenergic responses in rats were examined in adrenal medulla, the primary source of circulating epinephrine (Epi). Irrespective of duration, immobilization (IMMO) increased adrenal corticosterone to the same extent. In contrast, epinephrine changed little, suggesting that Epi synthesis replenishes adrenal pools and sustains circulating levels for the heightened alertness and physiological changes required of the "flight or fight" response. IMMO also induced the epinephrine-synthesizing enzyme, phenylethanolamine N-methyltransferase (PNMT). The rise in its mRNA and protein was preceded by increases in Egr-1 and Sp1 mRNA, protein and protein-DNA binding complex formation. With repeated and prolonged stress, PNMT protein did not reflect the magnitude of change in mRNA. The latter suggests that post-transcriptional, in addition to transcriptional mechanisms, regulate PNMT responses to stress. To further reveal molecular mechanisms underlying stress-induced changes in adrenergic function, the effects of hypoxia on PNMT promoter-driven gene expression are being examined in adrenal medulla-derived PC12 cells. Hypoxia activates the PNMT promoter to increase PNMT promoter-driven luciferase reporter gene expression and endogenous PNMT in PC12 cells. Induction of both appear mediated via activation of multiple signaling pathways and downstream activation of hypoxia inducible factor (HIF) and PNMT transcriptional activators, Egr-1 and Sp1. Hypoxia generates both partially and fully processed forms of PNMT mRNA. The former reportedly is translated into a truncated, non-functional protein and the latter into enzymatically active PNMT. Together, findings suggest that stress does increase PNMT gene transcriptional activity but post-transcriptional regulatory mechanisms limit the biological end-point of functional PNMT enzyme and thereby, Epi.

Keywords

phenylethanolamine N-methyltransferase (PNMT); stress; gene regulation; transcription factors; post-transcriptional control

INTRODUCTION

Stress affects everyone on a daily basis to greater or lesser extents. The student complains about the constant and insatiable noshing that occurs when they are preparing for their exams. The ice hockey player feels his heart pounding as he races down the rink with puck in tow, readying to slap in the winning goal. The speaker feels his/her heart pounding upon approaching the podium preparing to utter those first few sentences to engage the audience, hoping the quiver in his/her voice won't give away their nervousness. These are all classic symptoms of the adrenaline rush, the massive surge of epinephrine into the bloodstream, that permits us to meet the challenge of a stress and conquer it; the "fight or flight" mechanism described by Walter Cannon in the early 1900s ¹. Cortisol is co-released with Epi and together these stress hormones initiate a series of physiological and behavioral responses, including activation of the stress axis, the hypothalamic-pituitary adrenal (HPA) axis. CRF is synthesized and released from the paraventricular nucleus in the hypothalamus, stimulating the production and release of ACTH from the anterior lobe of the pituitary gland. ACTH, in turn, induces glucocorticoid production and release from the adrenal cortex. The first tissue to be bathed by newly synthesized corticosteroids is the adrenal medulla, the major source of circulating Epi. Elevation of glucocorticoids stimulates production of the Epi-synthesizing enzyme, phenylethanolamine N-methyltransferase (PNMT) and consequently, Epi is produced and released from the adrenal medulla into the circulation. In response to stress, the sympathetic nervous system is also activated, and acetylcholine and pituitary adenylate cyclase activating polypeptide (PACAP), neurotransmitters released from the splanchnic nerve innervating the adrenal medulla ², also contribute to PNMT activation and Epi production and release into the circulation. Eventually, homeostasis must be restored with the return of Epi and corticosteroids to basal expression. Epi is an autoregulator of its own production via inhibition of its biosynthetic enzyme PNMT ³. Throughout the HPA axis inhibitory feedback loops restore normal stress axis function and consequently, basal Epi and corticosteroid levels. Clearly, Epi is an important component of well-being in response to stress but excesses of stress and Epi can also be detrimental. Sustained stress and elevated Epi are considered major contributing factors in many illnesses, including cardiovascular disease, immune dysfunction and psychiatric disorders. Epi has pressor effects, increasing blood pressure and heart rate. The heart must work harder to maintain cardiac activity, and thus, sustained elevation of Epi can have dire consequence for cardiovascular function. Similarly, Epi affects many components of the immune system. If the latter is pre-activated, then it becomes more difficult to mount an immune response when necessary. Finally, altered Epi expression has been associated with behavioral disorders. Adrenergic cells in the C1, C2 and C3 regions of the brainstem send forward projections to the forebrain and midbrain, where Epi can be released to interact with alpha 1b receptors ⁴. These receptors lie proximal to dopaminergic, serotonergic and noradrenergic neurotransmitter centers that have been associated with psychiatric illness and are thought to modulate their function. These same centers are known targets for drugs effective in the treatment of behavioral disorders.

One of our long-term interests is to reveal how stress regulates Epi via its biosynthesis by PNMT, with the goal of developing therapeutics to sustain its positive effects while minimizing the negative effects that may lead to illness. In particular, we have been investigating whether genetic regulation of PNMT expression is an important mechanism for controlling adrenal medullary Epi levels, and hence, circulating Epi, in circumstances of stress.

It has previously been demonstrated that stress elevates the expression of PNMT mRNA ⁵. Specifically, in the adrenal medulla of rats, single and repeated immobilization (IMMO) stress markedly and rapidly increases PNMT message ⁶⁻⁸. Induction appears to be

predominantly due to corticosteroid activation of PNMT gene transcription as changes in PNMT mRNA expression can be prevented by the RNA synthesis inhibitor actinomycin D 7 and requires an intact stress axis 9. As duration of the stress, time to sacrifice and repeated IMMO increases, so does PNMT mRNA 10. However, this pattern of change, at least in part, likely reflects temporal changes in message expression as well. Certainly, in response to a variety of stimuli, maximum changes in PNMT mRNA occur between 6–8 hours 11–14.

To date, six transcriptional activators of the PNMT gene have been identified, the activated glucocorticoid receptor (GR) 15, 16, the immediately early gene transcription factor Egr-1 17, the constitutively expressed transcription factor and transcription initiator Sp1 18, the neural crest developmental factor AP-2 19, 20, glial cell missing-like factor GCM₁ 21 and the tumorigenic, cMyc-activating factor MAZ 22. Examination of their expression in nuclear extracts isolated from adrenal medulla of rats subjected to IMMO provides further evidence supporting PNMT gene activation. We have demonstrated that induction of both Egr-1 and Sp1 occurs and precedes the rise in PNMT message, consistent with what one would expect for transcription factors stimulating the target gene 10. Specifically, levels of their mRNA determined by ribonuclease protection assay or reverse transcription-polymerase chain reaction (RT-PCR) as well as their protein content in the nucleus determined by western analysis and the ability of their nuclear protein component to form protein-DNA complexes determined by gel mobility shift assay (GMSA) increases with duration of IMMO, time to sacrifice and repeated exposure to IMMO 10. However, the rise in Sp1 mRNA, protein and protein-DNA complex is not perfectly matched, suggesting that post-transcriptional regulation may influence PNMT gene transcription. Sp1 and Egr-1 are proteins that are post-translationally modified by phosphorylation. In the case of Egr-1, phosphorylation does not alter its interaction with its consensus sites within gene promoters but when bound does enhance its ability to induce transcription. In contrast, phosphorylation of Sp1 is required for both binding to its consensus sequences and gene activation. When PNMT protein is assessed by western analysis, a mismatch with mRNA changes is also apparent. Repeated IMMO leads to an inverse pattern of change in protein compared to message with PNMT mRNA rising with duration of stress and time to sacrifice and PNMT protein expression declining. This discordance in PNMT mRNA and protein again points to the import of post-transcriptional regulatory controls in determining the final outcome of stress-induced expression of functional PNMT protein. As well, the reduction in PNMT protein expression relative to mRNA may also be indicative of the onset of desensitization to repeated IMMO stress, despite the fact that marked corticosterone activation still occurs.

To further identify molecular mechanisms underlying stress-induced activation of PNMT, we are currently utilizing a hypoxic stress model. Certainly, it is well established that different stressors vary in their effects on circulating Epi levels and the catecholamine biosynthetic enzymes depending on the intensity of the stress and exposure duration 23, 24. However, we hypothesize that for those stressors activating Epi and PNMT, there are both unique and common factors. In the case of our hypoxic stress paradigm, we are utilizing adrenal medulla-derived PC12 cells 25. Our approach is two-fold. First, the PNMT promoter-luciferase reporter gene construct pGL3RP893, a construct containing 893 bp of the rat PNMT promoter upstream of the firefly luciferase gene, can be inserted into the cells by phenylethylamine transfection 26. The oxygen concentration to which the transfected cells are exposed is then reduced from normoxic conditions (21% oxygen) to hypoxic conditions (5% oxygen). Five percent oxygen is considered moderate hypoxia, but further reduction in oxygen appears to lead to cell death. Assessment of PNMT promoter-driven luciferase expression, determined by luminescence assay 27, then serves as an indirect measure of PNMT gene expression. Second, it is well established that PC12 cells express endogenous PNMT 28, 29. Hence, the cells can simply be exposed to 5% oxygen and total RNA and cytosolic and nuclear protein extracts isolated. PNMT and transcription factor

mRNA can be quantified by RT-PCR and corresponding proteins assessed by western analysis. In the case of the transcription factors, protein-DNA complex formation can further be examined by GMSA using the nuclear protein extracts.

Preliminary findings demonstrate that exposure of PC12 cells transfected with the PNMT promoter-luciferase reporter gene construct rapidly and markedly elevates PNMT promoter-driven luciferase expression. Elevation of luciferase activity is observed at 1.5 hr, with maximum induction apparent at 6 hr (~5-fold induction) and sustained through at least 72 hours (Table 1, data shown through 24 hr).

We have also begun to investigate potential signaling pathways that may be associated with hypoxic responses of the PNMT promoter. To this end, the PC12 cells transfected with the PNMT promoter-luciferase reporter gene construct pGL3RP893 are pre-treated with a variety of signaling pathway inhibitors. Since the integrity of signaling pathways is critical to cell survival, we have titrated all inhibitors used to date to make sure that the concentrations employed are not toxic to the cells. Following pretreatment with the inhibitors, the cells are exposed to hypoxia and after 6 hours, luciferase activity determined. Preliminary findings indicate that two major signaling pathways are critical to hypoxic activation of the PNMT promoter, that of cAMP and phospholipase C. However, every inhibitor that we have examined thus far abrogates hypoxic induction of the promoter to varying extents. So at present we are left to assort downstream signaling mechanisms and to ascertain whether any crossover between signaling subsequently occurs.

Cell signaling, of course, subsequently leads to transcription factor activation. In the case of hypoxia, the transcription factor family that is consistently induced is that of the hypoxia inducible factors (HIFs). HIFs are an interesting class of proteins because they appear to be constitutively expressed in most cells 30. However, their cellular activity is limited by hydroxylation. Specifically, hydroxylation of critical asparagine residues at the carboxy-terminus of the HIF molecule prevents HIF activation by precluding interaction with the transcriptional co-activator CBP/p300 while hydroxylation of critical proline residues leads to ubiquitinylation of HIF and rapid proteosomal degradation. To investigate the potential role of HIF in hypoxic activation of the PNMT promoter, we have begun to examine the effects of HIF over expression by performing co-transfection assays with HIF constructs (courtesy of Dr. H. Franklin Bunn, Division of Hematology, Harvard Medical School) paired with our PNMT promoter-luciferase construct in the PC12 cells. Initial findings indicate that HIF does play a role in hypoxia-induced PNMT transcriptional activation. When a HIF1 α , truncated HIF1 α (active portion of the molecule) or HIF1 β expression construction is co-expressed with the wild-type PNMT pGL3RP893 construct, a marked activation of luciferase activity is observed under both normoxic and hypoxic conditions, with highest expression driven by HIF1 α . In fact, HIF1 α is the predominant HIF isoform expressed in chromaffin cells in the adrenal medulla. Consistent with the latter, we also have preliminary evidence that the HIF1 α stimulators, cobalt chloride and desferoxamine activate PNMT promoter-driven luciferase expression to the same extent as the HIF1 α expression construct. Moreover, hypoxia induces HIF1 α expression in the nuclear protein component of PC12 cells (Fig. 1A)

If there are common transcriptional proteins participating in hypoxic activation of the PNMT promoter, what might those be among known transcription factors underlying PNMT gene activation? From PC12 cells exposed to 5% oxygen, total RNA and cytosolic and protein nuclear extracts have been isolated, and RT-PCR, western analysis and GMSA are being used to assess their contributions to hypoxic induction of PNMT. Evidence to date again suggests that Egr-1 and Sp1 are important (Fig. 1B). Western analysis of nuclear protein extracts demonstrates a rapid and transient rise in Egr-1 expression. Elevation is

apparent at 30 min, peaks between 45–60 min and then rapidly declines so that at 24 hours, levels of Egr-1 do not appear significantly different from basal expression in the PC12 cells. While the rise in Sp1 is rapid as well, in contrast to Egr-1 its elevation is sustained at least through 24 hours. Both the 105 and 95 kDa phosphorylated forms of Sp1 are expressed with the former being more abundant, suggesting that hypoxic stress leads to more extensive phosphorylation of Sp1 protein. When we compare the pattern of change in Egr-1 mRNA to Egr-1 protein, it would appear that while mRNA is highest at 1 hr, protein is highest at 6 hours. Whereas there is a significant decline in Egr-1 protein by 24 hours, the decline in mRNA at the same time point is not as marked. Finally, when hypoxic changes in PNMT mRNA and protein are examined, PNMT message is most elevated at 6 hours. As described previously, this finding is consistent with temporal induction of PNMT mRNA in response to a variety of stimuli. By 24 hours, it would appear that there is some decline in PNMT message but qualitatively, levels are equivalent to those observed with 1 hour of exposure to hypoxia, where PNMT mRNA is approximately about 2-fold higher than basal values. Interestingly, PNMT protein elevation induced by hypoxic stress also shows maximum induction at 6 hours with a slight decline at 24 hours. However, levels at 24 hours appear about 2-fold higher than those observed at 1 hour. The striking finding is that maximum expression of PNMT protein occurs at 6 hr. PNMT is a slow turnover protein, and a variety of stimuli have not been shown to maximally up-regulate its expression until 24 hours.

The above-described changes in PNMT transcriptional activators and PNMT provide correlative evidence of the role of these transcription factors in hypoxic induction of PNMT. If indeed they do participate in hypoxic transcriptional regulation of this enzyme, then mutation of the activation sites to which they bind in the PNMT promoter should abrogate the response. Using one of our PNMT promoter-luciferase reporter gene constructs in which the predominant Egr-1 site in the rat PNMT promoter has been inactivated by site directed mutagenesis 17, we have compared the effects of 5% oxygen on PNMT promoter-driven luciferase expression in PC12 cells transfected with that construct versus the wildtype construct with intact promoter. Reduction of oxygen completely prevented PNMT promoter stimulation by hypoxia in cells harboring the mutant construct.

In recent years, two forms of PNMT messenger RNA have been identified in PC12 cells 28 as well as rat and mice heart 31 and rat adrenal medulla 32. The “long” form represents partially processed primary PNMT transcript where one of two introns is not removed. The consequence of the latter is the introduction of a stop codon. Thus, when the “long” form of PNMT mRNA is used as a template for protein translation, a truncated PNMT protein is produced, which appears to be inactive. The “short” form of message represents fully processed PNMT mRNA from which full length, functionally active PNMT enzyme is derived upon its translation. When conditions for separating PNMT cDNA amplicons on polyacrylamide gels were optimized, both the “long” and “short” forms of message were detected. It would appear that although hypoxia may induce transcriptional activation of PNMT gene expression, post-transcriptional controls may limit the amount of mRNA template generating active enzyme. In earlier studies, we have previously shown that hormones can also control the rate of degradation of PNMT protein 33. They appear to do so by regulating levels of the methyl donor and co-substrate, S-adenosylmethionine (AdoMet), used in the PNMT catalyzed conversion of norepinephrine to epinephrine 34. Binding of AdoMet to PNMT apparently protects the enzyme from proteolytic degradation.

In summary, it is apparent from both studies in rats and PC12 cells, that stress can stimulate PNMT, and thereby Epi expression, via transcriptional activation of the PNMT gene. Fig. 2 schematizes present knowledge of signaling pathways and transcription factors associated with hypoxic stress activation of the PNMT gene as suggested by the findings included

herein. However, as importantly, post-transcriptional regulatory mechanisms exert important additional controls on the final biological endpoint of functionally active PNMT enzyme.

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Figure 1.

Hypoxia-induced changes in HIF-1 α and PNMT transcriptional activators Egr-1 and Sp1 in PC12 cells. A. PC12 cells, untransfected or transfected with a HIF-1 α expression construct (courtesy of Dr. H. Franklin Bunn, Division of Hematology, Harvard Medical School), were exposed to 5% oxygen for 24 hours. Nuclear protein extracts were prepared and analyzed for HIF-1 α protein by western analysis (HIF-1 α antibody, Santa Cruz Biotechnology, Santa Cruz, CA). B. Untransfected PC12 cells were exposed to hypoxia for 24 hours. Nuclear protein extracts were prepared and analyzed for Egr-1 and Sp1 protein by western analysis (Egr-1 and Sp1 antibodies, Santa Cruz Biotechnology, Santa Cruz, CA).

Figure 2. Hypoxia-induced transcriptional activation of PNMT. Schematic of hypothesized molecular mechanism associated with hypoxia-induced activation of the PNMT gene.

TABLE 1

PC12 cells transfected with the PNMT promoter luciferase reporter gene construct pGL3RP893 were exposed to 5% hypoxia and luciferase activity determined after different times of exposure to reduced oxygen by luminescence assay 17. Luciferase activity remained elevated through 72 hr. Data shown through 24 hr.

TIME (hours)	LUCIFERASE ACTIVITY	±SEM
0	1.42	0.08
1.0	1.63	0.22
6.0	6.54	0.37
24.0	6.22	0.64