Autonomic control after blockade of the norepinephrine transporter: a model of orthostatic intolerance

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Carson, Robert P., André Diedrich, and David Robertson. Autonomic control after blockade of the norepinephrine transporter: a model of orthostatic intolerance. J Appl Physiol 93: 2192–2198, 2002. First published August 23, 2002; 10.1152/japplphysiol.00033.2002.—Orthostatic intolerance (OI) is a common autonomic problem affecting >500,000 Americans (17, 21). The criteria for the definition of OI includes a rise in heart rate (HR) of >30 beats/min with upright posture, prolonged (>6 mo) and disabling orthostatic symptoms, and standing plasma norepinephrine (NE) levels of >600 pg/ml (17). Although many studies of OI have focused on an increased release of NE from the nerve terminal, an alternative explanation of elevated sympathetic NE would be through abnormalities in NE clearance. Impairment of the NE transporter (NET) would result in decreased NE clearance, which functionally might be manifest as increased sympathetic outflow and increased NE release. Our laboratory’s report (24) of an association of OI and NET deficiency warrants a fuller examination of the role the NET plays in cardiovascular regulation.

Multiple mechanisms exist for clearance of NE from the synaptic cleft. NE can be recycled into the neuron by the NET, enzymatically metabolized by catechol-O-methyltransferase, transported into nonneuronal cells by the extraneuronal transporter, or simply diffused away from the synapse into the bloodstream. In organs such as the heart, most NE clearance is through the NET.

The NET is a Na/Cl-dependent 12-transmembrane domain protein that resides on presynaptic nerve terminals. NE removed from the synaptic cleft via the transporter may be repackaged into vesicles in the sympathetic nerve terminal or metabolized by monoamine oxidase. The NET is responsible for clearance of ~50–90% of released NE from the synaptic cleft (11). Deletion of the NET gene in mice elicited a reduction in NE levels of 55–70% in multiple brain regions, despite elevations in tyrosine hydroxylase activity, suggesting that reuptake plays a major role in keeping noradrenergic neurons supplied with NE (33).

Much of our understanding of NET physiology has emerged from use of pharmacological agents, such as desipramine (DMI), that bind to the NET and block NE reuptake. DMI is a tricyclic molecule that has seen extensive use in treating central nervous system (CNS) disorders and is one of the most potent blockers of NE reuptake identified (28). The integrated effects of NET blockade on cardiovascular regulation remain controversial. Postural hypotension without dramatic tachycardia has been reported in some subjects after NET blockade (16). Postural tachycardia in humans after tricyclic antidepressants or the selective NET blocker reboxetine (9), postural tachycardia in patients with NET deficiency (25), and an excessive tachycardia after a vasodilatory stimulus in DMI-treated rabbits (7) suggest that baroreflex sensitivity may be altered after NET blockade.

The aims of this study are to determine the role of NET on basal blood pressure, HR, and baroreflex function in the rat. A secondary goal is to generate an animal model of NET deficiency that could be used as a platform for testing therapeutic strategies.

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METHODS

Animals. The experimental protocol was approved by the Vanderbilt University Animal Care and Use Committee before implementation. Twelve control and twelve DMI-treated female Sprague-Dawley rats (Sasco), 10–12 wk of age at the onset of the experiment, were housed at 23°C with a 12:12-h light-dark cycle and were provided standard rodent chow and tap water ad libitum. Sickness, loss of venous patency, or loss of the telemetry signal were grounds for removal of animals from the study. Survival analysis revealed no significant differences in the percentages of animals able to complete the study (28 days) between DMI-treated (67%) and vehicle-treated (75%) groups (P = 0.6806). At the end of the experimental protocol, animals were anesthetized with halothane and killed by exsanguination.

Drugs. DMI hydrochloride, NE bitartrate, phenylephrine (PE) hydrochloride, sodium nitroprusside (NP), and tyramine were obtained from Sigma Chemical (St. Louis, MO). All agents were dissolved in 0.9% saline and administered as bolus intravenous injections.

Preparation of animals. Animals were prepared as described previously (5). Briefly, rats were anesthetized with ketamine/acepromazine (75 mg·2.5 mg·kg−1·kg−1). Female rats were used because OI appears to be considerably more common in women than in men (18). The abdominal aorta was exposed by using aseptic technique. The catheter attached to the radio-transmitter (TA11PA-C40, Data Sciences, St. Paul, MN) was inserted into the abdominal aorta, and the site was sealed with vascular adhesive (Vetbond, Data Sciences). The catheter attached to the radio-transmitter (TA11PA-C40, Data Sciences, St. Paul, MN) was inserted into the abdominal aorta, and the site was sealed with vascular adhesive (Vetbond, Data Sciences). The transmitter was sutured to the inner abdominal wall, and the skin was closed with staples. To allow venous access, a heparinized catheter (PhysioCath, Data Sciences) was inserted into the jugular vein and tunneled under the skin to exit dorsally, via a polyurethane button at the level of the neck. The venous access was filled with heparin (50 units) and flushed daily. Animals received a subcutaneous injection of ampicillin (60 mg/kg) and were allowed a minimum of 7 days to recover before experimentation.

NET blockade. Ten to fourteen days after surgery, animals were intravenously administered 5 mg/kg DMI in 0.9% saline or saline vehicle (200 μg/kg at 100 μl/min.) twice daily, immediately after onset of the light cycle and within 60 min before onset of the dark cycle.

Data collection. To examine long-term changes in basal blood pressure and HR before and during DMI administration, 30-s intervals of blood pressure were recorded continuously every 10 min. The average of hemodynamic variables during dark cycle collected from midnight to 2 AM were used to determine daily values. Pharmacological data were collected during the light cycle while the animal was resting quietly in its home cage. Pharmacological agents were administered intravenously through PE-50 tubing attached to the catheter on the animal’s neck. Beat-to-beat blood pressure data were acquired by using telemetry and analyzed by using the ART Gold software (Data Sciences). HR and mean arterial blood pressure (MAP) were derived from the blood pressure waveform.

Spectral analysis. While animals were resting comfortably during the light cycle, beat-to-beat blood pressure was collected for 15 min in 5-min intervals. The average HR, diastolic blood pressure (DBP), and systolic blood pressure (SBP) were determined from the average of each 3-min interval for a total of 15 min of beat-to-beat data. SBP, DBP, and interbeat (RR) interval were calculated from the blood pressure waveform (ART Gold, Data Sciences). Estimation of the power spectral density was done by the Fast Fourier transform-based Welch method (19). Intervals of ~90 s, free from blood pressure changes due to behavior, i.e., ambulation, eating, grooming, were interpolated, low-pass filtered out (0–3 Hz), equidistant resampled by 6 Hz, and detrended by linear regression method. A Hanning window was applied before estimation of the power spectral density. Frequency resolution was 0.012 Hz. The integrated power in the frequency ranges for low frequencies (LF: 0.25–0.6 Hz), and high frequencies (1.0–2.0 Hz) was calculated for each interval (6, 8, 15, 20). The average of five spectra was used for the final determination of power spectral density. Spectral data collection and pharmacological testing dates were timed at 4-day intervals to minimize potential estrous-related cardiovascular effects. Vaginal cytology revealed that animals were cycling normally, although not all animals were studied at the same estrous stage (data not shown).

Pharmacological testing. α1-Receptor sensitivity was examined with the selective agonist PE (2.5 μg/kg; 5 μg·kg−1·min−1) and with NE (0.25 μg/kg; 0.5 μg·kg−1·min−1). To elicit sympathetic activation, nitroprusside (4.0 μg/kg; 8.0 μg·kg−1·min−1) was administered. Baroreflex sensitivity was determined by analysis of the slope of the change in RR interval for a total of 15 min of beat-to-beat data. Spectral data were collected during the blood pressure and HR before and during DMI administration. Pharmacological testing occurred 1 day before DMI administration was begun, 3 days after onset of DMI, and at 8-day intervals thereafter through 27 days.

Plasma catecholamines and plasma DMI. Two groups of four female rats were administered either saline vehicle or 5 mg/kg DMI twice daily for 11 days. Animals were anesthetized with halothane and exsanguinated by cardiac puncture. Blood was collected into tubes containing EGTA and reduced glutathione. Plasma was isolated and stored at −70°C until analyzed. NE and dihydroxyphenylglycol (DHPG) levels were determined by high-performance liquid chromatography. Plasma for DMI determination was collected as described above with the exception that blood was collected into tubes containing EDTA. DMI levels were determined by Specialty Labs (Santa Monica, CA).

Statistical methods. Data are presented as means ± SE. Data were analyzed by repeated-measures ANOVA followed by Dunnett’s post hoc test, comparing data within DMI- or vehicle-treated groups. P < 0.05 was considered significant.

RESULTS

NET block. Chronic administration of 5 mg/kg DMI (twice daily) to female Sprague-Dawley rats resulted in plasma DMI levels of 344 ± 51 ng/ml (n = 4), which were consistent with those encountered during DMI therapy in humans (32). To verify that DMI was blocking NET and to quantitate the magnitude of NET blockade, animals were administered 250 μg/kg tyramine. Tyramine is an indirect noradrenergic agonist that enters nerve terminals through the NET and releases NE. Initial sensitivity was examined with the selective agonist PE (2.5 μg/kg; 5 μg·kg−1·min−1) and with NE (0.25 μg/kg; 0.5 μg·kg−1·min−1). To elicit sympathetic activation, nitroprusside (4.0 μg/kg; 8.0 μg·kg−1·min−1) was administered. Baroreflex sensitivity was determined by analysis of the slope of the change in RR interval for a total of 15 min of beat-to-beat data. Spectral data were collected during the blood pressure and HR before and during DMI administration. Pharmacological testing occurred 1 day before DMI administration was begun, 3 days after onset of DMI, and at 8-day intervals thereafter through 27 days.

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the selective α1-agonist PE and NE. Pressor responses to both PE and NE (Fig. 2, A and B) were not dramatically altered by vehicle treatment, whereas in DMI-treated animals the pressor responses to both PE and NE were elevated over that of vehicle or the pre-DMI response (Fig. 2). The dramatic loss of a tyramine response in the context of increased NE potency suggests a near complete loss of NET transport activity.

Plasma catecholamines. To biochemically verify inhibition of NET activity by DMI, plasma catecholamines and their metabolites were analyzed. DHPG, the major intraneuronal metabolite of NE, is produced through oxidation of NE by monoamine oxidase. NE, which leaks from vesicle stores or undergoes uptake by the NET, can be converted to DHPG, which can diffuse out of the nerve terminal into the plasma. Administration of DMI led to elevated plasma NE levels and decreased plasma DHPG levels, significantly reducing the plasma DHPG/NE ratio (Table 1). The reduction in the plasma DHPG/NE ratio lends further support to the effectiveness of this dose of DMI at inhibiting neuronal uptake of NE.
the duration of the study, demonstrating an 8% difference in body mass after 27 days of DMI administration.

MAP and HR. MAP and HR were determined in female rats during the dark cycle, a time at which animals are active. Initial MAP and HR values were 94 ± 2 mmHg and 405 ± 7 beats/min, respectively, for the vehicle group and 88 ± 2 mmHg and 407 ± 7 beats/min for DMI-treated animals. After chronic treatment with DMI, both MAP and HR were significantly elevated in NET-blocked animals relative to vehicle-treated animals (Fig. 3), despite decreased activity in the DMI-treated animals (data not shown.) Similar effects on MAP and HR were observed during the light cycle when animals were resting quietly (P = 0.016 and P < 0.0001, respectively).

Sympathetic outflow. Stimulation of \( \alpha_2 \)-receptors in the brain stem with NE or its congeners, such as clonidine, can lead to reductions in sympathetic outflow (13, 30, 31). To investigate cardiovascular effects of DMI action in the CNS, alterations in sympathetic outflow were quantitated indirectly by using spectral analysis. Studies in both conscious and anesthetized rats have shown a high coherence between 0.4-Hz blood pressure variability and sympathetic nerve activity (4, 29), suggesting that variability in the 0.4-Hz frequency range may provide a useful index of sympathovagal activity (4, 29), suggesting that variability in the range of 0.4 Hz (LF) is associated with DMI administration. Spectral analysis was used to examine changes in blood pressure variability before (pre) and after (post) NET blockade. Variability in the range of 0.4 Hz (LF) is associated with sympathetic outflow. A: in a representative systolic blood pressure (SBP) spectra from a female rat before and after DMI treatment, a clear peak is observed at 0.4 Hz before (black trace) but not after DMI administration (gray trace). B: spectral variability of SBP was measured before and during chronic DMI or vehicle administration. LF variability was dramatically reduced in DMI-treated animals (gray bars) by 3 days of DMI treatment and remained attenuated throughout the study. LF variability was not altered with vehicle treatment (black bars). Data are means ± SE and were analyzed by ANOVA with Dunnett’s post test (n = 8–12). *P < 0.05 vs. pre-DMI; **P < 0.01 vs. pre-DMI.

Baroreflex function. Sympathetic outflow is known to be modulated in the CNS by catecholamines. To address whether chronic NET inhibition may result in alterations of the sympathoexcitatory arc of the baroreflex, baroreflex sensitivity was tested through administration of the vasodilator NP. To stimulate baroreflex-mediated sympathetic outflow, animals were treated with the NO donor and vasodilator sodium nitroprusside (4 \( \mu \)g/kg). The overall reduction in MAP after NP was slightly enhanced after DMI administration relative to pre-DMI treatment and to vehicle treatment, with the peak reduction occurring after 3 days of DMI treatment. Surprisingly, the relative HR increase in response to hypotension was somewhat attenuated in NET-blocked animals, which translated to a reduction in baroreflex slope (Fig. 5A). Administration of the \( \alpha_1 \)-agonist PE resulted in increases in MAP that were greater in NET-blocked animals than in controls, although the magnitude of the baroreflex bradycardia was proportional to the increase in MAP, which suggests that the sensitivity of the baroreflex to elevations in MAP was not altered (data not shown). Administration of NP leads to decreased vascular tone and reduced MAP. As MAP returned to baseline, there was a slight blood pressure overshoot that averaged <5 mmHg in control animals (Fig. 5B). Treatment of animals with DMI magnified the overshoot, further sug-

Fig. 3. Heart rate (HR) and MAP are elevated after chronic DMI treatment. MAP and HR were determined in female rats during the dark cycle, a time in which animals are active. Both MAP and HR became significantly elevated in NET-blocked animals relative to vehicle-treated animals, despite decreased activity in DMI-treated animals (data not shown). Data are means ± SE and were analyzed by ANOVA (n = 8–12).

Fig. 4. Low-frequency (LF) spectral power is significantly depressed with DMI administration. Spectral analysis was used to examine changes in blood pressure variability before (pre) and after (post) NET blockade. Variability in the range of 0.4 Hz (LF) is associated with sympathetic outflow. A: in a representative systolic blood pressure (SBP) spectra from a female rat before and after DMI treatment, a clear peak is observed at 0.4 Hz before (black trace) but not after DMI administration (gray trace). B: spectral variability of SBP was measured before and during chronic DMI or vehicle administration. LF variability was dramatically reduced in DMI-treated animals (gray bars) by 3 days of DMI treatment and remained attenuated throughout the study. LF variability was not altered with vehicle treatment (black bars). Data are means ± SE and were analyzed by ANOVA with Dunnett’s post test (n = 8–12). *P < 0.05 vs. pre-DMI; **P < 0.01 vs. pre-DMI.
suggestive of an impaired ability to clear NE from the synapse.

DISCUSSION

The primary goal of this study was to evaluate the role of NET in both basal and reflex-mediated cardiovascular control to determine whether CNS regulation of sympathetic outflow would be altered after exposure to chronically elevated levels of NE. Our results demonstrate that, in rats, a near complete loss of NE reuptake leads to elevations in plasma NE and a decreased DHPG/NE ratio. Despite evidence of decreased sympathetic outflow, HR and MAP were elevated. Furthermore, the reflex tachycardia in response to a vasodepressor stimulus was attenuated, consistent with the concept that elevation in the CNS level of NE is leading to an attenuation in sympathetic outflow and to suppression of the baroreflex, thus obscuring or preventing the expression of the peripheral manifestations of impaired NE reuptake.

To examine the cardiovascular effects of loss of NE reuptake, female Sprague-Dawley rats were chronically administered a therapeutically relevant dose of DMI (32). The reduction in the pharmacological response to tyramine demonstrated that the dose of DMI utilized in this study led to a nearly complete loss of NET activity. Analysis of catecholamine levels revealed an increase in plasma levels of NE, consistent with a loss of NE clearance. Because plasma NE levels are dependent not only on clearance of NE but on NE release, plasma NE alone is not a good marker of sympathetic activity. As plasma DHPG reflects concentrations of an intraneuronally derived metabolite of NE, comparison of the ratio of plasma DHPG to plasma NE can provide an index of neuronal reuptake. The reduction in the ratio of plasma DHPG to NE indicates that the dose of DMI used in this study was effective in inhibiting NE reuptake.

Administration of NE demonstrated the functional effect of impaired NE reuptake. The potency of NE was increased by greater than threefold in DMI-treated animals, presumably as a result of decreased NE clearance through the NET. Interestingly, the pressor response of PE was also elevated, although not to the same extent as was seen with NE. It is noteworthy that PE is not a substrate for the NET. A variety of mechanisms could potentially explain this result. Alterations in postsynaptic \( \alpha_1 \)-receptor density may increase the relative potency of PE, although in the context of elevated plasma NE levels one would anticipate a functional decrease in \( \alpha_1 \)-receptor density. Alternatively, impaired baroreflex-mediated sympathoinhibition could result in enhanced pressor responses. In an animal with impaired peripheral NET activity, sympathetic activity would be functionally enhanced through impaired ability to remove NE from the synaptic cleft, leading to prolonged end organ stimulation. Thus an inability to decrease HR and reduce sympathetic activity in the context of increased arterial pressure could potentiate a pressor response, an effect observed in humans with baroreflex failure (22, 22) or after administration of a ganglionic blocking agent (26). The observation that the magnitude of the baroreflex-mediated bradycardia after PE is proportional to the MAP response suggests that vagal reflexes are intact and would argue against such a mechanism affecting HR in DMI-treated rats.

The effects of impaired NET function on sympathetic outflow were analyzed by using spectral techniques. Chronic administration of DMI resulted in dramatic reductions in LF spectral power in rats. Many studies have reported an association of LF oscillations of blood pressure and sympathetic outflow (4, 29). Stimulation of \( \alpha_2 \)-receptors in the brain stem with clonidine, an intervention known to reduce sympathetic outflow, led to reduced LF power in rats, which is consistent with a relationship of LF power to sympathetic outflow (2, 14). The results from the present study are consistent with the view that decreased NE clearance after DMI administration leads to increased levels of NE in the brain stem and to increased stimulation of inhibitory \( \alpha_2 \)-receptors. The inhibitory effect of NE is believed to be mediated through inhibition of glutamate release at the level of the rostral ventrolateral medulla (15). Previous studies have been interpreted as demonstrating reduced sympathetic nerve activity after DMI admin-

![Graph A](image1.png)  
**A** Baroreflex sensitivity is decreased with NET blockade. Animals were treated with the nitric oxide donor and vasodilator sodium nitroprusside (4 \( \mu \)g/kg). The vasodilatory effect of sodium nitroprusside was augmented in animals treated with DMI (data not shown), whereas the baroreflex-mediated increase in HR was attenuated, thus resulting in a reduction in baroreflex slope (A) in animals treated with DMI (gray bars) relative to vehicle-treated animals (black bars). The return to basal values of MAP was accompanied by a slight blood pressure overshoot, presumably due to excess NE in the synaptic cleft. The magnitude of the MAP overshoot in DMI-treated animals is elevated over that of vehicle-treated animals (B). Data are means ± SE and were analyzed by ANOVA (n = 8–12).

![Graph B](image2.png)  
**B** The effects of impaired NET function on sympathetic outflow were analyzed by using spectral techniques.
istration in both humans and rabbits (10, 12). Furthermore, the persistent loss of LF power throughout the present study suggests that, despite potential elevations of synaptic NE levels due to decreased clearance, the CNS α2A-receptors exposed to elevated NE may not be dramatically desensitized. Both in vitro analysis of receptor signaling and in vivo studies support a resistance of α2A-receptors to desensitization (9, 23). Examination of NE kinetics in NET (−/−) mice revealed a 60% reduction in NE release and a sixfold reduction in NE clearance, with the net effects being a twofold increase in extracellular NE levels. Although elevations in NE levels led to reductions in α2A-receptor binding in the hippocampus, α2A-receptor density in the spinal cord was not altered (3).

Alternatively, the loss of LF spectral power may be associated with alterations in the expression of sympathetic nerve traffic due to impaired NE kinetics. A previous study examining the blood pressure effects of acute DMI in rats demonstrated that spectral power associated with sympathetic activity was reduced after NET blockade as a result of a shift of LF power from 0.4 to 0.1 Hz (1). Although the data in the present study are consistent with human data that have demonstrated both a reduction in sympathetic nerve traffic (12) and LF spectral power (24) after NET blockade, loss of LF power due to altered baroreflex cycling secondary to peripheral reductions in NE kinetics cannot be ruled out.

To determine the combined effect of both central and peripheral blockade of NE reuptake on baroreflex mechanisms, a baroreflex-mediated increase in sympathetic outflow was stimulated by reducing blood pressure with the NO donor NP. NP administration led to a reduction in MAP and a concomitant baroreflex-mediated tachycardia that was present in both control and DMI-treated animals. Although an excessive tachycardia after NP was predicted, administration of NP led to an attenuated tachycardia despite enhanced reductions in MAP. These seemingly paradoxical data are again consistent with a central reduction in sympathetic nerve outflow. As a result of blunted sympathetic nerve traffic, the amount of NE available at peripheral synapses (cardiac) is reduced, leading to a functional attenuation of the baroreflex and reduced baroreflex gain. Alternatively, a left shift in the MAP-HR set point as a result of the elevations in basal HR may alter the baroreflex slope in response to a depressor stimulus independent of changes in sympathetic outflow.

Some aspects of this study in rats were similar to those seen previously in human NET deficiency and in a recent study with the selective NET antagonist reboxetine (24, 25). Patients heterozygous for a nonfunctional NET transporter exhibited a volatile supine HR, orthostatic tachycardia, decreased NE clearance, and reduced tyramine responsiveness. Biochemical data revealed a reduced DHPG/NE ratio. As elevated NE in the CNS decreases sympathetic outflow, it is expected that loss of NET function should decrease sympathetic tone, yet, interestingly, in patients with OI, sympathetic tone appears to be normal. In contrast, spectral data in this study and in a human study using reboxetine suggest an overall reduction in sympathetic tone (24). Furthermore, the attenuated baroreflex response to NP in rats suggests a functional inhibition of sympathetic outflow, whereas the presence of orthostatic tachycardia in patients with NET deficiency or after reboxetine treatment suggests an enhancement of sympathetic outflow. A likely explanation for these differences may lie in the partial nature of the NET defect in the heterozygote patients and the magnitude of NET blockade with acute reboxetine compared with the near complete blockade of NET achieved with the high dose of DMI used in this study.

In this study, the role of NET in modulation of autonomic function was investigated. These data demonstrated that, despite a decrease in sympathetic outflow and concomitant reduction of effective baroreflex sensitivity, loss of NE clearance by NET results in elevated plasma NE levels and an elevation in HR. This supports our observations in humans that dysregulation of NE uptake is a mechanism that may result in autonomic dysfunction.

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