Neuropeptide Y antagonism reduces reflex cutaneous vasoconstriction in humans

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Skin blood flow is controlled through two branches of the sympathetic nervous system: a vasoconstrictor system and an active vasodilator system of unknown neurotransmitter (21). The vasodilator system is not tonically active but is engaged during periods of increased internal temperature. Previous studies suggest this system to be cholinergic and to involve a nitric oxide-mediated vasodilation (30, 31). These studies indicate that NPY can exert physiological effects in humans and that maneuvers that increase reflex sympathetic activity can also increase plasma NPY.

The purpose of this study, therefore, was to test whether NPY participates in the reflex control of the cutaneous circulation. We hypothesized that application of an antagonist of NPY Y1 receptors would attenuate the cutaneous vasomotor response to whole body cooling. Furthermore, we hypothesized that simultaneous antagonism of α-adrenergic, β-adrenergic, and NPY Y1 receptors would completely block the reflex cutaneous vasomotor response to whole body cooling. Our results indicate that norepinephrine and NPY are the principal mediators of reflex cutaneous vasoconstriction.

METHODS

This study was approved by the Institutional Review Board of The University of Texas Health Science Center (San Antonio, TX). The 15 subjects in these studies were all young (19–33 yr) healthy men, free of medications for 24 h and free of caffeine and alcohol for at least 12 h before the study. All were of normal height and weight, none was taking chronic medication, and all were nonsmokers. Average mean

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arterial blood pressure at rest immediately before whole body cooling was 79.6 ± 4.4 mmHg in protocol 1 and 83.4 ± 4.2 mmHg in protocol 2. Each subject provided written informed consent to participate in this study after being verbally briefed regarding its methods, risks, and aims. The protocols in this study conformed to the standards of the Declaration of Helsinki.

**Protocols.** The aim of this study was to test whether NPY contributes to reflex cutaneous vasosconstriction. We reasoned that if the persistent vasosconstriction in the presence of combined α- and β-adrenergic receptor antagonists observed previously (37, 38) was an independent effect of NPY, then selective blockade of NPY receptors would attenuate the non-noradrenergic portion of reflex cutaneous vasosconstriction induced by whole body cooling. Also, the addition of such blockade to combined α- and β-adrenergic blockade would further inhibit or abolish reflex cutaneous vasosconstriction.

The subjects reported to the laboratory between 7:30 and 9:30 AM. Depending on which experimental protocol was performed, either two or four microdialysis membranes were placed in the venarial aspect of the forearm using sterile techniques. The probes were fabricated in our laboratory and have been described previously (9, 24). They consist of 1 mm of microdialysis tubing (18-kDa molecular mass cutoff) with borosilicate tubing attached at both ends and reinforced with a stainless steel wire. A 15-cm² ice pack was applied to the venarial surface of the forearm for approximately 10 min for a temporary anesthetic effect. The site was then wiped clean with an alcohol pad. A 25-gauge needle was inserted into the dermis for anesthetic effect. The site was then wiped clean with an alcohol pad. A microdialysis probe was threaded through the needle. The needle was then removed, leaving the probe in place. The external portions of the probe were taped to the skin to prevent movement, and one end was attached to the syringe and pump. This process was repeated for the insertion of all probes, which were all oriented parallel to the long axis of the forearm.

Ninety minutes after the last probe was implanted, the subject was instrumented with thermocouples placed at six representative sites on the body surface (40) to measure whole body skin temperature (TSK). For the control of TSK, the subject dressed in a water-perfused suit that covered the torso and legs but not the areas of blood flow measurement (37, 38). The subject tested supine for the duration of the study. To minimize initial tonic vasosconstrictor nerve activity (21), TSK was held at 34.5 °C for the baseline period. Ringer solution was infused at 4 μl/min for 15–20 min into all microdialysis probes at the beginning of the study. In protocol 1, Ringer solution at one site was replaced with a solution containing 10.5 μM BBP-3226 to test the effects of NPY Y₁ receptor blockade on reflex cutaneous vasosconstriction. A total of 10 subjects participated in this protocol.

Protocol 2 was designed to test whether the combination of NPY Y₁ receptor antagonism with noradrenergic receptor antagonism had a greater inhibition of the reflex vasosconstriction than did noradrenergic receptor blockade alone. This series included eight subjects, two of whom participated in protocol 1. In those two subjects, studies were separated by at least 6 wk. In protocol 2, one site was treated with a solution of 5 mM yohimbine plus 1 mM propranolol to antagonize the effects of α- and β-adrenergic receptors (15, 37, 38). Two other sites were treated with identical solutions of 5 mM yohimbine plus 1 mM propranolol plus 10.5 μM BBP-3226 to antagonize α-adrenergic, β-adrenergic, and NPY Y₁ receptors (11, 37, 38). Data from those sites were averaged. Concentrations of yohimbine and propranolol were based on our earlier studies (37, 38). Those concentrations of yohimbine were found to antagonize the vasoconstrictor effects of exogenous norepinephrine in keeping with a nonspecific α-adrenergic blockade and in accord with other studies showing that high concentrations of yohimbine are nonspecific with respect to α-adrenergic receptor subtypes (15). The concentration of BBP-3226 was based on preliminary studies. In both protocols, after 40 min of antagonist infusion and provided that blood flow had been stable for at least 10 min, a whole body cooling ramp similar to that used previously (37, 38) was performed by progressively cooling the water perfusing the suit by 2°C/min until the target whole body TSK of 31.5°C was reached. Antagonist infusion continued throughout this portion of the protocol, which typically lasted 15 min. TSK was then returned to 34.5°C over a period of ~10 min. In protocol 2, antagonist solutions were replaced with antagonist solutions in the same concentrations with norepinephrine (10 μM) added. This combination of norepinephrine with the antagonists was applied to test the adequacy of blockade of the effects of norepinephrine. After norepinephrine was applied with the antagonists for 15 min, all solutions were replaced with sodium nitroprusside (25 mM) to cause maximal cutaneous vasodilation (24). All infusions were performed at 4 μl/min with a four-channel syringe pump (Harvard Instruments).

**Measurements.** Skin blood flow was measured by laser Doppler flowmetry (Moor, MBF3D) with a probe centered over each of the microdialysis membranes. Blood pressure was measured at the finger by the Penaz method (Finapres, Ohmeda) (32). Mean arterial pressure was obtained by electrical integration of the pulsatile blood pressure signal. All data were collected at 1 sample/s with a laboratory computer using LabView software (National Instruments; Austin, TX) and stored as 20-s averages.

BBP-3226 (10.5 μM) and norepinephrine (10 μM, Sigma; St. Louis, MO) were prepared in Ringer solution and filtered through a microprobe filter (Acrodisc, Pall; Ann Arbor, MI). Yohimbine (5 mM), propranolol (1 mM), and sodium nitroprusside (25 mM, Sigma) were prepared in Ringer solution and filtered through a micropore filter as described above. All drugs were mixed immediately before use and were protected from the light. Ascorbic acid (1 mg/ml) was added to prevent spontaneous oxidation of norepinephrine and sodium nitroprusside and therefore was added to all solutions as a control.

**Data analysis.** Cutaneous vascular conductance (CVC) was calculated from the ratio of skin blood flow to mean arterial pressure. CVC was expressed as a percentage of the baseline before each intervention by normalizing the data to the average of the 3 min immediately before whole body cooling or to the 3 min immediately before the application of norepinephrine. Because CVC collected during whole body cooling was analyzed as the percent reduction from baseline, it was important to demonstrate that the baseline CVC values were not significantly different, i.e., CVC values began declining from similar starting points. Thus, to test for baseline differences, we normalized the CVC values at each site to the maximum CVC induced by sodium nitroprusside infusion. For this analysis, baseline CVC values were expressed as a percentage of each site’s maximum CVC as determined from the average of the CVC values during the final 5 min of sodium nitroprusside perfusion (24). CVC data (%baseline) from each site during whole body cooling were analyzed by one-way ANOVA with repeated measures followed by a Dunnett’s posttest when a significant difference was detected. To find whether the treatment by any antagonist solution (BBP-3226, yohimbine + propranolol, or yohimbine + propranolol + BBP-3226) significantly attenuated the reflex response to whole body cooling, for each 0.5°C decrement in TSK CVC values from the Ringer solution- and antagonist-treated sites were compared by two-way ANOVA with repeated measures followed by a Bonferroni post test when a significant difference was detected. To find whether combined blockade of α-adrenergic, β-adrenergic, and NPY Y₁ receptors completely blocked reflex vasosconstriction, values for CVC during whole body cooling were analyzed by one-way ANOVA with repeated measures and with a Dunnett’s post test when a significant difference was detected. Statistical significance was accepted at P < 0.05.

**RESULTS**

The design of this study included using 34.5°C as the initial TSK to minimize or abolish tonic sympathetic activity before the period of body cooling. Table 1 shows CVC, relative to maximal, before and 40 min after the beginning of antagonist delivery. CVC was not significantly changed by the application...
Table 1. CVC levels before and after antagonist administration

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Before Antagonist</th>
<th>After Antagonist</th>
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</thead>
<tbody>
<tr>
<td>Protocol 1</td>
<td>Ringer solution</td>
<td>20.3 ± 2.6</td>
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<tr>
<td></td>
<td>Ringer solution</td>
<td>17.8 ± 4.8</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>Ringer solution</td>
<td>12.8 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Yoh + Pro</td>
<td>15.3 ± 2.4</td>
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<tr>
<td></td>
<td>Yoh + Pro + BIBP-3226</td>
<td>10.6 ± 1.2</td>
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Values are means ± SE expressed as a percentage of maximal cutaneous vascular conductance (CVC) for sites treated with the neuropeptide Y1 antagonist (BIBP-3226), with α- and β-adrenergic antagonists (yohimbine (Yoh) + propranolol (Pro)), or with combined blockade (Yoh + Pro + BIBP-3226). Data are from the period before antagonist infusion and after antagonist infusion before the beginning of the whole body cooling protocol. Ringer solution sites received only that infusion throughout.

of BIBP-3226 (protocol 1) or by yohimbine plus propranolol or the combination of antagonists (protocol 2, all P > 0.05), indicating low or zero levels of tonic sympathetic vasoconstrictor activity (21).

In protocol 1, whole body TSK was reduced from 34.5 ± 0.04 to 31.7 ± 0.02°C (Fig. 1) over a period of 15 min. CVC at sites treated with Ringer solution or with BIBP-3226 was significantly reduced at a TSK of 34.0 ± 0.03°C and remained significantly reduced throughout the remainder of cooling (P < 0.05; Fig. 1). Two-way ANOVA detected a significant difference in CVC between sites treated with Ringer solution and those treated with BIBP-3226 at the three lowest levels of whole body TSK analyzed (P < 0.05). At the lowest whole body TSK analyzed, CVC at Ringer solution-treated sites averaged 55.1 ± 5.6% baseline and at the BIBP-3226 treated sites averaged 65.9 ± 5.0% baseline.

In protocol 2, which involved combined blockade of noradrenergic receptors and of NPY Y1 receptors, TSK was reduced from 34.3 ± 0.1 to 31.7 ± 0.1°C (Fig. 2) over a period of 15 min. CVC at the sites perfused with Ringer solution was significantly reduced from baseline at TSK of 34.0 ± 0.01°C (CVC = 87.9 ± 2.6% baseline) and remained reduced throughout the remainder of body cooling, reaching 67.4 ± 4.3% of baseline (P < 0.05). At the sites treated with yohimbine plus propranolol, CVC was significantly reduced from baseline at TSK of 32.5 ± 0.01°C (CVC = 84.7 ± 4.1% baseline) and below during whole body cooling, reaching 81.3 ± 4.4% baseline at the lowest TSK analyzed (P < 0.05). CVC from sites treated with the combination of yohimbine, propranolol, and BIBP-3226 was not significantly reduced from baseline at any TSK analyzed during whole body cooling, reaching 90.3 ± 3.5% baseline at the lowest TSK (P > 0.05). After whole body cooling, norepinephrine was applied via microdialysis in solution with the antagonists to confirm blockade of α-adrenergic and β-adrenergic receptors. These results are shown in Fig. 3. The concentrations of the antagonists were the same as those delivered during whole body cooling. Average CVC at sites treated with Ringer solution was significantly reduced from baseline by minute 8 of application of norepinephrine (CVC = 81.4 ± 4.0% baseline), reaching 49.5 ± 7.1% baseline by minute 15 (P < 0.05). In contrast, CVC at yohimbine plus propranolol-treated sites was not significantly reduced during application of exogenous norepinephrine until minute 13, reaching 89.6 ± 3.9% baseline (P < 0.05). CVC at sites treated with the combination of yohimbine, propranolol, and BIBP-3226 was not significantly reduced from baseline at any point during the 15-min application of norepinephrine (P > 0.05; CVC = 97.4 ± 4.9% baseline at minute 15).

**DISCUSSION**

The major finding from this study is that an NPY Y1 antagonist significantly inhibits reflex cutaneous vasoconstriction, strongly indicating that NPY contributes to the vasoco-

Fig. 1. Average responses in cutaneous vascular conductance (CVC) during whole body cooling during protocol 1. Sites were treated with Ringer solution or 10.5 μM BIBP-3226 delivered via microdialysis in 10 healthy men. CVC values at both sites were significantly reduced at 34.0°C and below (P < 0.05). *Points at which CVC at Ringer solution- and BIBP-3226-treated sites were significantly different by two-way ANOVA and Bonferroni post test (P < 0.05). These data indicate that neuropeptide YNP (NPY) antagonist significantly attenuates reflex cutaneous vasoconstriction during body cooling.

Fig. 2. Average responses in CVC as a percentage of baseline (means ± SE) from protocol 2. Sites were treated with Ringer solution; yohimbine plus propranolol; or yohimbine, propranolol, and BIBP-3226 during whole body cooling in 8 healthy men. *Significant reduction from baseline (P < 0.05). At sites treated with yohimbine, propranolol, and BIBP-3226, CVC was not significantly reduced at any point in this cooling protocol (P > 0.05). These data indicate the vasomotor response to whole body cooling is mediated largely if not entirely by norepinephrine and NPY. P values indicate significant differences in the response between Ringer solution and yohimbine plus propranolol (P < 0.001) and between yohimbine plus propranolol plus BIBP-3236 and the other two sites (P < 0.01).
striction induced by whole body cooling. Earlier, we observed a persistent vasoconstriction that occurred during whole body cooling despite antagonism of the effects of norepinephrine (37, 38). In the present study, we found that at sites with selective NPY Y1 receptor antagonism with BIBP-3226, reflex vasoconstriction during whole body cooling was attenuated at the lowest whole body Tsk analyzed. This attenuation indicates that NPY makes a significant contribution and is consistent with the magnitude of the persistent vasoconstriction after noradrenergic receptor blockade seen here as well as previously (37, 38). Second, we tested whether combined blockade of the vasomotor effects of both norepinephrine and NPY would completely abolish the reflex response to whole body cooling. We observed that with combined antagonism of α-adrenergic, β-adrenergic, and NPY Y1 receptors, there was no detectable reflex cutaneous vasomotor response to whole body cooling. These data also indicate that norepinephrine and NPY are the major if not sole mediators of the reflex cutaneous vascular response to whole body cooling. The role for the nonnoradrenergic mechanism is substantial. Of the total reflex vasoconstrictor response to cooling, ~40% is due to something other than norepinephrine and is consistent with our previous results (37, 38).

In testing for the identity of the nonnoradrenergic mechanism of vasoconstriction, we believed NPY was the best candidate because it is a well-known vasoconstrictor and has been identified in the perivascular sympathetic nerves of human skin (26). Furthermore, NPY participates in the regulation of skin blood flow in several animal models (17, 18, 29). Others have shown that NPY participates in vasomotor responses in human cutaneous and subcutaneous resistance vessels in vitro (16, 30, 34). It is possible, however, that another cotransmitter makes a contribution to reflex vasoconstriction, e.g., ATP (4). Although reports in the literature indicate that ATP induces cutaneous vasodilation in humans (8, 13), it is nevertheless possible that ATP or other transmitters make contributions. Indeed, the possibility of redundancy and of interaction among multiple neurotransmitters cannot be excluded, nor can the possibility that more pronounced cooling could lead to the release of other cotransmitters through higher sympathetic nerve activity.

Any conclusion that NPY participates in the reflex control of the cutaneous circulation depends on the effectiveness and selectivity of BIBP-3226 in blocking the vasoconstrictor effects of NPY. The effects of NPY as either a potent vasoconstrictor or as a potentiator of the effects of norepinephrine are likely mediated by NPY Y1 receptors (11, 12, 30, 35). As an antagonist, BIBP-3226 has been shown to be highly selective for NPY Y1 receptors versus NPY Y2 receptors in vitro preparations (12, 35). Second, BIBP-3226 has no measurable affinity for adrenergic receptors (35). Third, BIBP-3226 does not influence the physiological response evoked by norepinephrine in either whole animal or isolated organ preparations, thus illustrating that BIBP-3226 does not influence α-adrenergic receptors (7, 20, 28). Finally, BIBP-3226 antagonizes the NPY-induced constriction of cutaneous blood vessels from both humans and animals (16, 30, 34). Thus any effect of BIBP-3226 on reflex cutaneous vasoconstriction should be through antagonism of NPY Y1 receptors. Such antagonism could be through inhibition of direct vasoconstrictor effects of NPY or through inhibition of NPY-mediated potentiation of noradrenergic vasoconstriction. In either case, it is a Y1 receptor-initiated mechanism. These results indicate that if NPY is causing cutaneous vasoconstriction via NPY Y1 receptors, then application of BIBP-3226 should attenuate reflex vasoconstriction in vivo in humans.

In several vascular beds, the primary role of NPY is as a potentiator of the effects of norepinephrine rather than as an independent mediator of vasoconstriction (14, 41). The intracellular signaling pathways for NPY Y1 and α1-adrenergic receptors on vascular myocytes converge at the level of inositol (1,4,5)-trisphosphate and diacylglycerol (36). This convergence can lead to an amplification of the signal to increase intracellular Ca2+ concentrations, which would affect vasoconstriction. NPY can also recruit α1-adrenergic receptors to the plasma membrane, thus increasing the sensitivity of the cell to extracellular norepinephrine (19). Thus NPY can potentiate the effects of norepinephrine through an increase in smooth muscle sensitivity or an amplified response to norepinephrine. However, Clarke et al. (6) found that intra-arterial infusion of a vasoconstricting dose of NPY did not significantly increase the forearm vasoconstrictor response to either lower body negative pressure or to exogenous norepinephrine. These results suggest that the vasoconstrictor effect of NPY is not through potentiation of reflex sympathetic vasoconstriction in the human forearm. It is important to note that the potentiating effects of NPY are dependent on the presence of α1-adrenergic receptors. It does not appear that α2-adrenergic receptor signaling is affected by NPY (36).

The findings from the present study do not clearly indicate whether the effects of BIBP-3226 on reflex vasoconstriction are due to inhibition of direct vasoconstrictor actions of NPY or to interference with potentiation of α-adrenergic receptor-mediated vasoconstriction. The small but significant vasoconstriction seen with norepinephrine application at yohimbine plus propranolol-treated sites at minutes 13 and 14 (see Fig. 3) was not seen when BIBP-3226 was added. This could be taken
as evidence for the removal of a potentiation. However, we are reluctant to reach any firm conclusions on that basis for two major reasons. First, the vasconstriction did not persist to minute 15. Second, for BBP-3226 to have that effect, tonic release of NPY in conditions of normothermia (Tsk of 34°C) would be required. The distinction between direct and potentiating roles for NPY and their antagonism by BBP-3226 is an important issue and one that deserves further study.

In normothermic conditions, internal temperature is regulated by subtle changes in sympathetic vasoconstrictor tone and in skin blood flow. Although changes in skin blood flow may be small, and the influence of NPY even less, it is important to understand that small changes of blood flow over the surface of the human body can have profound effects. For example, although a change of skin blood flow of only 10% of baseline skin blood flow is significant.

In conclusion, we observed that blockade of NPY Y1 receptors significantly attenuates the non-noradrenergic component of the reflex cutaneous vasconstrictor response to mild whole body cooling. Furthermore, the combined blockade of the effects of norepinephrine and those of NPY Y1 receptors further inhibited this reflex vasconstriction. These data strongly suggest that the non-noradrenergic mechanism of reflex vasconstriction reported earlier (37, 38) is mediated to a significant extent by NPY. Finally, it appears that norepinephrine and NPY are the major mediators of the reflex cutaneous vasconstrictor response to body cooling.

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REFERENCES


