Neuropeptide Y-Mediated Pressor Responses Following High-Frequency Stimulation of the Rat Sympathetic Nervous System

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ABSTRACT

Neuropeptide Y (NPY) is a potent pressor agent that is stored in the sympathetic nerves. In several species, NPY release is augmented when sympathetic impulse frequencies increase. We investigated the extent to which NPY contributes to the pressor response to high- and low-frequency electrical stimulation. Rats were pithed, and the sympathetic trunk was stimulated at either 20 or 3 Hz in the presence or absence of antagonists of NPY and alpha and beta adrenergic receptors. The 20-Hz stimulation raised plasma NPY levels, but the 3-Hz stimulation did not. The 20-Hz stimulation caused marked pressor responses that were maintained for several minutes after the end of stimulation regardless of whether rats were pretreated with adrenergic blockers. The NPY antagonists BIBP 3226 and 1229U91 reduced the size of the pressor response that followed 20 Hz stimulation by 50%. The rapid blood pressure spikes that occur during electrical stimulation are attenuated by alpha adrenergic but not by NPY antagonists. There is a prolonged pressor response after high-frequency stimulation of the sympathetic trunk in pithed rats that begins after 1 to 2 min of stimulation and lasts ~10 min after the end of stimulation. At least half of this pressor response is mediated by NPY.

The vascular system is highly innervated with sympathetic nerves. Firing of these nerves causes vasoconstriction and, in the absence of compensatory responses, increases BP. NE is believed to be the primary neurotransmitter inducing vasoconstriction during sympathetic activation. However, NPY can be coreleased with NE (Lundberg et al., 1984, 1986; Pernow et al., 1988) and is a potent vasoconstrictor.

NPY is stored in sympathetic nerve terminals often at one-150th of the levels of NE (on a molar basis) (Dahlof et al., 1994). The potency of NPY as a vasoconstrictor varies considerably in different vascular beds (Pernow et al., 1987), but it has been reported to be ≤25 times as potent as NE (Hellstrom et al., 1985). NE is stored in both small and large dense core vesicles in sympathetic nerve terminals, whereas NPY appears to be stored only in large dense core vesicles (Fried et al., 1985). In several species, fractional release of NPY has been reported to rise with increasing electrical impulse frequency (Allen et al., 1984; Lundberg et al., 1984, 1986; Pernow et al., 1988), probably due to selective release of large dense core vesicles at higher impulse frequencies (Lundberg et al., 1989). However, some studies have not found preferential NPY release at higher stimulation frequencies (De Potter et al., 1995). The extent to which NPY contributes to sympathetic pressor responses in vivo is uncertain and is the subject of this study.

Investigation of the contribution of NPY to sympathetic pressor responses has been limited by several factors. Until recently, there were no potent and specific NPY antagonists. Rudolf et al. (1994) developed BIBP 3226, a nonpeptide antagonist of NPY1 receptors, the receptors that primarily mediate the pressor actions of NPY (Modin et al., 1991). Even more recently, Daniels et al. (1995) discovered a potent peptide antagonist of NPY1 receptors, which has been designated 1229U91. Another difficulty has been that the main species used for hypertension research, the rat, has exceedingly high platelet NPY levels (Myers et al., 1988). Even slight contamination of plasma with platelets can dramatically alter plasma NPY levels. This has discouraged some researchers from using the rat to study NPY physiology and others from using rat plasma NPY levels as an index of NPY release.

In the present study, we examined the effects of high- and low-frequency electrical stimulation of the sympathetic nervous system on NPY release and pressor effects.

ABBREVIATIONS: NPY, neuropeptide Y; NE, norepinephrine; BP, blood pressure; MIPR, mean integrated pressor response; MAP, mean arterial pressure; ANOVA, analysis of variance.
Methods

The procedures in this study were performed on male Sprague-Dawley rats (Harlan Laboratories) weighing 300 to 375 g and were approved by the University of California San Diego Animal Subjects Committee.

Preparation of pithed rats and measurement of BP. Rats were anesthetized with halothane, and the trachea was cannulated. Soon afterward, the rats were pithed by insertion of a steel rod into an eye socket and through the length of the spinal column. Rats were then connected to a ventilator (SAR 530-p, CWE Inc., Ardmore, PA) and resuscitated at a rate of 55 breaths/min. A rod similar to the pithing rod was inserted underneath the skin along the entire length of the body. A jugular vein was then catheterized with PE-50 tubing, and the rats were injected with gallamine triethiodide (Sigma Chemical Co., St. Louis, MO) at 20 mg/kg. A carotid artery was also catheterized with PE-50 tubing that was connected to a BP transducer. Rats were allowed to stabilize for ≥20 min after pithing before BP was recorded. BP measurements were collected at a rate of 128 Hz using a Sleeptrace 2000 (Sleeptrace, Palo Alto, CA) computerized recorder.

Electrical stimulation. The sympathetic outflow of the pithed rats was stimulated by connecting the two pithing rods to leads from a Grass S44 stimulator (Grass Medical Instruments, Quincy, MA). Rats were stimulated at a frequency of 3 or 20 Hz. Regardless of stimulation frequency, stimulation periods lasted 30 sec, and each impulse lasted 1 msec. All stimulated rats received the treatments at 30-sec intervals: 7.5 V, rest, 10 V, rest, 15 V, rest, 20 V and rest.

Effect of stimulation at 3 and 20 Hz on plasma catecholamines and NPY. Five rats were injected with phenoxybenzamine (20 mg/kg intraperitoneal). At ∼30 min later, they were pithed and catheterized. At ≥1 hr after the phenoxybenzamine injection, a blood sample was collected, and the fluid was replaced with the same volume of saline. Two minutes later, the rats were stimulated in the usual manner at 3 Hz. A second blood sample was drawn at ∼7 min later, and the fluid loss was replaced with saline. At 15 min later, a third blood sample was drawn with saline replacement. At 2 min later, the rats were stimulated at 20 Hz in the usual way, and a blood sample was taken after 7 min, with saline replacement. A final blood sample was collected 15 min later. To control for any sequence effects, the same protocol as above was repeated in five additional rats except that the 20-Hz stimulation was performed before the 3-Hz stimulation.

To avoid contamination of plasma with platelet NPY, all 0.5-ml blood samples were immediately transferred to iced microfuge tubes containing 55 μl of acid citrate dextrose as anticoagulant and platelet stabilizer. Soon afterward, the blood was spun in a refrigerated microfuge for 2 min at 11,000 × g. The plasma was then separated and stored at −70 degrees until assayed for catecholamines according to the radioenzymatic method of Kennedy and Ziegler (1990) and for NPY by radioimmunoassay (Brown et al., 1989). Effect of NPY antagonists on pressor response to [Leu²¹,Pro³⁴]NPY. To test the potency of our NPY antagonist drugs, 17 rats were injected with phenoxybenzamine (20 mg/kg intraperitoneal; Ciba-Giegy). Approximately 30 to 45 min later, each of these rats was pithed and catheterized. Approximately 30 min later, 5 of the rats were injected with 0.4 ml of saline, 7 rats were injected with BIBP 3226 (20 mg/kg intravenous) and at ∼30 min later, they were pithed and catheterized. At ≥1 hr after phenoxybenzamine injection, 6 of the rats were injected intravenously with BIBP 3226 (0.6 mg/kg), and 5 were injected with 1229U91 (0.2 mg/kg); the remaining rats received saline. At 3 min later, the rats were stimulated at 20 Hz according to the standard protocol; BP was constantly measured.

For both 3- and 20-Hz-stimulated rats, the BP tracings generally consisted of four spikes during stimulation followed by BP decreases after each stimulation. During 20-Hz stimulation, BP usually did not fall to base line in the 30-sec interval between stimulations. We determined four main parameters from the tracings: (1) the highest MAP (above the initial prestimulation base-line MAP) attained during each 30-sec stimulation interval, (2) the lowest resting MAP that occurred during the 30 sec after each stimulation, (3) the highest MAP attained above the base-line MAP observed just before the start of each stimulation period, and (4) the MIPR after the final stimulation interval.

The fourth parameter, MIPR, was calculated by determining the peak systolic response during the first 30-sec stimulation (7.5 V), d2 is the diastolic response during the second stimulation (10 V), and so on; and s1 is the maximal systolic response during the first 30-sec stimulation (7.5 V), s2 is the systolic response during the second stimulation (10 V), and so on.
area of the systolic pressor response (in mm Hg/min) and the area of the diastolic pressor response. The response was considered to start the instant at which the final stimulation stopped and was considered to be finished when BP returned to prestimulation values. The MIPR was calculated according to the formula: MIPR = [(DPR × 2) + SPR]/3, where SPR is systolic pressor response and DPR is diastolic pressor response.

The MIPR of [Leu31,Pro34]NPY was calculated in a similar manner except that the start of the response occurred when BP rose detectably above base line and finished at return to base line.

The statistical significance of intergroup differences in pressor responses to drugs or electrical stimulation was generally determined by Student’s t test when two experimental groups were present. However, to determine the statistical significance of differences in pressor responses determined at several time points in control and drug-treated rats, a two-way mixed-model ANOVA was used. When three experimental groups were present, the significance of any intergroup differences was determined by one-way ANOVA and Tukey’s test. When repeated measurements were performed on a single group of rats, the results were analyzed by ANOVA for repeated measures and Tukey’s test. P < .05 was considered statistically significant.

### Results

Electrical stimulation at 20 Hz in phenoxybenzamine-treated pithed rats raised plasma levels of NPY by 2-fold, of NE by 6-fold and increased plasma epinephrine by >300 pg/ml (fig. 1). Stimulation at 3 Hz did not alter plasma NPY levels and significantly raised NE and epinephrine but to a much lesser extent than 20-Hz stimulation (fig. 1).

NPY has a more prolonged action than catecholamines. In accord with this, we found that the pressor response to 20-Hz stimulation was qualitatively different than that of 3-Hz stimulation. After the final 3-Hz stimulation, MAP declined rapidly and steadily to base-line levels. In contrast, after the final 20-Hz stimulation, MAP typically dropped about half-way to base-line levels but then rose slowly for 1 to 2 min before gradually returning to base line at ~10 min later (fig. 2).

After alpha blockade with phenoxybenzamine, the difference between the pressor effects of 3- and 20-Hz stimulation was even more dramatic. Once the final stimulation at 3 Hz stopped, the BP of alpha-blocked rats immediately and permanently returned to base line. In contrast, after the final 20-Hz stimulation, BP also typically dropped quickly to base line, but within 1 to 2 min, a pressor response occurred that lasted ~10 min (fig. 2).

The 20-Hz stimulation markedly elevated plasma epinephrine levels. In alpha-blocked rats, epinephrine is a vasodilator, so we investigated the pressor actions of 20-Hz stimulation in rats that were both alpha and beta blocked. The alpha plus beta blockade dramatically increased pressor responses during and between successive 20-Hz stimulation periods (fig. 2). With alpha blockade, MAP returned to base line between successive 20-Hz stimulations. After alpha plus beta blockade, resting MAP continually rose after successive stimulations such that during the 30-sec interval after the final stimulation, MAP was 51 ± 6 mm Hg above base-line BP. Also, the mean integrated pressor response after the final stimulation period in these alpha- plus beta-blocked rats was nearly double those of rats receiving alpha blockade alone (table 2).

To determine the contribution of NPY to the prolonged post-20-Hz pressor responses, we treated rats with the NPY₁ antagonists BIBP 3226 and 1229U91. Both NPY₁ antagonists reduced the integrated pressor response to 5 μg of [Leu31,Pro34]NPY by ≥85% (P < .001) in alpha-blocked and/or -unblocked pithed rats (table 3). BIBP 3226 also reduced the post-20-Hz pressor response in unblocked, alpha-blocked and alpha- plus beta-blocked rats (table 2, fig. 2). BIBP 3226 reduced the mean integrated arterial poststimulation pressor response in rats without adrenergic blockade by 53% (P < .001) in alpha-blocked rats by 59% (P < .05) and in alpha- plus beta-blocked rats by 65% (P = .013). The NPY antagonist 1229U91 was given only to alpha-blocked rats and reduced the post-20-Hz stimulation by 53% (P < .05).

BIBP 3226 also reduced MAP in rats without adrenergic blockade during and after the four successive 30-sec 20-Hz
studies. Also, BIBP 3226 did not reduce MAP to a greater extent in rats during sympathetic stimulation than it did between or after stimulations (fig. 3).

**Discussion**

High-frequency (20-Hz) electrical stimulation of the rat sympathetic nervous system preferentially increased NPY release into the circulation and induced a prolonged secondary pressor response, which did not follow low-frequency (3-Hz) stimulation. The pressor response during and after 20 Hz was attenuated by NPY antagonists, suggesting mediation by NPY. To our knowledge, this is the first report showing that NPY antagonists markedly reduce whole-body pressor responses to sympathetic activation in mammals.

Our results extend to the rat the previous observations in dog, pig and cow that high-frequency electrical stimulation selectively enhances NPY release from sympathetic nerves. Resting plasma NPY levels in our rats were at or below those reported by others when precautions were taken to prevent contamination with platelet NPY (Myers et al., 1993; Zukowska-Grojec et al., 1991). The 20-Hz stimulation doubled plasma NPY from resting levels in pithed rats, whereas 3-Hz stimulation did not alter NPY. It is conceivable that the apparent lack of effect of 3-Hz stimulation on NPY levels is due simply to the ~7-fold lower rate of nerve depolarization at this frequency. However, even an NPY response that is one seventh as large as that produced by 20-Hz stimulation should have been detected with our assay, but it was not. In contrast to NPY, plasma NE and epinephrine increased significantly after 3-Hz stimulation.

The 20-Hz stimulation also differed from 3-Hz stimulation

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TABLE 2

Integrated mean arterial pressor response after termination of electrical stimulation of pithed rats

<table>
<thead>
<tr>
<th>Adrenergic blockade</th>
<th>Electric impulse frequency</th>
<th>3 Hz</th>
<th>20 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>311 ± 21*</td>
<td>147 ± 21*</td>
</tr>
<tr>
<td>Alpha</td>
<td>1229U91</td>
<td>N.D.</td>
<td>44 ± 13b</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of 5 to 8 determinations.

a P < .01 vs. saline 20 Hz unblocked by t test.

b Intergroup differences in alpha-blocked rats: 
P < .013 by ANOVA, P < .05 vs. saline by Tukey’s test.

* P = .013 vs. saline 20 Hz alpha- and beta-blocked by t test.

N.D., not determined.

TABLE 3

Integrated mean arterial pressor response to an intravenous injection of 5 μg of [Leu31, Pro34]NPY in pithed rats

<table>
<thead>
<tr>
<th>Adrenergic blockade</th>
<th>None</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>153 ± 20</td>
<td>171 ± 28</td>
</tr>
<tr>
<td>BIBP 3226</td>
<td>17 ± 3a</td>
<td>13 ± 3b</td>
</tr>
<tr>
<td>1229U91</td>
<td>N.D.</td>
<td>23 ± 6b</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of five to eight determinations.

a P < .01 vs. saline by t test.

b Intergroup differences in alpha-blocked rats: 
P < .01 by ANOVA, P < .05 vs. saline by Tukey’s test.

N.D., not determined.

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**Fig. 3.** Pressor responses of 5 control rats and 8 BIBP 3226-treated rats during 20-Hz stimulation (STIMULATED) and in the same rats between and after stimulations. Periods of 20-Hz stimulation are denoted by heavy lines in the top left. The slope of the STIMULATED BIBP 3226 line was significantly lower than that of the STIMULATED CONTROL line (P = .017). The MAP of the BIBP 3226-treated rats during the four rest periods immediately after stimulations was significantly lower than that of the resting controls (P = .025). The mean integrated pressor response after the final stimulation was significantly smaller in BIBP 3226-treated rats than controls (P < .001).

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**TABLE 4**

Peak responses of MAP during electrical stimulation

<table>
<thead>
<tr>
<th>Adrenergic blockade</th>
<th>Electric impulse frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Hz</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Alpha</td>
<td>Alpha plus beta</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>BIBP 3226</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>1229U91</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of five to eight rats.

N.D., not determined.
in the nature of the pressor response it induced. After 20-Hz stimulation ended, an initial rapid fall in BP was typically followed by a rise for 1 to 2 min and then a gradual return to base line within 10 min. After 3-Hz stimulation, only a rapid BP fall to base line occurred. The prolonged post-20-Hz pressor response resembles that occurring with intravenously injected NPY (Lundberg and Tatemo, 1982).

Pretreatment with an NPY antagonist reduced the size of the post-20-Hz stimulation pressor response in adrenergically unblocked rats by >50%. BBP 3226 has also been reported to inhibit sympathetically mediated vasoconstriction by >50% in certain vascular beds of the pig (Lundberg and Modin, 1995; Modin and Lundberg, 1995).

The alpha adrenergic blockade accentuated differences between 3-Hz and 20-Hz poststimulation pressor responses. After 3-Hz stimulation of alpha-blocked rats, BP returned almost immediately to base line. In contrast, after 20-Hz stimulation, BP typically fell immediately to base line, but soon afterward, a marked pressor response lasting 10 to 15 min occurred. The size of this BP response was reduced by more than half by NPY antagonists.

Phenoxybenzamine and other adrenergic blockers can enhance NPY release from sympathetic nerves, possibly by actions at presynaptic receptors. However, these actions are unlikely to account for our results because the pressor responses to 3-Hz stimulation differed qualitatively from those for 20-Hz stimulation even in the absence of adrenergic blockade.

The addition of beta blockade to alpha blockade prevented MAP from dropping between stimulations, thus causing successive stimulations to have additive effects on MAP. The beta blockade also nearly doubled the size of the pressor response after 20-Hz stimulation relative to alpha blockade alone. An NPY antagonist reduced the pressor response after 20-Hz stimulation by more than half in these alpha- plus beta-blocked rats.

We attribute the enhancement of the pressor response in the beta-blocked rats to antagonism of epinephrine-induced vasodilation. Epinephrine stimulates vasodilating beta-2 receptors, and 20-Hz stimulation markedly elevated circulating epinephrine. In alpha- plus beta-blocked rats and in rats without adrenergic blockade, there was a shorter interval before the NPY pressor response was apparent than in alpha-blocked rats. This may be because in alpha-blocked rats, epinephrine-induced vasodilation masked NPY pressor responses sufficiently to momentarily delay the onset of a detectable BP rise.

Our results suggest that at least half of the post-20-Hz pressor response is mediated by NPY. The incomplete blockade of post-20-Hz pressor responses by BBP 3226 and 1229U91 is probably not due to poor blockade of NPY1 receptors as these drugs inhibited the pressor responses to intravenous [Leu31,Pro34]NPY by >85%. It is conceivable that our NPY antagonists did not reach high concentrations at intrasympathetic NPY receptors. However, in vitro, low concentrations of the NPY antagonist BBP 3226 reduce pressor responses to electrically induced contraction of the guinea pig vena cava by 90% (Malmstrom and Lundberg, 1995). Additional neurotransmitters may contribute to the pressor response that follows 20-Hz stimulation.

The results suggest that the pressor actions of NPY are more gradual in onset than those of NE but are still detectable within 2 min of stimulation. NPY antagonists had no effect on the height of the rapid MAP spikes that occurred during sympathetic stimulation. The height of these spikes was dramatically reduced by phenoxybenzamine, suggesting their mediation by NE and not by NPY. During the initial 30 sec of 20-Hz stimulation and the subsequent 30-sec rest interval, pressor responses were not reduced by an NPY antagonist. However, by the fourth stimulation interval, highly significant inhibition occurred. We attribute the increased inhibition to a greater fractional contribution of NPY to the pressor responses. Results similar to ours have been reported by Hegde et al. (1995), who found no inhibition by an NPY antagonist of pressor responses in pithed rats during a 30-sec stimulation period at frequencies of ~16 Hz. However, when they extended the stimulation interval to 2 min, they found a modest but significant inhibition of the pressor response.

The present results may help us understand the role NPY plays in regulating human cardiovascular function. In humans, as in other mammals the NPY-to-NE release ratio varies greatly depending on the nature of the stress that initiates sympathetic activation. Changing from a recumbent to a standing position typically doubles plasma NE but does not alter NPY. In contrast, plasma NPY levels rise after strenuous exercise (Pernow et al., 1986). Increased NPY release during exercise may be due to the higher sympathetic firing frequencies induced by heavy exercise (Saito et al., 1993). Even though generalized sympathetic activation at 20-Hz has not yet been reported in intact mammals, frequencies as high as 35 Hz have been recorded from human sympathetic nerves (Hallin and Torebjork, 1974).

The doubling of plasma NPY that we observe after 20-Hz stimulation is dramatic, and it is conceivable that some NPY may have come from platelets. However, Zukowska-Grojec et al. (1991) reported a doubling of plasma NPY levels after exposure of male rats to a combination of handling, novel environment and ice water. This suggests that NPY may induce substantial pressor responses not only in pithed rats but also in intact mammals after certain types of stress.

In conclusion, our results suggest that high-frequency stimulation of the sympathetic nervous system of pithed rats releases enough NPY to cause a gradually increasing pressor response lasting ~10 min. This prolonged pressor response can be attenuated by antagonists of NPY1 receptors but not of adrenergic receptors.

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References


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