Cutaneous constitutive nitric oxide synthase activation in postural tachycardia syndrome with splanchnic hyperemia

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Cutaneous constitutive nitric oxide synthase activation in postural tachycardia syndrome with splanchnic hyperemia. Am J Physiol Heart Circ Physiol 301: H704–H711, 2011. First published June 3, 2011; doi:10.1152/ajpheart.00171.2011.—Models of microgravity are linked to excessive constitutive nitric oxide (NO) synthase (NOS), splanchnic vasodilation, and orthostatic intolerance. Normal-flow postural tachycardia syndrome (POTS) is a form of chronic orthostatic intolerance associated with splanchnic hyperemia. To test the hypothesis that there is excessive constitutive NOS in POTS, we determined whether cutaneous microvascular neuronal NO and endothelial NO are increased. We performed two sets of experiments in POTS and control subjects aged 21.4 ± 2 yr. We used laser-Doppler flowmetry to measure the cutaneous response to local heating as an indicator of bioavailable neuronal NO. To test for bioavailable endothelial NO, we infused intradermal acetylcholine through intradermal microdialysis catheters and used the selective neuronal NOS inhibitor L-nitroarginine (NLA, 10 mM), the selective inducible NOS inhibitor aminoguanidine (10 mM), the nonspecific NOS inhibitor nitro-l-arginine (NLA, 10 mM), or Ringer solution. The acetylcholine dose response and the NO-dependent plateau of the local heating response were increased in POTS compared with those in control subjects. The local heating plateau was significantly higher, 98 ± 1% maximum cutaneous vascular conductance (%CVCmax) in POTS compared with 88 ± 2%CVCmax in control subjects but decreased to the same level with NLA (46 ± 5%CVCmax in POTS compared with 49 ± 4%CVCmax in control) or with NLA (45 ± 3%CVCmax in POTS compared with 47 ± 4%CVCmax in control). Only NLA blunted the acetylcholine dose response, indicating that NO produced by endothelial NOS was released by acetylcholine. Aminoguanidine was without effect. This is consistent with increased endothelial and neuronal NOS activity in normal-flow POTS.

nitrergic; laser-Doppler flowmetry; acetylcholine; cutaneous blood flow

ORTHOSTATIC INTOLERANCE is defined by symptoms and signs relieved by recumbency. Symptoms include dizziness, fatigue, exercise intolerance, headache, memory problems, palpitations, nausea, blurred vision, pallor, and abnormal sweating while upright, which improve with recumbence and which have no other medical explanation. Postural tachycardia syndrome (POTS) is the predominant form of chronic orthostatic intolerance (17, 28, 38, 52, 53) and is defined by symptoms of orthostatic intolerance associated with the physical sign of excessive upright tachycardia (38). We previously described three subsets of POTS patients.

Low-flow POTS is characterized by pallor, absolute hypovolemia, reduced stroke volume, blunted orthostatic vascular responses, increased plasma angiotensin II, and decreased bioavailability of cutaneous nitric oxide (NO) of neuronal NO synthase (nNOS) origin (46) related to increased plasma angiotensin II (44) consequent to angiotensin-converting enzyme 2 deficiency (50). High-flow POTS is characterized by normovolemia, peripheral vasodilation, and increased peripheral blood flow, cardiac output, and microvascular filtration accounting for postural tachycardia (42, 43). Evidence indicated a mechanism of defective adrenergic-mediated vasoconstriction, possibly associated with a postviral peripheral neuropathy (16, 27).

In regard to normal-flow POTS, less well characterized are, perhaps, the majority of our recent POTS patients who have normal peripheral blood flow, heart rate, and vascular resistance when supine. Upright, splanchnic vasodilation causes splanchnic hyperemia, resulting in a redistributive reduction in central blood volume and reflex tachycardia because of excessive splanchnic pooling (45, 49).

Interestingly, splanchnic hyperemia also occurs in real and simulated microgravity and is often associated with orthostatic intolerance, adrenergic hyporeactivity (2, 26, 32).

We therefore hypothesized that microvascular NO production would be upregulated in normal-flow POTS subjects with splanchnic hyperemia. To test this hypothesis, we examined the plateau phase of the cutaneous response to local heat that is largely NO dependent (19, 27) and acetylcholine-induced increase in cutaneous blood flow that is due, in part, to endothelial NO (46). We used different doses of acetylcholine alone and then combined with NO synthase (NOS) inhibitors nitro-l-arginine (NLA), 1-Nω-nitroarginine-2,4-L-diamino-butyric amide (Nω, 10 mM), and aminoguanidine (AG) to elicit changes in skin blood flow responses thought to represent local signaling by NO.

METHODS

To test this hypothesis, we used skin as a surrogate microvascularity (14) and stimulated the production of NO in two ways: 1) via the local cutaneous heating response reported by Kellogg et al. (21) and Minson et al. (29), which reaches a plateau that is highly sensitive to NOS inhibition; and 2) using the acetylcholine dose response, which is partly dependent on receptor-mediated endothelial NO production.

In both cases we tested our hypothesis by employing a highly selective, isospecific nNOS inhibitor Nω, a highly specific inducible NOS (iNOS) inhibitor AG, and a nonisoform-specific NOS inhibitor NLA to examine individual roles for nNOS, endothelial NOS (eNOS), and iNOS in the regulation of microvascular function in normal-flow POTS patients.

Subjects

Enrolled POTS patients were referred to the Hypotension Center for investigation of signs and symptoms of chronic orthostatic intolerance lasting at least 3 mo. Orthostatic intolerance was defined by the presence of dizziness, fatigue, exercise intolerance, headache, mem-
ory problems, palpitations, nausea, blurred vision, pallor, and abnormal sweating while upright, relieved by recumbency. The diagnosis of POTS was made in these patients during a screening upright tilt table test to 70° for a maximum of 10 min. POTS was diagnosed by symptoms of orthostatic intolerance during tilt associated with an increase in sinus heart rate of >30 beats/min or to a rate of >120 beats/min during the first 10 min of tilt (28, 36). During the same visit, POTS patients were partitioned on the basis of supine calf blood flow measured by venous occlusion strain-gauge plethysmography (12) into either normal-flow, high-flow, or low-flow POTS as we have previously defined (48). In the current study, we only enrolled subjects with normal-flow POTS. Normal-flow POTS subjects are defined as those who had a minimum calf blood flow of 1.2 ml·100 ml tissue \(^{-1}\)·min\(^{-1}\) and a maximum calf blood flow of 4.0 ml/100 ml tissue \(^{-1}\)·min\(^{-1}\). This is based on the range of calf blood flow that we have measured in healthy volunteers from determinations in over 150 healthy subjects. Splanchnic hyperemia was confirmed in these patients (45, 49).

Using these criteria, we recruited 14 normal-flow POTS patients (10 women, 4 men; all Caucasian; age, 15.5–24.1 yr; and median age, 21.3 yr). Twelve healthy Caucasian volunteers subjects were also recruited (8 women, 4 men; all Caucasian; age, 17.0–25.7 yr; and median age, 21.5 yr) and were studied after a screening upright tilt at 70° demonstrated normal orthostatic response. Volunteer subjects served as a control group and were recruited from among adolescents and young adults referred for innocent heart murmur. Subjects with a history of syncope or orthostatic intolerance were specifically excluded.

All subjects were free from systemic, cutaneous, and cardiovascular diseases. Subjects refrained from alcohol and caffeinated beverages for 24 h before study and were not taking any medications. There were no smokers or competitive athletes. Informed consent was obtained from all participants, and the Committee for the Protection of Human Subjects (Institutional Review Board) of New York Medical College approved all protocols. Female subjects were enrolled without regard to the phase of their menstrual cycle except that none were menstruating during testing procedures.

Protocols

Two sets of experiments were performed in which changes in cutaneous blood flow were measured.

The first series of experiments investigated the cutaneous response to local heating in POTS patients compared with healthy volunteers in the presence and absence of NOS inhibition. We employed the highly selective nNOS (NOS-1) inhibitor N\(^{\omega}\) (10 mM), the highly selective iNOS (NOS-2) inhibitor AG (10 mM), and the nonselective NOS inhibitor NLA (10 mM). There is no highly selective eNOS (NOS-2) inhibitor, and eNOS inhibition was assessed by inference (e.g., suppression by NLA but not by N\(^{\omega}\) or AG) and by exploiting eNOS-specific stimulation using acetylcholine.

Past experiments have demonstrated that N\(^{\omega}\) decreased cutaneous vascular conductance (CVC) during local heating by an amount equivalent to the largest reduction produced by NLA (46). We have therefore used the local heating response as a bioassay for nNOS and do so in the current experimental design.

The second series of experiments investigated the response to increasing doses of acetylcholine, a receptor-mediated eNOS agonist. This was measured in the presence and absence of NOS inhibition.

Experiment 1: the effect of NOS inhibitors on local heat-mediated vasodilation. We compared the effects of the isoform-selective nNOS inhibitor N\(^{\omega}\) to a maximally plateau-suppressing dose of NLA, a nonselective NOS inhibitor and to AG, a selective iNOS inhibitor. N\(^{\omega}\) is a highly specific nNOS inhibitor that does not bind the iNOS isoform and has ~1,500-fold selectivity for nNOS over eNOS (15). AG is a highly specific iNOS inhibitor that has ~100-fold selectivity for iNOS over nNOS or eNOS (57). All NOS inhibitors were dissolved in Ringer solution and, each having a low molecular weight,
Subjects then received perfusate containing Ringer solution through catheter insertion, baseline LDF values were obtained. Integrative laser-Doppler probes as in polarizing factor, and the influence of local axon reflexes. Acetylcholine response because there are effects of other local vaso-in skin blood flow responses thought to represent local signaling by sodium nitroprusside to obtain CVC\text{max}.

Only the last 5 min of data were averaged at each acetylcholine dose. Continued and each dose was administered for 20 min during which this agonist through microdialysis catheters (24, 41). LDF monitoring based on previous determinations in human skin using the delivery of the range of concentration of acetylcholine used (0.01–100 mM) was combination with NOS inhibitors through each catheter at 2 min. 2.5 mM of AG through catheter 4, and 10 mM of AG through each catheter at 2 µl/min.

Subjects were instrumented with four microdialysis catheters and integrative laser-Doppler probes as in experiment 1. Following recovery from catheter insertion, baseline LDF values were obtained. Subjects then received perfusate containing Ringer solution through catheter 1 (control), 10 mM NLA through catheter 2, 10 mM N\textsuperscript{ω} through catheter 3, and 10 mM of AG through catheter 4, each perfused at 2 µl/min for 45 min during a drug run-in phase. When run-in was complete, subjects received perfusate containing acetylcholine, dissolved in Ringer solution containing identical NOS inhibitors (or Ringer alone) as received during run-in. Acetylcholine was perfused in ascending doses (0.01, 0.10, 1.0, 10, and 100 mM) in combination with NOS inhibitors through each catheter at 2 µl/min. The range of concentration of acetylcholine used (0.01–100 mM) was based on previous determinations in human skin using the delivery of this agonist through microdialysis catheters (24, 41). LDF monitoring continued and each dose was administered for 20 min during which steady-state values of LDF were achieved. For purposes of analysis, only the last 5 min of data were averaged at each acetylcholine dose.

At the end of experiments, all catheters were perfused with 28 mM sodium nitroprusside to obtain CVC\text{max}.

Statistics

We used two-way analysis of variance (2 × 4) to compare the plateau phases of the local heating response treatment of POTS with those in control subjects receiving N\textsuperscript{ω}, NLA, AG, or Ringer solution in experiment 1. We used two-way analysis of variance with repeated measures to compare dose-response curves of acetylcholine alone (acetylcholine + Ringer), acetylcholine + NLA, acetylcholine + N\textsuperscript{ω}, and acetylcholine + AG for POTS and control subjects. The results were calculated using SPSS (Statistical Package for the Social Sciences) software version 14.0. Apart from representative figures, text, graphic, and Table 1, results are reported as means ± SE. Significance requires $P < 0.05$.

Table 1. Dimensions and supine hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>POTS</th>
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<tbody>
<tr>
<td>$n$</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Age, yr</td>
<td>22 ± 1.5</td>
<td>22 ± 1.8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>70 ± 3</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171 ± 2</td>
<td>169 ± 3</td>
</tr>
<tr>
<td>Body surface area, m$^2$</td>
<td>1.80±.04</td>
<td>1.76±.05</td>
</tr>
<tr>
<td>Supine heart rate, beats/min</td>
<td>68 ± 2</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Supine systolic blood pressure, mmHg</td>
<td>120 ± 3</td>
<td>124 ± 3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>67 ± 2</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>55 ± 2</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Venous occlusion calf blood flow, ml·100 ml$^{-1}$·min$^{-1}$</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Calf arterial resistance, ml·100 ml$^{-1}$·mmHg$^{-1}$</td>
<td>35 ± 4</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>Maximum laser-Doppler flow with sodium nitroprusside, pfu</td>
<td>183 ± 10</td>
<td>167 ± 9</td>
</tr>
<tr>
<td>Baseline laser-Doppler flow, pfu</td>
<td>21 ± 5</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Baseline, %CVC\text{max}</td>
<td>12 ± 1.1</td>
<td>11 ± 1.3</td>
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Values are means ± SE; $n$, number of subjects. POTS, postural tachycardia syndrome; %CVC\text{max}, percent maximum cutaneous vascular conductance; pfu, perfusion units.

RESULTS

As shown in Table 1, POTS and control subjects had similar height, weight, supine heart rate, systolic blood pressure, diastolic blood pressure, pulse pressure, calf blood flow, and calf arterial resistance. The baseline LDF, the maximum LDF elicited with 28 mM sodium nitroprusside, and the calculated baseline %CVC\text{max} were the same in POTS and control subjects. Microdialysis of drugs had no effect on systemic hemodynamics (heart rate, and arm and leg blood pressure) in any patient.

Experiment 1: The Effect of NOS Inhibitors on Local Heat-Mediated Vasodilation

The local heat response is increased in normal-flow POTS. Figure 1 shows representative heating responses for a POTS patient and a control subject during local heating while perfused with Ringer solution (no NOS inhibition). The values for %CVC\text{max} of the heat-induced plateau measured in the POTS subjects were nearly equal to 100%, whereas that of control subjects was significantly less.

NLA, N\textsuperscript{ω}, and AG have no effect on baseline cutaneous blood flow. As shown in Fig. 2, and as previously reported for NLA (51) and N\textsuperscript{ω} (46), the inhibition of NOS had no effect on the baseline cutaneous blood flow compared with Ringer alone when measured before the application of local heat. This was also found for AG, which had no effect on baseline flow, compared with that measured with Ringer solution alone. When each of these NOS inhibitors was tested, there was also no difference between baseline flows comparing control to POTS subjects.

NLA and N\textsuperscript{ω} are equipotent inhibitors of local heating in normal-flow POTS and control; AG has no effect on the local heating response. Figure 2 shows heat responses averaged over all subjects for each NOS inhibitor in POTS and control subjects. On average, the plateau-phase conductance while

Fig. 1. Local heating response in a representative normal-flow postural tachycardia syndrome (POTS) patient (gray) and in a healthy volunteer control subject (black). Key points along the curve are marked. The heat response plateau, dependent on bioavailable nitric oxide (NO), is near maximum vasodilation in POTS patients, whereas the response in control subjects is significantly smaller. %CVC\text{max}, percent maximum cutaneous vascular conductance.
receiving Ringer solution was 88 ± 2% CVC\textsubscript{max} for control compared with 98 ± 1% CVC\textsubscript{max} for normal-flow POTS (P < 0.01); the plateau phase conductance while receiving NLA was 47 ± 4% CVC\textsubscript{max} for control compared with 45 ± 3% CVC\textsubscript{max} for normal-flow POTS; the plateau phase conductance while receiving N\textsuperscript{w} was 49 ± 4% CVC\textsubscript{max} for control compared with 46 ± 5% CVC\textsubscript{max} for normal-flow POTS; and the plateau phase conductance while receiving AG was 86 ± 2% CVC\textsubscript{max} for control compared with 97 ± 2% CVC\textsubscript{max} for normal-flow POTS (P < 0.025).

Conductance was significantly increased during local heating in normal-flow POTS, and this increase was unaffected by AG. NLA and N\textsuperscript{w} reduced the plateau conductance during local heating to a similar degree. During the perfusion of Ringer solution alone, the plateau conductance in normal-flow POTS patients was larger than the plateau conductance in control subjects. Consequently, perfusion with either NLA or N\textsuperscript{w} reduced the NO-sensitive plateau by a larger amount in POTS compared with control subjects.

There were no effects of selective nNOS and iNOS inhibitors on the acetylcholine dose response. There were large reductions of nonisoform selective NOS inhibition with NLA on the acetylcholine dose response.

**DISCUSSION**

**Summary and Discussion of Findings**

Our main findings are that cutaneous nNOS- and eNOS-mediated production of NO are both increased in normal-flow POTS patients compared with control subjects.

**Experiment 1: nNOS activity is increased in normal-flow POTS.** The administration of a nonselective NOS inhibitor blunts the NO-dependent plateau of the local heating response. A selective nNOS inhibitor is equally effective in blunting this response at a dose that should exert a minimal effect on eNOS. AG has no effect on local heating, indicating a lack of influence of iNOS under these experimental conditions. These findings indicate that the local heating plateau can be used as a bioassay for nNOS activity. The local heating response is enhanced in normal-flow POTS compared with control subjects, reaching conductances close to CVC\textsubscript{max}. This suggests...
that there is increased NO derived from nNOS in normal-flow POTS.

The dependence of the local heating response on nNOS is controversial. Kellogg et al. (22) have maintained that the local heating response is dependent on eNOS rather than nNOS. Those conclusions were based on observations made using 7-nitroindazole as a selective nNOS inhibitor and N\textsubscript{G}H9275-o-nitro-L-arginine as a selective eNOS inhibitor (22). However, 7-nitroindazole has no selectivity for nNOS in vitro (the IC\textsubscript{50} values for inhibition of nNOS and eNOS are 0.71 and 0.78 M, respectively) and exhibits modest selectivity in vivo. N\textsubscript{G}H9275-o-nitro-L-arginine inhibits nNOS with \textit{K}_i values of 15 nM and eNOS with \textit{K}_i value of 39 nM and cannot be regarded as eNOS selective.

Experiment 2: eNOS activity is increased in normal-flow POTS. Our past work showed that acetylcholine increases cutaneous blood flow and that this increase is due, in part, to NO (46). Our current results are similar and thus support the association between increased blood flow and NO. While NLA does not completely eliminate the response to increasing doses of acetylcholine, it attenuates this increase by some 50% (Fig. 3).

The administration of a sufficient amount of the nonselective NOS inhibitor NLA blunts the response to acetylcholine. Neither the selective nNOS inhibitor N\textsuperscript{G} nor the selective iNOS inhibitor AG affects this acetylcholine-mediated response. These findings indicate that the blunting of the heat response by NLA is an indicator of local eNOS activity. The response to acetylcholine is enhanced in normal-flow POTS compared with control subjects. This suggests that there is increased NO of endothelial origin in normal-flow POTS. Other investigators have reported an increase in endothelial NO production during intradermal acetylcholine administration (5). In the current work, we found no effect of N\textsuperscript{G} at any dose of acetylcholine used.

Significance of Increased Bioavailable Constitutive NO

Systemic illnesses typically have cutaneous manifestations. In particular, prior cutaneous findings in other variants of POTS (50) have been substantiated by systemic measurements (30). The skin is therefore a useful surrogate of the microcirculation that is likely reflective of systemic disease (14) in POTS.

Both nNOS and eNOS are expressed constitutively (8) and require calcium-calmodulin for activation. nNOS was first discovered in the brain and therefore was initially associated with neurons, whereas eNOS was first discovered in endothelial cells. However, nNOS and eNOS have been found in diverse tissues. Thus, for example, nNOS has been found in keratinocytes (3); cardiac, skeletal muscle; and splanchnic, renal, and neurovascular tissue (6, 7, 18, 20), whereas eNOS has been found in every organ containing endothelium. nNOS has been shown to be important in the regulation of human basal forearm microvascular and coronary blood flow (39, 40).

Single nucleotide polymorphisms of eNOS have been explored in POTS and seem to be loosely related to disease (10), although studies did not subgroup patients on the basis of vascular physiology. Similar studies for nNOS single nucleotide polymorphisms and POTS have not yet been performed.
However, genetic differences are not necessary to alter the expression of constitutive NOS as evidenced in hepatic cirrhosis where both nNOS and eNOS isoforms are increased and sustain NO-mediated vasodilation and the hyperdynamic circulation (35). Changes in the expression of nNOS and eNOS have been reported following conditions of simulated microgravity and occur in both bed-rested humans and the rat hindlimb suspension models and are associated with splanchnic dilation (2, 56) and orthostatic intolerance (2, 54).

Increased NO can contribute to peripheral vasodilation through cGMP-mediated vasodilation (37), through its blunting of central sympathetic nerve activity (25) and through peripheral modulation of sympathetic neurotransmission. Modulated sympathetic neurotransmission may result from the inhibitory effects of NO on the release of norepinephrine (11), chemical deactivation of norepinephrine by NO reducing its activity (23), or both. Of particular interest are the effects of nNOS on the central sympathetic nervous system and on nitrergic nerves innervating mesenteric vasculature (13, 34). Nitricergic nerves contain nNOS and are distributed with cholinergic parasympathetic nerves. nNOS may even colocalize with acetylcholine and vasoactive intestinal peptide in the perivascular parasympathetic fibers (33) and modulate vascular tone and blood flow (31, 55).

**Limitations**

We studied the cutaneous circulation, which has unique autonomic control. Our recent work indicates that flow regulation abnormalities in POTS occur throughout the circulatory system (49) and that the flow abnormalities that occur in skin may be used as representation of systemic diseases (14).

We studied the calf cutaneous circulation. While flow abnormalities are widespread in POTS, whenever peripheral blood flows are studied, the most significant results occur in the lower extremities. It may be that dependence and gravitational exposure are important to the observed findings. There may be inherent differences in forearm versus calf cutaneous microvascular control that may explain the role of various NOS isoforms thought to be responsible for the mediation of the local heat response (22, 46).

Whereas NOS has well-documented in vitro selectivity for nNOS over eNOS (15), in vivo selectivity has not been well established. Thus there could be additional actions of this agent on choline transport, acetylcholineesterase, butyrocholineesterase, muscarinic, prostaglandin, or NO mechanisms other than its effects of NOSs.

We studied women without regard to menstrual cycle. The phase of the menstrual cycle can exert important effects on NO-dependent mechanisms (1). Recent evidence suggests a relationship between phases of the menstrual cycle and changes in POTS symptoms and signs during a 2-h stand because of altered response of the renal-angiotensin-aldosterone system that affects hemodynamics during orthostasis (9). Since our determinations are made while the subject is supine, it is unlikely that hormone-related differences play a major role in the response of skin to local heat; however, this issue remains a concern.

**REFERENCES**

7. Dawson D, Lygate CA, Zhang MH, Hulbert K, Neubauer S, Casadei B. nNOS gene deletion exacerbates pathological left ventricular remodel-
Constitutive NOS in POTS


