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# Upregulation Of Inducible Nitric Oxide Synthase Contributes To Attenuated Cutaneous Vasodilation In Essential Hypertensive Humans

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Dan E. Berkowitz, and Lacy A. Holowatz

## Abstract

Essential hypertension is a proinflammatory, proconstrictor disease coinciding with endothelial dysfunction and inward vessel remodeling. Using the skin circulation, our aim was to determine whether inducible NO synthase (iNOS) upregulation attenuates NO-dependent cutaneous vasodilation in hypertensive humans. We hypothesized that, with hypertension, localized iNOS inhibition would restore vasodilation in response to NO-dependent stimuli, and iNOS expression would be increased and phosphorylated vasodilator-stimulated phosphoprotein would be decreased. For, *in vivo* protocols, 4 intradermal microdialysis fibers were placed in 9 hypertensive and 10 normotensive men and women (systolic blood pressure:  $146 \pm 4$  versus  $113 \pm 2$  mm Hg;  $P < 0.001$ ). Microdialysis fibers served as control, iNOS inhibited (1400 W), neuronal NO synthase inhibited (N $\omega$ -propyl-L-arginine), and nonselective NOS inhibited (NG-nitro-L-arginine methyl ester). Cutaneous vascular conductance was calculated (percentage of sodium nitroprusside) during standardized local heating ( $42^\circ\text{C}$ ) and acetylcholine dose-response protocols (0.01, 0.10, 1.00, 5.00, 10.00, 50.00, 100.00 mmol/L). The NO-dependent local heating response was attenuated at control ( $95 \pm 2\%$  versus  $76 \pm 2\%$  cutaneous vascular conductance;  $P < 0.05$ ) and neuronal NO synthase-inhibited sites ( $94 \pm 4\%$  versus  $77 \pm 3\%$  cutaneous vascular conductance;  $P < 0.01$ ) in hypertensives. iNOS inhibition augmented the NO-dependent local heating response ( $93 \pm 2\%$  versus  $89 \pm 10\%$  cutaneous vascular conductance). Acetylcholine-induced vasodilation was attenuated in control sites at doses  $\geq 0.1$  mmol/L of acetylcholine in hypertensives and was restored with iNOS inhibition (0.1 mmol/L,  $P < 0.05$ ; 1, 5, and 10 mmol/L,  $P < 0.001$ ; 50 and 100 mmol/L,  $P < 0.01$ ). *In vitro* iNOS expression was increased ( $P = 0.006$ ) and phosphorylated vasodilator-stimulated phosphoprotein was decreased in skin from hypertensive humans ( $P = 0.04$ ). These data suggest that iNOS is upregulated in essential hypertensive humans and contributes to reduced NO-dependent cutaneous vasodilation.

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# Upregulation of Inducible Nitric Oxide Synthase Contributes to Attenuated Cutaneous Vasodilation in Essential Hypertensive Humans

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**Abstract**—Essential hypertension is a proinflammatory, proconstrictor disease coinciding with endothelial dysfunction and inward vessel remodeling. Using the skin circulation, our aim was to determine whether inducible NO synthase (iNOS) upregulation attenuates NO-dependent cutaneous vasodilation in hypertensive humans. We hypothesized that, with hypertension, localized iNOS inhibition would restore vasodilation in response to NO-dependent stimuli, and iNOS expression would be increased and phosphorylated vasodilator-stimulated phosphoprotein would be decreased. For, in vivo protocols, 4 intradermal microdialysis fibers were placed in 9 hypertensive and 10 normotensive men and women (systolic blood pressure:  $146\pm 4$  versus  $113\pm 2$  mm Hg;  $P<0.001$ ). Microdialysis fibers served as control, iNOS inhibited (1400 W), neuronal NO synthase inhibited ( $N^{\omega}$ -propyl-L-arginine), and nonselective NOS inhibited ( $N^G$ -nitro-L-arginine methyl ester). Cutaneous vascular conductance was calculated (percentage of sodium nitroprusside) during standardized local heating ( $42^{\circ}\text{C}$ ) and acetylcholine dose-response protocols (0.01, 0.10, 1.00, 5.00, 10.00, 50.00, 100.00 mmol/L). The NO-dependent local heating response was attenuated at control ( $95\pm 2\%$  versus  $76\pm 2\%$  cutaneous vascular conductance;  $P<0.05$ ) and neuronal NO synthase-inhibited sites ( $94\pm 4\%$  versus  $77\pm 3\%$  cutaneous vascular conductance;  $P<0.01$ ) in hypertensives. iNOS inhibition augmented the NO-dependent local heating response ( $93\pm 2\%$  versus  $89\pm 10\%$  cutaneous vascular conductance). Acetylcholine-induced vasodilation was attenuated in control sites at doses  $\geq 0.1$  mmol/L of acetylcholine in hypertensives and was restored with iNOS inhibition (0.1 mmol/L,  $P<0.05$ ; 1, 5, and 10 mmol/L,  $P<0.001$ ; 50 and 100 mmol/L,  $P<0.01$ ). In vitro iNOS expression was increased ( $P=0.006$ ) and phosphorylated vasodilator-stimulated phosphoprotein was decreased in skin from hypertensive humans ( $P=0.04$ ). These data suggest that iNOS is upregulated in essential hypertensive humans and contributes to reduced NO-dependent cutaneous vasodilation.

**Key Words:** NO ■ inducible NO synthase ■ skin blood flow ■ hypertension ■ microvascular dysfunction

Hypertension-related vascular dysfunction involves a complex interaction of inflammation, endothelial dysfunction, including a loss of endothelial NO, and an upregulation of proconstrictor pathways.<sup>1-3</sup> The pathogenesis of hypertension-associated microvascular dysfunction occurs simultaneously in multiple vascular beds.<sup>4,5</sup> The cutaneous circulation is an accessible, representative vascular bed for the assessment of mechanisms underlying microvascular dysfunction with essential hypertension.<sup>5-10</sup> Deficits in microvascular reactivity, including a loss of NO signaling, and inward vessel remodeling are clearly evident in essential hypertensive human skin and parallel vascular changes that occur in the renal circulation with essential hypertension.<sup>5</sup> However, the precise mechanisms leading to this dysfunction are unclear.

One putative mechanism underlying hypertension-related microvascular dysfunction is through inducible NO synthase

(NOS; iNOS).<sup>11,12</sup> iNOS generates high concentrations of NO, which is easily converted to peroxynitrite and superoxide in the pro-oxidant environment characteristic in essential hypertension. In addition, iNOS also upregulates arginase activity, which limits NO production through endothelial NOS (eNOS)<sup>13</sup> by preferentially using the common substrate L-arginine and is capable of inducing NOS uncoupling.<sup>14</sup> We have demonstrated that acute localized arginase inhibition augments NO-dependent vasodilation in essential hypertensive human skin<sup>15,16</sup>; however, alterations in NO production and the role of iNOS are unknown. Therefore, the purpose of the present study was to examine the contributions of constitutively expressed and iNOS isoforms in the cutaneous vasculature of essential hypertensive humans using both physiological and pharmacological stimuli to induce endothelium-dependent vasodilation.<sup>17</sup> We hypothesize that in vivo attenuated vascular reactivity in essential hypertensive hu-

**Table 1. Participant Characteristics**

Characteristic	Normotensive	Hypertensive
Subjects, men, women	3, 7	7, 2
Age, y	52±1	53±2
Body mass index	25±1	27±1
Systolic blood pressure, mm Hg	113±3	146±4*
Diastolic blood pressure, mm Hg	73±2	93±2*
Mean arterial pressure, mm Hg	86±2	111±3*
Glucose, mg · dL <sup>-1</sup>	90±3	94±3
High-density lipoprotein, mg · dL <sup>-1</sup>	62±5	57±4
Low-density lipoprotein, mg · dL <sup>-1</sup>	110±4	106±7

\* $P < 0.01$  difference from the normotensive age-match control group.

mans results from compromised eNOS-derived NO-dependent dilation and that acute iNOS inhibition would augment NO-dependent vasodilation in hypertensive humans induced by local heating of the skin or perfusion of the endothelium-dependent agonist acetylcholine (Ach). We further hypothesized that in vitro iNOS expression would be increased and the downstream indicator of NO vasodilatory function in vascular smooth muscle, vasodilator-stimulated phosphoprotein (VASP), would be decreased in skin samples obtained from humans with essential hypertension.

## Methods

### Subjects

Experimental protocols were approved by the institutional review board at Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent were voluntarily obtained from all of the subjects before participation. Subject characteristics are presented in Table 1. Blood pressure status was determined in accordance with the guidelines set forth by the American Heart Association<sup>18</sup> and further explored using 24-hour ambulatory blood pressure monitoring. Subjects underwent a complete medical screening and were otherwise healthy with the exclusion of stage 1 hypertension and were not taking any medications, including antihypertensives. Seven of the 8 essential hypertensive subjects were naive to antihypertensive pharmacotherapy, and 1 subject had previously been taking antihypertensive drugs but had not taken the drug for more than a year. All of the premenopausal women (N=2) were studied on days 2 to 7 of their menstrual cycle, and postmenopausal women (N=8) reported that it had been  $\geq 1$  year since the cessation of their last menses. No perimenopausal women were studied.

### In Vivo Vasoreactive Studies

Protocols were performed in a thermoneutral laboratory with the subject semisupine and the experimental arm at heart level. Four intradermal microdialysis fibers (MD 2000, Bioanalytical Systems) were inserted into the forearm skin, as described previously.<sup>19</sup> Microdialysis sites were perfused with ringers to serve as control, 0.1 mmol/L of 1400 W {*N*-[3-(aminomethyl)benzyl] acetamide, AG Scientific} to inhibit iNOS, 5 mmol/L of *N*<sup>ω</sup>-propyl-*L*-arginine (Tocris) to inhibit neuronal NOS (nNOS), and 20 mmol/L of *N*<sup>G</sup>-nitro-*L*-arginine methyl ester (L-NAME; Tocris) to nonselectively inhibit all of the NOS isoforms (FDA IND 105 572) at a rate of 2  $\mu$ L/min (Bioanalytical Systems Beehive and Baby Bee micro-infusion pumps, West Lafayette, IN). All of the drugs were mixed just before usage, dissolved in lactated Ringer solution, and sterilized (Acrodisc, Pall, Ann Arbor, MI). The efficacies of the isoform-specific antagonism and concentrations of the pharmacological agents used in this study have been demonstrated in other microdi-

alysis studies (1400 W inhibition constant = 7 nmol/L; *N*<sup>ω</sup>-propyl-*L*-arginine inhibition constant = 57 nmol/L).<sup>13,20–25</sup>

### Local Heating Protocol

After subsidence of initial insertion trauma (60–120 minutes), local skin temperature was clamped at 33°C and laser Doppler probes attached over each site. After baseline measurements, a standardized local skin warming protocol was performed to induce NO-dependent vasodilation.<sup>26</sup> This protocol induced cutaneous vasodilation that is predominantly ( $\approx 70\%$ ) mediated by the production of NO from eNOS.<sup>17,27,28</sup> No subjects reported pain or a burning sensation during skin heating. After skin blood flow reached an established plateau (30–40 minutes), 20 mmol/L of L-NAME was perfused to quantify NO-dependent vasodilation in all of the sites. A representative tracing illustrating the phases of the local heating response from a normotensive and a hypertensive subjects' control sites are illustrated in Figure 1. This figure shows the phases of the local heating response including the initial peak and nadir, which are primarily mediated by sensory nerve mechanisms with a small NO contribution, followed by the predominantly NO-dependent plateau as illustrated by the infusion of L-NAME to quantify the functional production of NO.<sup>26</sup> After a new post-L-NAME stabilization in skin blood flow, local temperature was increased to 43°C and 28 mmol/L of sodium nitroprusside was perfused to induce maximal cutaneous vasodilation (CVC<sub>max</sub>).<sup>21,29</sup> In our previous work and in pilot work this combination of heat and high concentration of sodium nitroprusside has been shown to induce maximal vasodilation.<sup>21</sup>

### Ach Dose-Response Protocol

Subsidence of insertion trauma and baseline measurement followed the same procedure as for the local heating. Protocols were performed on the same arm and separated by  $\geq 1$  week to allow the skin to fully heal between trials. After baseline measurement, local skin temperature remained clamped at 33°C during perfusion of 7 ascending concentrations of Ach for 5 minutes each: 0.01, 0.10, 1.00, 5.00, 10.00, 50.00, and 100.00 mmol/L of Ach. This amount of time allowed for a plateau in skin blood flow at each concentration of Ach. Each Ach concentration was mixed with the appropriate isoform-specific NOS inhibitor and perfused at 2  $\mu$ L/min. After completion of the Ach dose response, local skin temperature was increased to 43°C and 28 mmol/L of sodium nitroprusside was perfused through all of the sites at a rate of 4  $\mu$ L/min to induce CVC<sub>max</sub>.<sup>21,29</sup>

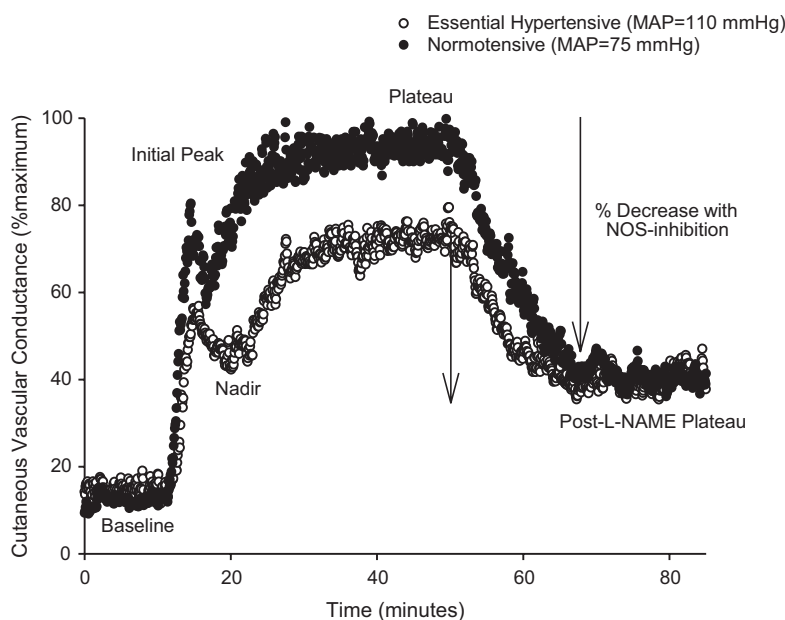
### In Vitro Skin Sample Analysis

Ventral forearm skin samples were obtained on a separate day from the in vivo functional assessment of vasoreactivity and on the opposite arm. Using sterile technique, two 3-mm-diameter skin samples were obtained after anesthetization using 2% lidocaine without epinephrine. Samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### Western Blot Analysis

After centrifugation of skin homogenates twice at 15 000g at 4°C for 20 minutes, protein concentration was determined using a Bio-Rad DC protein assay. For Western blot analysis, 25- $\mu$ g proteins were fractionated by SDS/PAGE and electrotransferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences). The membranes were blocked for 1 hour at room temperature (5% nonfat dry milk, in Tris-buffered saline containing 0.1% Tween 20) and incubated with a primary antibody to eNOS (Santa Cruz Biotechnology; 1:1000); nNOS (BD Bioscience, 1:1000); iNOS (BD Bioscience, 1:1000); and phosphorylated vasodilator-stimulated phosphoprotein (1:1000; Cell Signaling). Bound antibody was detected with horseradish-peroxidase-conjugated IgG secondary antibody (1:1000; Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence. Next, the phosphorylated vasodilator-stimulated phosphoprotein (pVASP) blot was stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific) and reprobed with VASP antibody (Cell Signaling). GAPDH was used as loading

## Local Heating Representative Tracings



**Figure 1.** Representative skin blood flow tracings during local heating for a normotensive and essential hypertensive subject. The characteristic phases of the local heating response are labeled: initial peak, nadir, local heating plateau, and post-*N*<sup>G</sup>-nitro-*L*-arginine methyl ester (*L*-NAME) plateau. The difference between the plateau and the post-*L*-NAME plateau indicates the vasodilation attributed to the production of NO.

control. Densitometry analyses were performed using Image J software (National Institutes of Health).

### Data and Statistical Analyses

Skin blood flow data were digitalized at 40 Hz, recorded, and stored for offline analysis using Windaq software and Dataq data acquisition system (Windaq; Dataq Instruments, Akron, OH). Data were normalized to a percentage of  $CVC_{max}$ . CVC data were averaged over a stable 5 minutes of baseline, local heating plateau, post-*L*-NAME plateau, and maximal vasodilation. The initial peak and nadir CVC were visually identified as the highest and lowest values and averaged over 10 seconds. The vasodilation attributed to NO was calculated from the difference between the plateau and the post-*L*-NAME plateau (Figure 1). Absolute  $CVC_{max}$  in each site was calculated as the average of a stable plateau in laser-Doppler flux during 28 mmol/L of sodium nitroprusside infusion and local heating to 43°C divided by mean arterial pressure (Table 2). Because the late plateau phase of the local heating response is primarily dependent on NOS function, whereas the early phase has contributions from both sensory nerves and NOS, analysis and further discussion focus on the later phase of the cutaneous vasodilatory response.

Student unpaired *t* tests were used to compare physical characteristics between subject groups and to examine potential differences in the densitometry analysis of the Western blots. Absolute  $CVC_{max}$  data were analyzed using a 2-way repeated-measures ANOVA (group\*pharmacological site, SPSS 19). % $CVC_{max}$

**Table 2. Absolute Maximum CVC at Each Drug Site for Normotensive and Hypertensive Subjects During the Local Heating Protocol**

Group	Maximum CVC			
	Control	nNOS Inhibited	iNOS Inhibited	NOS Inhibited
Normotensive	1.79±0.28	1.70±0.19	1.86±0.23	1.81±0.12
Hypertensive	1.56±0.15	1.90±0.28	1.53±0.25	1.56±0.34

CVC indicates cutaneous vascular conductance; nNOS, neuronal NO synthase; iNOS, inducible NO synthase; NOS, NO synthase. There were no differences between groups or with localized microdialysis drug interventions (2-way repeated-measures ANOVA). Values are presented as mean±SEM.

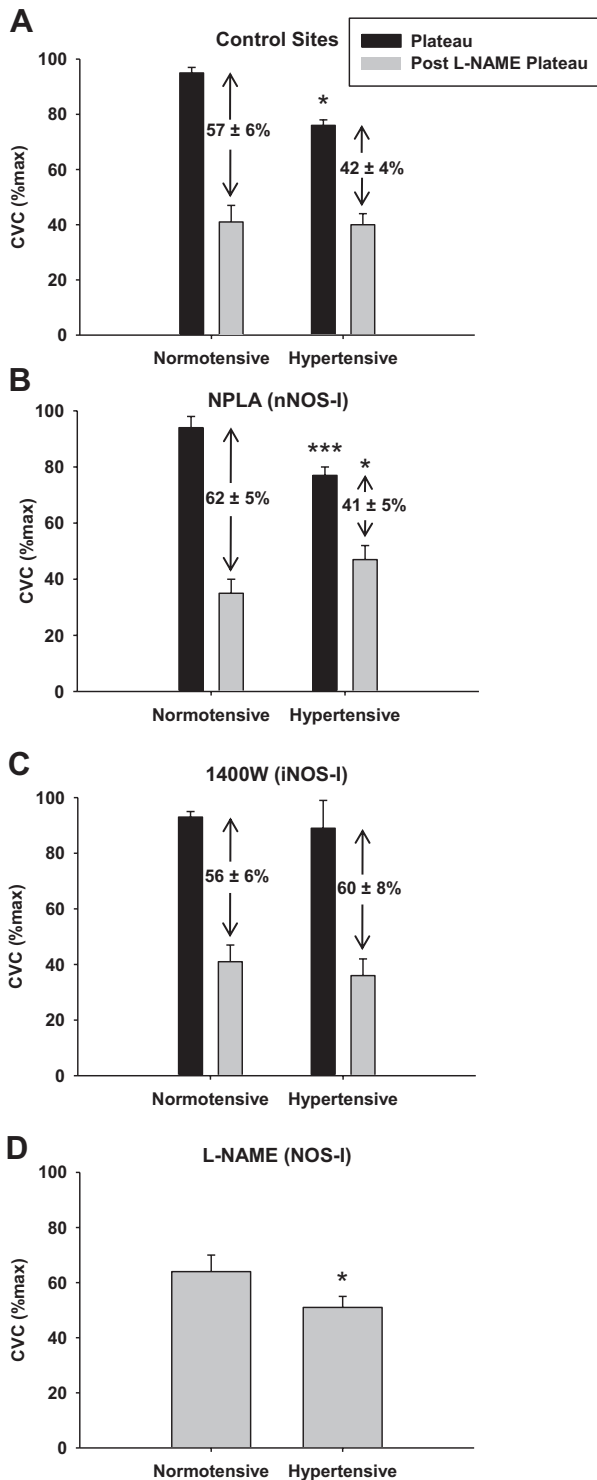
were analyzed using 3-way, mixed model, repeated-measures ANOVA (group\*pharmacological site\*local heating phase or Ach dose; proc mix SAS 9.2). Specific planned comparisons were performed when appropriate to determine where differences between groups and pharmacological sites occurred with appropriate Bonferroni correction. Significance was set at  $\alpha=0.05$ . Values are presented as mean±SEM.

### Results

The physical characteristics of the subjects are presented in Table 1. Subjects were matched for age, body mass index, total cholesterol, and high- and low-density lipoproteins. Hypertensive subjects showed a significantly higher resting systolic, diastolic, and mean arterial blood pressure compared with normotensive controls ( $P<0.001$ ).

Figure 1 illustrates representative skin blood flow tracings during local heating for a normotensive and essential hypertensive subject. The characteristic phases of the local heating response are labeled.

Figure 2 illustrates the % $CVC_{max}$  at the NO-dependent plateau and after NOS inhibition with the nonspecific NOS inhibitor *L*-NAME during local skin warming at different pharmacological sites in normotensive controls and essential hypertensive subjects. The difference between the plateau and the post-*L*-NAME plateau is a quantification of within-site NO-dependent vasodilation during local heating and is labeled numerically on the graph. The NO-dependent plateau was attenuated in the hypertensive (HT) group at control (HT: 79±2% versus the normotensive group [NT]: 95±2%  $CVC_{max}$ ;  $P<0.024$ ) and nNOS inhibited (HT: 77±3% versus NT: 94±4%  $CVC_{max}$ ;  $P=0.0004$ ) sites compared with the normotensive group but was restored in the hypertensive group at the iNOS-inhibited sites (HT: 89±9% versus NT: 93±2%;  $P=0.39$ ). A greater decrease in % $CVC_{max}$  with NOS inhibition was present in the normotensive group at the control (HT: 42±4% versus NT: 57±6%  $CVC_{max}$ ;  $P=0.039$ ) and nNOS-inhibited (HT: 41±5% versus NT: 62±5%  $CVC_{max}$ ;



**Figure 2.** Cutaneous vascular conductance (%CVC<sub>max</sub>) at the plateau in skin blood flow during local warming and after NO synthase (NOS) inhibition with *N*<sup>2</sup>-nitro-*L*-arginine methyl ester (L-NAME) in normotensive and essential hypertensive subjects in the (A) control site, (B) the neuronal NOS (nNOS)-inhibited site, (C) the inducible NOS (iNOS)-inhibited site, and (D) the nonspecific NOS-inhibited site. The black bars indicate %CVC<sub>max</sub> at the plateau and the gray bar indicates %CVC<sub>max</sub> at the post-L-NAME plateau. NO-dependent vasodilation during local heating is the difference between the bars and is shown (mean ± SEM). Values significantly different from the normotensive group: \**P*<0.05; \*\**P*<0.001.

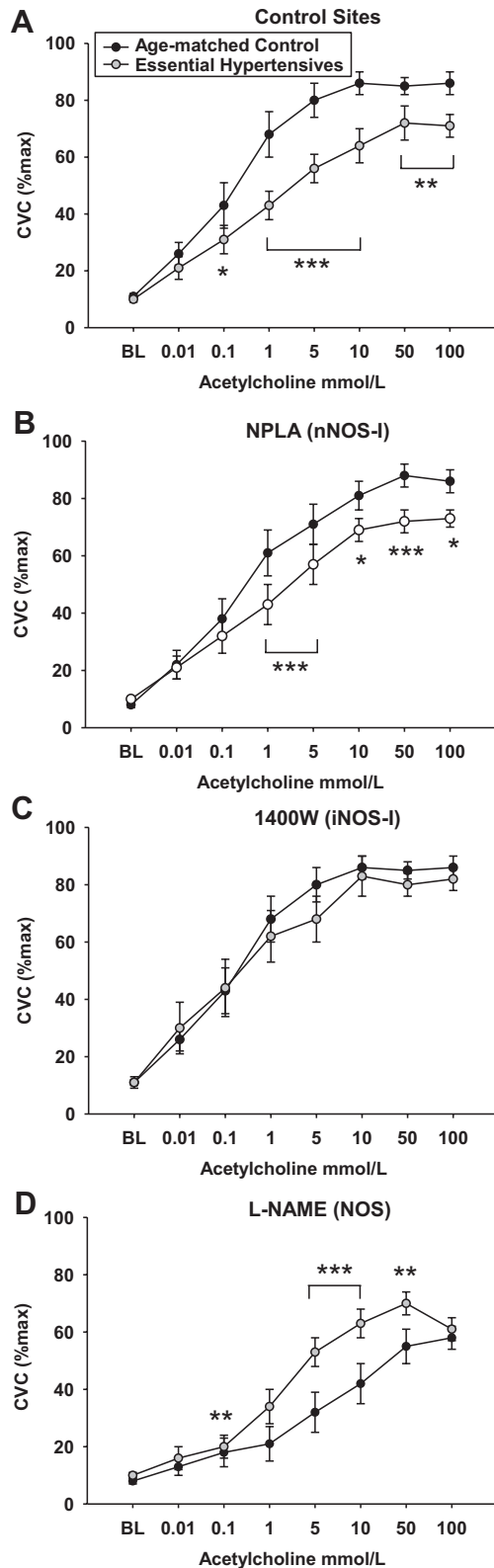
*P*<0.001) sites, but no difference was present at the iNOS-inhibited site (HT: 60 ± 8% versus NT: 56 ± 6% CVC<sub>max</sub>; *P*=0.39). The plateau in %CVC<sub>max</sub> during local heating at the continuous L-NAME site (nonspecific NOS inhibited) was greater in the normotensive compared with the hypertensive group (Figure 2D; *P*<0.001). A significant difference in %CVC<sub>max</sub> between groups was observed during local heating at the iNOS-inhibited site (1400 W) during the initial peak (HT: 60 ± 10% versus NT: 49 ± 4% CVC<sub>max</sub>; *P*=0.04) and nadir (HT: 48 ± 8% versus NT: 30 ± 3% CVC<sub>max</sub>; *P*=0.0002). No differences in %CVC<sub>max</sub> were present between groups at any of the other pharmacological sites for baseline, initial peak, or nadir during local heating. There were also no differences in absolute maximal CVC with localized microdialysis treatment or between groups (Table 2) (*P*>0.05).

Figure 3 shows the mean Ach dose-response curves for both groups in each drug treatment site. Ach-induced vasodilation was attenuated at all of the concentrations of Ach >0.1 mmol/L in the control sites and >1 mmol/L of Ach in the nNOS-inhibited sites in the hypertensive compared with the normotensive group (all *P*<0.05; see Figure 3). Ach-induced vasodilation was restored with iNOS inhibition in the hypertensive group compared with their control sites. In addition, there were no longer differences between the hypertensive and the normotensive groups in %CVC<sub>max</sub> in the sites where iNOS was inhibited. Vasodilation was attenuated at all of the Ach concentrations with nonspecific NOS inhibition in both groups compared with their respective control sites, but vasodilation was greater in the hypertensive compared with the normotensive group at all doses from 1 to 50 mmol/L of Ach (1.0 mmol/L *P*<0.01, 5 and 10 mmol/L *P*<0.001, and 50 mmol/L *P*<0.01) but not 100 mmol/L of Ach.

Figure 4 shows densitometric analysis and representative Western blots of nNOS, iNOS, eNOS, and pVASP from the skin biopsy samples for both groups. There were no differences in eNOS or nNOS expression between the groups. iNOS expression was increased (*P*=0.006) and pVASP (*P*=0.046) was decreased in skin samples from the essential hypertensive group.

## Discussion

The major new findings of the present study were that cutaneous NO-dependent vasodilation to both physiological and pharmacological stimuli is attenuated in essential hypertensive human skin and acute iNOS inhibition with 1400 W increased NO-dependent vasodilation likely through eNOS-mediated mechanisms. In response to endothelium-dependent agonists, vasodilation was increased in the essential hypertensives when all of the NOS isoforms were inhibited compared with the normotensive group. In vitro analysis of skin biopsy tissue showed that iNOS protein expression was increased but there were no differences in the constitutively expressed NOS isoforms. Furthermore, pVASP was reduced in the hypertensive skin samples, suggesting that functional NOS activity at the level of the vascular smooth muscle is decreased. Together these findings suggest that reduced functional NO production from eNOS contributes to cutane-



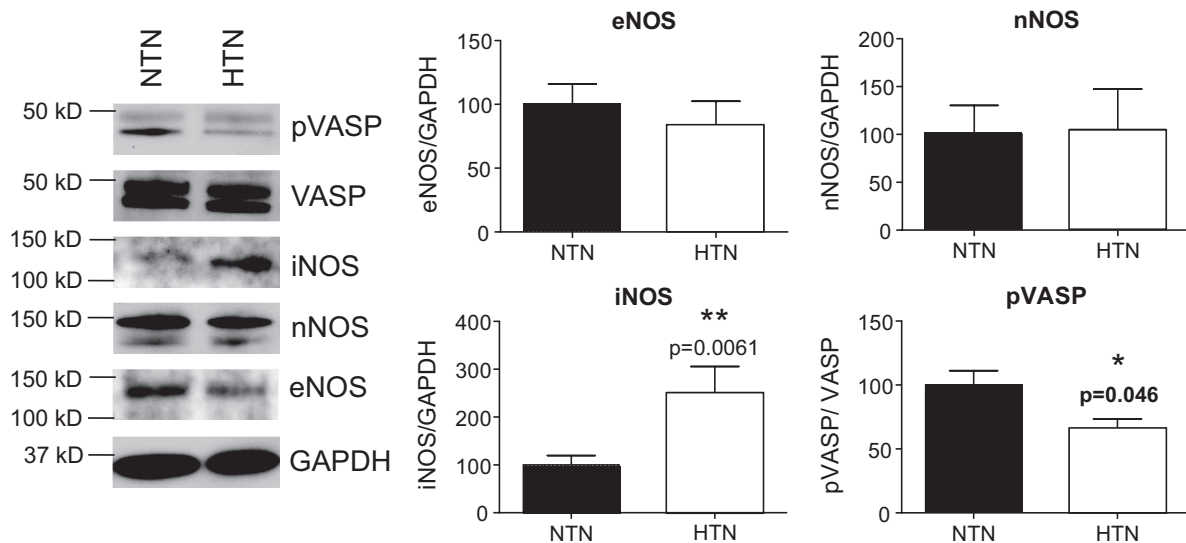
**Figure 3.** Cutaneous vascular conductance (%CVC<sub>max</sub>) in normotensive and essential hypertensive subjects during acetylcholine-induced vasodilation across a series of concentrations in the (A) control site, (B) the neuronal NO synthase (nNOS)-inhibited site, (C) the inducible NO synthase (iNOS)-inhibited site, and (D) the non-specific NOS inhibited site (*N*<sup>ω</sup>-nitro-*L*-arginine methyl ester [L-NAME]). Values are significantly different from the normotensive group: \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

ous microvascular dysfunction in essential hypertensive humans and may be related to an upregulation in iNOS.

The present data identify iNOS upregulation as a possible target contributing to attenuated NO-dependent vasodilation in essential hypertensive humans. One putative mechanism that may be contributing to hypertension-associated microvascular dysfunction is an iNOS-mediated upregulation of arginase activity. Arginase is the final enzyme of the urea cycle and competes with eNOS for the common substrate *L*-arginine, resulting in reduced NO bioavailability, and partially mediating eNOS uncoupling,<sup>14</sup> where eNOS produces superoxide instead of NO.<sup>30</sup> In animal models of essential hypertension, increased arginase activity contributes to endothelial dysfunction and inward vessel remodeling through the downstream production of polyamines.<sup>31,32</sup> Because the affinity of arginase for *L*-arginine is considerably lower than eNOS, direct substrate competition is unlikely in healthy individuals,<sup>33,34</sup> because such acute arginase inhibition is ineffective at increasing NO-dependent vasodilation in young and middle-aged healthy humans.<sup>35</sup> However, recent work has established an iNOS-dependent upregulation of arginase activity through S-nitrosylation as a mechanism for limiting NO production through eNOS. This posttranslational mechanism acts to stabilize arginase and alter its substrate affinity, allowing it to compete with eNOS for *L*-arginine.<sup>13,36</sup> Although we did not measure arginase activity in the present data because of the limited protein obtained from the skin biopsy samples, we have shown in other studies that acute arginase inhibition augments NO-dependent vasodilation in essential hypertensive human skin.<sup>19,37</sup> Further research into the link among augmented iNOS, arginase, and microvascular dysfunction is necessary.

In vitro analysis of the skin biopsies showed that the downstream vascular smooth muscle NO target pVASP was decreased in samples obtained from the hypertensive group. This is consistent with the functional in vivo findings of attenuated NO-dependent vasodilation during local heating and pharmacological perfusion of the endothelium-dependent agonist Ach. Because pVASP is a general downstream vasodilatory molecule, there are limits to the mechanistic interpretation of these data. However, these data together suggest that NO synthesized by iNOS is not having a vasodilatory effect on the vascular smooth muscle. Instead, in the pro-oxidant environment characteristic of hypertension, it is more likely that iNOS synthesized NO is rapidly oxidized and converted to peroxynitrite. This potential mechanism is supported by the increase in NO-dependent vasodilation observed when high concentrations of the nonspecific antioxidant ascorbate are acutely administered in hypertensive humans.<sup>19</sup>

In the present study we have initially focused on characterizing the involvement of the NOS isoforms in attenuated vasodilation with essential hypertension. Our current findings suggest that increased iNOS expression has potential downstream interaction through the constitutive NOSs (mainly eNOS). However, the initial stimulus (or stimuli) that increases iNOS in hypertensive cutaneous microvasculature remains unclear. Possible candidates include a host of immune modulators, including tumor necrosis factor- $\alpha$ , inter-



**Figure 4.** NO synthase (NOS) 1, NOS2, NOS3, vasodilatory-stimulated phosphoprotein (VASP), and phosphorylated VASP (pVASP). Expressions of the 3 NOS enzymes and VASP and pVASP activity were determined by Western blotting. GAPDH was used as loading control. Sample blot is shown in first panel. Densitometry analysis was performed using ImageJ software (National Institutes of Health). \* $P < 0.05$  difference from the normotensive group.

leukin 6, cyclooxygenase 2, and elevated angiotensin II.<sup>38</sup> As such, potential pharmacological targets for the treatment of hypertension-induced microvascular dysfunction include the angiotensin pathway and novel anti-inflammatory compounds, such as acromalin, which decreases iNOS and cyclooxygenase 2 expression by altering tumor necrosis factor- $\alpha$  and interleukin 6.<sup>39</sup>

An additional interesting finding from the present data set was that vasodilation was increased in the hypertensive group when all of the NOS isoforms were inhibited in response to the endothelium-dependent agonist Ach (compared with the normotensive group). There are many possible explanations for this finding in addition to decreased functional NO bioavailability. First, there may be other non-NO-dependent mechanisms that are upregulated with hypertension acting as a compensatory mechanism for decreased eNOS activity, including endothelium-derived hyperpolarization factors and both vasoconstrictor and vasodilatory products of the arachidonic acid pathway. Secondly, increased superoxide dismutase production from uncoupled eNOS and other hypertension-associated elevations in the activity of enzymes NAD(P)H oxidase and xanthine oxidase<sup>1,2</sup> may lead to a relative vasoconstriction. Superoxide is capable of inducing vasoconstriction through the Rho/Rho-kinase pathway in the vascular smooth muscle and has been identified mechanistically as one signal inducing vasoconstriction in human skin.<sup>40-42</sup> Furthermore, this mechanism is implicated in microvascular dysfunction with primary aging.<sup>43-45</sup> Thus, inhibiting the uncoupled eNOS with L-NAME would decrease superoxide production and result in increased blood flow with increasing concentrations of Ach.

In the present study we did not find a significant difference in absolute maximal cutaneous vascular conductance as we and others have demonstrated using similar techniques in unmedicated essential hypertensive humans.<sup>19,37</sup> This difference likely reflects the relatively young age and stage 1

hypertensive status of our population. Moreover, it suggests that the disease progression begins with a relative endothelial dysfunction and then moves on toward vascular smooth muscle hypertrophy and inward vessel remodeling.

We have used the cutaneous circulation to examine a potential mechanism underlying hypertension-induced microvascular dysfunction in humans. Using both physiologically relevant and pharmacological stimuli, our results are consistent with findings from other regional circulations<sup>46</sup> and demonstrate a globalized endothelial dysfunction. Although the cutaneous circulation is being recognized as a useful model for examining hypertension-induced microvascular dysfunction, it should be acknowledged that the sympathetic neural innervation is specialized for thermoregulatory functions and does not significantly contribute to overall blood pressure control. However, recent findings specific to essential hypertensive vascular pathology suggest that the changes occurring in the cutaneous circulation parallel those that occur in the renal vascular bed,<sup>5</sup> which is important in blood pressure regulation.

### Limitations

Men and women demonstrate differences in cardiovascular risk, and several studies have demonstrated important differences in endothelial function between men and women. In this study we included both men and women, but the groups were not evenly matched for sex, and 2 premenopausal women were included (1 in each group). It would have been ideal to match the groups evenly for sex and menopausal status. However, whereas sex differences have been observed in the reflex regulation of thermoregulatory skin blood flow because of differences in sympathetic control, little evidence exists for potential differences in the regulation of skin blood flow during local heating or the perfusion of endothelium-dependent agonists.

We did not measure a total index of direct NOS activity in the skin samples. Because of the limited number of skin

samples per subjects and protein recovery per sample, we were not able to successfully measure total NOS activity. One reason for this was that there are several endogenous enzyme-independent sources of NO in the skin that increase with UV light exposure,<sup>47</sup> leading to an unfavorable signal:noise ratio. Instead, we measured a downstream vascular smooth muscle indicator of NOS activity, demonstrating that it is decreased in samples from essential hypertensive humans. Finally, we attempted to probe for phosphorylated eNOS; however, because of the time lapse between the initial analysis and labile nature of these proteins, they were not detectable.

## Perspectives

Hypertension-associated vascular dysfunction is a complex, multifaceted condition that occurs simultaneously in multiple vascular beds. Attenuated NO-dependent dilation in the cutaneous circulation may precede alterations in conduit vessels,<sup>9,19,37,48–51</sup> making the skin an easily accessible, generalizable vascular bed for assessing in vivo vascular function and dysfunction in preclinical and cardiovascular disease groups.<sup>4,10,52,53</sup> The present data indicate a mechanistic link between inflammation and attenuated NO-dependent vasodilation in hypertensive humans. Because iNOS inhibition restored locally mediated vasodilation in the hypertensive group, iNOS may be a potential molecular target in the treatment of essential hypertension-induced vascular dysfunction.

In summary, cutaneous NO-dependent vasodilation to both physiological and pharmacological stimuli is attenuated in essential hypertensive human skin. iNOS expression was increased in skin samples from essential hypertensive humans and acute iNOS inhibition restored NO-dependent vasodilation, likely through eNOS mechanisms. In addition, the downstream functional NO target pVASP was reduced in hypertensive skin samples. Together these findings suggest that reduced functional NO production from eNOS contributes to cutaneous microvascular dysfunction in essential hypertensive humans and may be related to an upregulation in iNOS.

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## Disclosures

D.E.B. is the scientific founder and consultant for Arginetix Inc, a biotechnology company dedicated to the development of therapeutics targeting arginase in diseases in which endothelial dysfunction is an important contributing factor.

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