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Immobilization Stress Elevates Intron Containing Transcripts for Tyrosine Hydroxylase in Rat Superior Cervical Ganglia Indicating Transcriptional Activation

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Abstract

While both the adrenal medulla and sympathetic nervous system are important in mediating the catecholaminergic response to stress, there are crucial differences in the mechanism. Stress elevates tyrosine hydroxylase (TH) protein and mRNA levels in both the adrenal medulla and sympathetic ganglia. In the adrenal medulla, transcription of the TH gene is rapidly induced with immobilization stress (IMO). Here, we examine whether IMO also increased TH transcription in the superior cervical ganglia (SCG). Quantitative real-time RT-PCR was used to determine the changes in TH mRNA and in transcripts containing intron 2. As expected in the adrenal medulla, following repeated IMO TH mRNA and intron containing transcripts were elevated about 5-fold. In SCG, significant increase in TH mRNA was observed following repeated 2 hr IMO for 2 or 6 days, but not with single IMO. The intron 2 containing transcripts were elevated about 50% above controls with even single IMO, and were at similarly elevated levels after the 2nd or 6th repeated daily IMO. The results indicate, for the first time, that transcriptional mechanisms are involved in mediating the IMO stress triggered elevation in TH gene expression in the SCG.

Keywords

mRNA; sympathetic nervous system; stress; ganglia; transcription; tyrosine hydroxylase

Introduction

Originally the sympathoadrenal system comprised of the sympathetic nervous system and the adrenal medulla was considered as one system mediating the “fight or flight” response to stress (Cannon 1929). Early studies showed that a variety of stressors increase activity of tyrosine hydroxylase (TH) the major rate limiting enzyme in catecholamine biosynthesis in both the adrenal medulla and sympathetic ganglia (Thoenen et al. 1969; Kvetnansky et al. 1970; Thoenen 1970; Kvetnansky et al. 1971). The stress triggered elevation of TH activity in response to cold and IMO stress is associated with elevated TH immunoreactive protein and mRNA levels [reviewed in (Sabban and Kvetnansky 2001)].

However, it has become clear in recent years that the adrenal medulla and sympathetic nervous system can be regulated differently. The magnitude of the stress triggered changes and the time course of changes in expression of TH and DBH genes differed between these tissues (Sabban et al. 2004). In addition, the involvement of the HPA axis in the stress- triggered increase in

TH and DBH gene expression appears to differ between the adrenal medulla and sympathetic ganglia. Activation of the HPA axis and specifically the elevation of ACTH may play a crucial, perhaps direct, role in the regulation of TH and DBH in sympathetic ganglia (Nankova et al. 1996; Nankova et al. 2003). Injections of rats with ACTH for 7 days triggers a marked elevation of TH and DBH mRNA levels in rat superior cervical ganglia (SCG) and TH mRNA in the SCG of the ACTH-treated animals were as high as observed after single IMO. In contrast, these ACTH injections had little effect on TH and DBH mRNA levels in adrenal medulla (Nankova et al., 1996). In line with these results the elevation of TH gene expression in the SCG was observed in adrenalectomized animals further suggesting a direct effect of ACTH (Serova et al. 2008).

Transcription is an important pathway for regulation of gene expression of CA biosynthetic enzymes in adrenal medulla in response to stress (Osterhout et al. 1997; Nankova et al. 1999). However, the mechanism in sympathetic ganglia is less clear. It has been difficult to definitely determine whether or not the stress triggered elevation in TH mRNA levels are due to decreased turnover rate or to increased transcription.

Detection of changes in intron containing transcripts can be used to determine that changes observed in mRNA levels is mediated by transcription. RNase protection and *in situ* hybridization with intron 2 specific probe were shown to provide a sensitive indication of changes in TH transcription rate (Chang et al. 2000). Semiquantitative RT-PCR of changes in TH RNA primary transcripts that express intron 2 sequences in the adrenal medulla and in cell culture led to results that were comparable with those attained with nuclear run-on assays of transcription (Sun et al. 2004).

In this study we use determination of changes in intron 2 expressing TH transcripts to examine the effect of IMO stress on changes in transcription in the adrenal medulla and SCG. The results indicate that IMO triggers increased transcription in SCG, but the elevation is much smaller than observed in the adrenal medulla.

Methods

Animal Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals. Male Sprague Dawley rats (275–300 g) obtained from Taconic Farms (Germantown, NY, USA) were housed four per cage at 24 °C in a humidity controlled room, on a 12 h light/dark cycle (lights on at 6 A.M. and off at 6 P.M.) in an isolated and sound proof room to minimize background stress. Food and water were available ad libitum. Immobilization stress (IMO) was accomplished by taping the forelimbs and hind limbs of the rat in a prone position with surgical tape to metal mounts attached to a board as previously described (Kvetnansky and Mikulaj 1970; Sabban et al. 2006). Animals (seven per group) were immobilized for 2 h (1× IMO), 2 h daily for 2 days (2× IMO), or 2 h daily for 6 days (6× IMO). Rats were sacrificed immediately after stress. Control rats were not subjected to stress. All animal manipulations were performed between 7 A.M. and 1 P.M.

Animals were euthanized by decapitation and both adrenal medulla and superior cervical ganglia were dissected from the animal (Sabban et al. 2006). Tissue samples were frozen immediately in liquid nitrogen and kept at –80 °C.

RNA Isolation and Real Time Quantitative RT-PCR

Total RNA was isolated by using RNA-STAT 60 (Tel-Test, Friendswoods, TX, USA) and treated with RNase-free DNase (Ambion, Austin, TX, USA) to remove the genomic DNA

contamination according manufacturer's protocol. The concentration of RNA was measured by Quan-itTM Ribo-Green[®] RNA assay kit (Molecular Probes, Eugene, OR, USA). RT reactions were performed in 5µl PCR mixture (1×AMV buffer, 10mM dNTP, 8 units RNase inhibitor, 1.25 units AMV, 10 µM reverse primer, and 300 ng of template RNA). Reverse transcription was carried out at 42°C for 1 hour followed by 10 minutes at 85°C.

Real-time quantitative PCR was performed as described previously (Cheng et al. 2005) by using LightCycler[®] 1.2 System (Roche Applied Science) with LightCycler[®] FastStart DNA Master SYBR Green I (Roche Applied Science). The primers for amplification of intron 2 containing transcripts (Sun et al. 2004) and TH mRNA with position from NC-005100 were as follows. Note the TH reverse primer spans two exons.

TH mRNA	Reverse transcription	5' TCAGGCTCCTCTGACAG 3' (5922–5938)
	Forward	5' TGAACCAATTCCCCATGTGG 3' (4009–4029)
	Reverse	5' TGAACCAGTACACCGTGGAGAG 3' (5419–5430; 5568–5578)
TH intron 2	Reverse transcription	5' TCGATTCTGGGAGCTC 3' (2441–2456)
	Forward	5' CCAGGGCTGAAATACGGCTG 3' (2232–2241)
	Reverse	5' CAGGTCACTAGCCAGAGCAAC 3' (2375–2395)

The denaturation program (95 °C for 7 min) was followed by a four-segment amplification and quantification program repeated 37 times (95 °C for 3s; 54 °C for 3 s for TH or 59°C for 3 s for TH intron 2; 72 °C for 18 s; 85 °C for 0 s for a single fluorescence measurement) and a melting curve program (70 °C to 99 °C) and, finally, a cooling program down to 40 °C. The threshold cycle was determined using the Fit Points method to provide optimal standard curve values (0.98–1.0). For quantification assays, a standard curve was used and produced by amplification of several 10-fold dilutions of linearized plasmids containing TH cDNA and TH intron 2 amplicons. The results of real-time PCR were normalized to the amount of total RNA used in the PCR reaction.

Statistical Analyses

Statistical significance was determined by Student's *t*-test for experiments with two groups or by performing an analysis of variance (ANOVA) followed by Tukey post-test examination for experiments with more than two groups. A level of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism and InStat Programs (GraphPad Software, Inc., San Diego, CA, USA).

Results

RT-PCR for Intron-2 containing transcripts

RNA was used in a broad range of concentrations for RT-PCR with the primers for intron 2 of the TH gene. They were found to be linear over a wide range of concentrations, from 10^2 to 10^8 copies. Melting curve analysis revealed that there was a single product with a melting point at about 86°C. No amplification was obtained when the RT step was omitted.

Comparison to standards revealed that basal TH mRNA levels in the rat adrenal medulla were mean $\pm$ SE of $(2 \pm 0.9) \times 10^6$ copies/ng RNA and $(6 \pm 2.6) \times 10^5$ copies/ng RNA for transcripts with intron 2. In SCG, basal TH mRNA levels were $(2.5 \pm 0.7) \times 10^5$ copies/ng RNA and $(1.8 \pm 0.2) \times 10^4$ copies/ng RNA for transcripts with intron 2.

Effect of IMO stress on TH mRNA levels and intron 2 transcripts in adrenal medulla and in the SCG

Changes in total TH mRNA levels and in intron 2 containing transcripts after treatment of samples with DNase were detected in adrenal medulla of rats exposed to IMO stress for 6 consecutive days (6× IMO) and euthanized immediately thereafter. As shown in Figure 1A, following repeated IMO stress (6× IMO), there was about a 5-fold increase in TH mRNA levels in the adrenal medulla. There was also a comparable, about 5-fold increase in transcripts containing intron 2 of the TH gene (Fig. 1B).

Total RNA was also isolated from the SCG of rats subjected to IMO stress for 2 hrs once (1× IMO) or for 2 hrs daily on two (2× IMO) or 6 (6× IMO) consecutive days and euthanized immediately afterwards and used to determine levels of TH mRNA (Fig. 2A). With single the about 50% increase in TH mRNA levels was not significantly different from the levels in control animals. TH mRNA levels in the SCG were significantly elevated in the rats exposed to IMO stress repeatedly for two or six days, and were about double basal levels. The levels of RNA transcripts containing intron 2 in the same samples are shown in Fig. 2B. Even a single exposure to IMO elevated transcripts with intron 2 by about 50%. A similar increase was observed following two and six daily exposures to IMO.

Discussion

The results of this study show, for the first time, that immobilization stress not only elevates TH mRNA levels but also significantly raises TH transcription in the SCG. The levels of RNA transcribed from the TH gene, with intron 2 not yet spliced out, were elevated following single as well as repeated IMO stress. The magnitude of transcriptional activation was much smaller in the SCG than in the adrenal medulla.

Our results in the adrenal medulla are very consistent with the previously published report (Sun et al. 2004). The authors found about a 5-fold increase in TH mRNA and in intron 2 containing transcripts after a week of daily exposure to 2 hr IMO. This finding is also consistent with the changes based on run-on assays of transcription following IMO daily for two or seven consecutive days (Nankova et al. 1999). Use of transgeneic mice with the TH promoter directing chloramphenicol acetyl transferase reporter activity demonstrated a similar effect, with TH reporter activity substantially elevated in the adrenal medulla with one, two, three and seven daily exposures to IMO stress (Osterhout et al. 1997).

However, to our knowledge this is the first study to determine that the IMO triggered elevation of TH mRNA is associated with increased TH transcripts with unspliced introns in the SCG. This likely reflects increased transcription rate, as it correlates with other measures of transcription in the adrenal medulla, as indicated above. However, we cannot rule out the possibility that the elevated intron 2 containing transcript might also reflect a stress triggered reduction in the rate of removal or splicing of this intron in the SCG. Run-on assays of transcription have been difficult to perform in the SCG, as it is harder to disrupt this tissue under conditions which preserve the nuclei intact. Previously, differences were also observed in involvement of AP-1 factors in these two locations. AP-1 is involved in trans-synaptic induction of TH gene expression by reserpine in the adrenal medulla but not in SCG (Trocme et al. 1997). In this regard, c-Jun-N-terminal kinase (JNK) is activated in the adrenal medulla at several time intervals of single and repeated IMO, while no statistically significant changes in JNK activity were observed in the SCG under the same conditions (Nankova et al. 1998).

The SCG also was found to differ from the adrenal medulla in terms of the transcription factors which bind proximal cAMP/Ca response element (CRE/CaRE) in the TH gene promoter following reserpine treatment. Reserpine led to enhanced binding of repressor ICER in the adrenal medulla, while in the SCG it enhances the binding of CREM factors to the CRE/CaRE motif on the TH promoter (Trocme et al. 2001). Our laboratory found increases in two complexes which bound to the CRE/CaRE motif of the TH promoter in electrophoretic mobility shifts assays with SCG of rats exposed to IMO stress. At least one of the complexes, contains CREB, or CREB immunoreactive protein (Sabban et al. 2004). We speculate that induction of CREB or other CRE binding protein may be involved in the slow IMO-triggered activation of TH transcription detected in the SCG.

In the adrenal medulla, following repeated stress the elevation in transcription, as determined by intron 2 containing transcripts, and in mRNA levels, are nearly identical at about 5-fold higher than in the controls. However, in the SCG with repeated stress the two fold rise in mRNA is somewhat greater than the observed rise in TH transcription, suggesting that TH mRNA stability might also be regulated by the stress. Alternatively, differences at the time of sampling may be due to different kinetics of changes in transcription and subsequent rise in mRNA.

The magnitude of the elevation in transcription observed in the SCG is only about 50% under all the conditions. This increase is not yet reflected in significant changes in TH mRNA following a single 2 hr IMO. Interestingly, the time course of changes in TH mRNA levels in stellate ganglia following IMO revealed that while there was no significant change immediately after 2 hr of a IMO, there was a prolonged steady rise in TH mRNA for at least a day after cessation of the stress (Micutkova et al. 2003; Kvetnansky et al. 2004). This would suggest that a prolonged sustained elevation of transcription may be responsible for chronic or sustained sympathetic activation, and consequently play an important role in disorders such as cardiovascular disease, metabolic syndrome or decreased immunity exacerbated by stress.

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Adrenal Medulla

Figure 1.

Effect of repeated IMO stress on TH mRNA levels and intron 2 containing transcripts in the adrenal medulla. The relative levels of TH mRNA and intron 2 containing transcripts were determined by Real-time RT-PCR in adrenal medulla from controls and rats exposed to 2 hr IMO daily for 6 days (6× IMO). Data are the mean ± S.E. values of TH mRNA or intron 2 levels relative to those in the control samples. The controls were taken as 100.* $p \leq 0.05$ compared to control.

Superior Cervical Ganglia

Figure 2.

Effect of IMO stress on TH mRNA levels and intron 2 containing transcripts in the superior cervical ganglia. The relative levels of TH mRNA and intron 2 containing transcripts was determined by Real-time RT-PCR in the SCG from controls and rats exposed to 2 hr IMO daily for 1 (1× IM), 2 (2×X IMO) or 6 consecutive days (6× IMO). Data are the mean $\pm$ S.E. values of TH mRNA or intron 2 levels relative to those in the control samples. The controls were taken as 100. * $p \leq 0.05$ compared to control.