Reactive oxygen species (ROS) from NADPH and xanthine oxidase modulate the cutaneous local heating response in healthy humans

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We have previously described the role of ANG II in the modulation of cutaneous blood flow using the vasodilation response of nonglabrous skin to local heating (39–41). This response has three distinct phases of cutaneous blood flow: an initial peak, a nadir, and an increase to a plateau which has been shown to be NO dependent (16, 24). When its effect on local skin blood flow was evaluated, ANG II exhibited vasconstrictive actions which are thought to be primarily mediated by ANG II receptor, type 1 (AT1R)-activation of oxidases (4, 10, 11, 16, 19). These reductions in cutaneous blood flow can be reversed by the ANG II receptor antagonist losartan (39–41) and are improved by intradermal administration of Ang-1(1–7) and the antioxidant ascorbate (39, 41). ANG II binds to the AT1 receptor at NADPH oxidase, which is thought to increase superoxide, $\text{H}_2\text{O}_2$ (4, 52) and peroxynitrite (28), thus increasing the level of local oxidative stress. In contrast, ascorbate, acting as an antioxidant, has been shown to reverse age-dependent decreases in cutaneous blood flow due to heat (14). Reactive oxygen species (ROS) are therefore likely involved in the regulation of skin blood flow and may modulate the response of skin blood flow to NO.

Our hypothesis, based on the effects of ANG II, is that the cutaneous response to local heat is mediated by products of oxidative stress. Therefore, to evaluate the role that ROS products play in the regulation of skin blood flow, we investigated the response to local heat as measured by laser-Doppler flowmetry in normal, healthy control subjects. Since some vasoactive actions of ANG II are mediated by AT1R-dependent activation of NADPH oxidase and xanthine oxidase causing increased ROS (7, 11, 19), we used allopurinol and apocynin, respectively, to inhibit these enzymes. These ROS include superoxide and $\text{H}_2\text{O}_2$ which exert important vasoactive and sympathetic effects (44, 46, 52); thus we reduced local cutaneous levels of superoxide (SO) with tempol and $\text{H}_2\text{O}_2$ with ebselen.

METHODS

Subjects. In the first series of experiments, we studied the effects of NADPH oxidase inhibition, xanthine oxidase inhibition, superoxide reduction, and $\text{H}_2\text{O}_2$ reduction on the cutaneous heat response in eight healthy volunteer subjects aged 24.5–29.5 yr, median age 27.15 yr (5 men and 3 women). In a second series of experiments, we studied the effects of ANG II on the heat response in the presence of NADPH oxidase inhibition, xanthine oxidase inhibition, superoxide reduction, and $\text{H}_2\text{O}_2$ reduction in the same study population. Subjects with a history of orthostatic intolerance were specifically excluded. Only subjects free from cutaneous, systemic, and cardiovascular diseases were eligible. Subjects were not taking any medications and refrained from alcohol and caffeinated beverages for at least 24 h before the study. There were no smokers or trained competitive athletes. Informed consent was obtained, 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.

Instrumentation. All testing was conducted in a temperature-controlled room (25°C) at least 2 h after a light breakfast. Skin temperature was continuously monitored by the laser-Doppler flow (LDF) probes used to make the skin blood flow measurements. Measurements were made in the left calf. Since all experiments were performed with the subject supine, the leg was at the level of the heart throughout all procedures. Subjects were instrumented in the dermal space of the lateral aspect of the left calf after hair was gently removed from the insertion site. Each site was cooled with ice before catheter insertion to reduce discomfort. Each probe (MD-2000 Linear Micro-
dialysis Probes; Bioanalytical Systems, West Lafayette, IN) has a 10-mm microdialysis membrane section that is placed in the intradermal space using a 25-gauge needle as an introducer. Catheters were randomly designated. The molecular mass cutoff is nominally 30,000 Da. Following placement, all catheters were initially perfused with Ringer’s solution at 2 μl/min. An integrating LDF probe (Probe 413; Perimed) containing seven individual probe tips (each contains a separate transmitting and receiving fiber) was then placed directly over each microdialysis catheter to measure skin blood flow, designated as LDF. LDF was thereafter recorded until values were similar to those measured over the same area before catheter insertion. The return of LDF to approximately preinsertion values indicated recovery from the trauma of the catheter emplacement and usually occurred by 60–90 min (1, 22). When necessary, longer times were allowed until preinsertion LDF was reached. Baseline untreated LDF was then recorded during local heating and 30 min post-heat recovery.

Local heating. Once baseline LDF values were obtained, the areas under each laser were gradually heated at 1°C/10 s to 42°C which was maintained for at least 30 min until a plateau was reached. The area underneath the heating unit is 3 cm². Heat was turned off to allow for recovery to baseline LDF.

Blood pressure measurements. Blood pressure was measured by finger plethysmography (Finometer, Arnhem, Netherlands), intermittently recalibrated against oscillometry. Mean arterial pressure was obtained by averaging the signal over 5 min and compared with oscillometry [using the formula mean arterial pressure = (systolic arterial pressure + 2 × diastolic arterial pressure)/3], as previously described (39). Finometer and oscillometric blood pressure were in agreement.

General protocol. The experiments were performed on 2 separate days on the same subjects. The order of the experimental days was randomized. During each day, four microdialysis catheters were placed to infuse drugs locally into the intradermal space of the calf. Before the microdialysis catheter insertion, LDF was measured over each of the four insertion sites to estimate baseline flows for later use in determining when the area had recovered from the trauma of catheter insertion. Laser probes were removed, and the four microdialysis catheters were inserted. After recovery, LDF was measured while perfusing the catheters with lactated Ringer’s solution, and values were recorded for 10 min. Following this, LDF was recorded during local heating at each site with continued perfusion of lactated Ringer solution. A recovery from heat period followed, requiring 30–60 min. After recovery, catheters were perfused with drugs dissolved in lactated Ringer’s solution, and local heating was repeated. The sequence of events for both experimental days is shown in Fig. 1.

On 1 day, the effects on the heating response of ROS inhibition using tempol, a superoxide dismutase mimic (10 μM), xanthine oxidase inhibition using allopurinol (10 μM), NADPH oxidase inhibition using apocynin (100 μM) and H2O2 inhibition plus 15-lipoxygenase/cyclooxygenase inhibition using ebselen (100 μM) was evaluated by perfusing each of these drugs through individual microdialysis catheters dissolved in lactated Ringer’s solution. After a 30-min run-in period, local heating and heating recovery were recorded at all sites. Following this, maximum blood flow and conductance were elicited by perfusing 28 mM sodium nitroprusside through each of the four microdialysis catheters.

The concentrations of tempol, allopurinol, apocynin, and ebselen we initially used in preliminary trials were based on studies in animals (26, 27, 53) and humans (23). We then performed preliminary experiments to determine appropriate concentrations for these inhibitors. In all cases, the concentrations used were the minimum dose required to elicit a maximal response. In addition, preliminary determinations confirmed that none of the inhibitors used influenced the response of skin blood flow to SNP; thus they did not alter maximal cutaneous vascular conductance (CVC).

Fig. 1. Sequence of events for experimental maneuvers. SNP, sodium nitroprusside; Apo, apocynin; Allo, allopurinol; Ebs, ebselen; Temp, tempol.
Allopurinol was perfused through the second catheter, 10−5 M tempol was perfused through each catheter for 30 min while taking baseline measurements. The effects of ANG II on LDF. As shown in Table 2, 10 μM ANG II caused significant reductions in all aspects of the cutaneous response to the application of local heat compared with Ringer’s alone (P < 0.05). This inhibition was evident over the entire time course measured (not shown) and resulted in a significant reduction of baseline, first heat peak, nadir, and heat plateau.

Use of heat-reheat assessment. In these experiments, we employed a heat-reheat protocol where we measured the cutaneous response to locally applied heat up to three times, while allowing for the skin blood flow to return to “baseline” in between, as we have employed in previous studies (40, 41). The utility of performing heat-and-reheat comparisons has been reported in detail elsewhere (40, 41) and shows that the variability between the plateau phase of the local heating response during sequential heat-reheat in each catheter is significantly less than that between local heating plateaus of different catheters placed in the same subject. This is in contrast to another study that showed repeated heating of the skin can affect its responses (6).

Data and statistical analysis. Laser-Doppler skin blood flows were measured in arbitrary perfusion units (pfu). Continuous LDF data were collected at a sampling rate of 200 Hz during experiments. Data from the lasers were multiplexed and interfaced to a personal computer through a analog-to-digital converter (DI-720; Dataq, Milwaukee, WI) using custom data acquisition software. LDF data were converted to units of CVC by dividing by the mean arterial blood pressure.

CVC measurements were then converted to a percentage of maximum CVC (%CVCmax) by dividing CVC by the CVCmax achieved after the administration of 28 mM sodium nitroprusside at the end of experiments. This fraction was converted to a percentile by multiplication by 100. Conductance data are therefore displayed as %CVCmax. Changes in baseline LDF before and after drugs were compared by two-way ANOVA. Results are shown and reported as means ± SE.

Table 1. Magnitudes of heat responses

<table>
<thead>
<tr>
<th></th>
<th>Ringer’s (n = 8)</th>
<th>Drug (n = 8)</th>
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<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apocynin</td>
<td>10.31 ± 2.04</td>
<td>16.36 ± 5.25</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>11.50 ± 2.29</td>
<td>17.91 ± 4.60</td>
</tr>
<tr>
<td>Ebselen</td>
<td>12.73 ± 2.45</td>
<td>10.07 ± 2.22</td>
</tr>
<tr>
<td>Tempol</td>
<td>9.40 ± 1.49</td>
<td>9.29 ± 1.88</td>
</tr>
<tr>
<td><strong>First thermal peak</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apocynin</td>
<td>49.75 ± 4.18</td>
<td>49.29 ± 8.33</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>42.38 ± 4.49</td>
<td>63.77 ± 3.70*</td>
</tr>
<tr>
<td>Ebselen</td>
<td>50.24 ± 2.78</td>
<td>45.78 ± 3.16</td>
</tr>
<tr>
<td>Tempol</td>
<td>54.72 ± 6.67</td>
<td>59.92 ± 3.87</td>
</tr>
<tr>
<td><strong>Nadir</strong></td>
<td></td>
<td></td>
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<tr>
<td>Apocynin</td>
<td>29.80 ± 4.57</td>
<td>31.84 ± 8.37</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>23.23 ± 3.78</td>
<td>42.60 ± 1.89†</td>
</tr>
<tr>
<td>Ebselen</td>
<td>33.36 ± 2.26</td>
<td>29.87 ± 2.88</td>
</tr>
<tr>
<td>Tempol</td>
<td>33.48 ± 7.40</td>
<td>40.47 ± 4.38</td>
</tr>
<tr>
<td><strong>Plateau</strong></td>
<td></td>
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<tr>
<td>Apocynin</td>
<td>74.07 ± 6.01</td>
<td>58.25 ± 10.01</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>73.46 ± 2.49</td>
<td>84.54 ± 3.08‡</td>
</tr>
<tr>
<td>Ebselen</td>
<td>80.08 ± 2.70</td>
<td>60.89 ± 4.68†</td>
</tr>
<tr>
<td>Tempol</td>
<td>78.06 ± 5.42</td>
<td>76.67 ± 4.87</td>
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</tbody>
</table>

Values are means ± SE and are shown as percentage of maximal cutaneous vascular conductance (%CVCmax). *P < 0.01, significantly different from value with Ringer alone, comparing %CVCmax. †P < 0.05, significantly different from value with Ringer alone, comparing %CVCmax. ‡P < 0.002, significantly different from value with Ringer alone, comparing %CVCmax.

Table 2. Effect of 10 μM ANG II on the cutaneous response to local heat

<table>
<thead>
<tr>
<th></th>
<th>Heat + Ringer’s</th>
<th>Heat + ANG II</th>
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</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>11.91 ± 1.14</td>
<td>8.31 ± 0.73</td>
</tr>
<tr>
<td><strong>First heat peak</strong></td>
<td>56.18 ± 1.83</td>
<td>13.19 ± 2.53</td>
</tr>
<tr>
<td><strong>Nadir</strong></td>
<td>35.26 ± 2.07</td>
<td>22.70 ± 1.68</td>
</tr>
<tr>
<td><strong>Plateau</strong></td>
<td>87.65 ± 2.45</td>
<td>45.58 ± 3.07</td>
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</table>

Values are means ± SE and are shown as %CVCmax.
was midway between and significantly different from heat alone, or heat plus ANG II.

The effects of ANG II, with and without ebselen scavenging H$_2$O$_2$ and hydroperoxides, are shown in Fig. 3. In contrast to that seen with apocynin and allopurinol, the heat response with ebselen plus ANG II was intermediate to and significantly different from heat alone and heat with ANG II, when comparing the first heat peak and nadir. The heat plateau values however were the same as heat plus ANG II and significantly less than with heat alone.

Last, to evaluate the effect of scavenging superoxide and decrease the formation of hydroxyl radicals, we performed similar determinations as described above but instead perfused the microdialysis catheters with tempol, an SOD mimetic agent. As shown in Fig. 4, the response to local heat with tempol plus ANG II was the same as heat alone for baseline, the first heat peak, and nadir, and therefore improved compared with that measured with ANG II alone. In a fashion similar to that seen with apocynin and allopurinol, the heat plateau values for tempol plus ANG II were intermediate between heat alone and heat plus ANG II.

DISCUSSION

In previous studies of the cutaneous response to local heat, we have shown that ANG II exhibits vasoconstrictive effects (39–41) mediated through the actions of the AT1R through activation of local oxidases. The primary effect of ANG II is thought to occur through direct activation of NADPH oxidase (4, 10–12) and secondarily through increased ROS via xanthine oxidase because of NADPH oxidase activation of a redox-sensitive pathway (19). The vasoconstrictive response to ANG II is largely due to increased ROS formation especially H$_2$O$_2$ (4, 43, 52), and NO scavenging through the binding of ANG II to NADPH and xanthine oxidases producing peroxynitrite (28, 43). With AT1Rs blocked, ANG II caused NO-independent vasodilation in control subjects, and infusion of the antioxidant sodium ascorbate, delivered through microdialysis catheters, increased the NO-dependent heat plateau in healthy subjects. This suggested a role for reactive oxygen species in the control of the local heat response (41).

In normal volunteers, we showed that the addition of ANG II is capable of attenuating the response to local heat in the skin (39–41). ANG II significantly attenuated baseline (unheated) blood flow as well decreasing the response to local heat by decreasing the magnitude of the first thermal peak, the nadir, and the final heat plateau. While there were no statistical differences between the values for baseline blood flow measured with and without drugs, apocynin and allopurinol resulted in noticeably higher values of %CVC compared with Ringer’s. If substantiated in future determinations, these findings might suggest a role for ROS in cutaneous thermoregulation under normothermic conditions.

Interestingly, of the inhibitors tested, only allopurinol resulted in a significant increase in all three phases of the heat response.
response, but had no significant effect on baseline blood flow. Ebselen caused a significant decrease in plateau blood flow compared with the heat response measured in the absence of drugs. While all of these inhibitors seemed able to partially mitigate the vasoconstrictive effects of ANG II, ebselen was the least effective. Perhaps this is because ebselen alone decreased the plateau heat response itself, and therefore was unable to effectively reverse the attenuated heat response caused by ANG II. It is possible, therefore, that H$_2$O$_2$ could increase the response to local heat. This is consistent with recent studies that have shown that H$_2$O$_2$ can cause NO-independent relaxation of the vasculature. These responses have been shown capable of being modified by the presence of antioxidants (8).

Since ANG II is thought to work through AT1R-dependent activation of NADPH oxidase and xanthine oxidase causing increased production of ROS (7, 11, 19), these oxygen species likely play a role in the regulation of the local heat response. Modulation of these products of oxidative stress may cause alterations in the response of skin to local heat and could be responsible for abnormalities of skin blood flow such as those described in variants of POTS (39–41). In addition to vasodilation, the vasoconstrictor response of skin to local cooling has also been shown to involve ROS and stimulation of the RhoA-Rho kinase pathway (42).

The response to local skin heating is characterized by its biphasic nature, the first heat peak being due to C-fiber nociceptor-mediated axon reflex (21, 30, 35), which results in vasodilatation presumed to occur through the local release of calcitonin gene-related peptide (CGRP) (30, 45), substance P (3, 45), and neuropeptide Y (13). The second increase in skin blood flow from local heat, characterized by the heat plateau, has been shown to be largely dependent (~70%) on NO (16, 24, 25). This is in contrast to the cutaneous vasodilatory response to whole body heat, which is thought to be due to cotransmission of acetylcholine and as yet unidentified neurotransmitter(s) mediated through sympathetic stimulation (17). However, even in whole body heating, NO is required to produce maximal cutaneous vasodilatation in young subjects, is thought to be responsible for ~30% of maximal flow, and acts in conjunction with histamine and vasoactive intestinal peptide to afford active vasodilatation (14, 38, 47, 49).

NO serves to regulate skin blood flow in a variety of situations. The bioavailability of NO in the skin is a function of the balance between its synthesis and degradation. Increased ROS has been shown to decrease available cutaneous NO either directly (2, 43) or indirectly (14, 18), possibly influencing the ability of skin to vasodilate in response to local heat (14, 41). In the present studies, apocynin and allopurinol were able to partially reverse the ANG II-induced suppression of the response to local heat. These agents are inhibitors of NADPH oxidase and xanthine oxidase, respectively, and therefore can result in decreased local production of ROS, likely resulting in increased availability of NO which can potentiate vasodilatation.

The finding that allopurinol increased the heat plateau above that measured in the absence of drug suggests that local ROS, produced by xanthine oxidase, may play a normal regulatory role in cutaneous vasoregulation. This observation is unique as it is thought that the response to ANG II elicits its effects largely through direct effects on NADPH oxidase (4, 11, 12, 44). However, some support for the role of xanthine oxidase is suggested by studies which have shown that ANG II can elicit an effect by causing NADPH-dependent changes in redox potential that result in increased local xanthine oxidase activity and augmented superoxide production (19).

Ebselen, a glutathione peroxidase mimetic, can result in lowered oxidative stress by decreasing local production of H$_2$O$_2$, which as above, may increase the availability of local NO, thus reversing the ANG II-induced suppression of the local heat response. Ebselen alone decreased the plateau heat value compared with the response in the absence of drug; the reason for this may be due to its additional actions as a lipoxygenase inhibitor and modulation of vasodilatory prosta-
glandins (34).

Tempol is also capable of reversing this response to local heat but is likely mediated by its ability as an SOD mimetic to decrease local ROS availability by promoting catalysis to other species (46). Superoxide decreases NO directly, or through production of peroxynitrite can uncouple enzymatic production of NO through oxidation of BH4 (2), possibly causing decreased NO and a blunted response to heat. Superoxide dismutase can also generate hydroxyl radicals and H$_2$O$_2$ (7), which themselves may be capable of affording effects. Therefore the effect of Tempol may be due to many factors.

A recent study has shown that TRPV-1 channel activation contributes to the axon reflex component (first heat peak) of local cutaneous heating. These investigators also showed that TRPV-1 channel activation directly contributes to the plateau phase as well (48). These data suggest activation of TRPV-1 channels, which are putative channels primarily located on cutaneous sensory nerves, may constitute a mechanism by which cutaneous sensory nerves are depolarized during local heating of the skin, and thus act as a local heat sensor. Numerous studies have demonstrated the effects of ROS on ion channel activity (5, 15, 37) and its effect on transmembrane potential (32, 37). Modification of ROS can result in alteration of vascular responsiveness and of blood pressure mediated centrally (7, 20, 29), directly within the kidney (9, 51), and at the level of local vasculature (7, 31, 33). Therefore, ROS may possibly have a direct effect on the TRPV-1 channel activity as well, and thus modulate the response to local heat. These findings are consistent with several recent studies which have shown that exogenous antioxidants can reverse the effects of aging on the response to both local heat (14) and cooling (50).

Thus, by showing that the response of skin to local heat, which is largely influenced by NO, can be influenced by products of oxidative stress, we suggest that endogenous ROS may play a regulatory role in local thermoregulation. This is based on our current findings, as well as those which have shown that locally administered ascorbate (14, 41, 50) can modify local cutaneous vasodilatation. This control may be afforded through two different mechanisms: the first through local vascular modification of NO by ROS (7, 31, 33), and the second through modulation of the response of local ion channels to heat by ROS. Together, these intermediaries of oxidative metabolism may subserve the function of controlling local vascular reactivity. Studies extending these observations in subjects with altered cutaneous responses, such as those with low-flow POTS are warranted, to evaluate which of these control mechanisms may be involved in altered hemodynamics measured in these subjects.
**Limitations.** We studied women without regard to menstrual cycle. The phase of the menstrual cycle can affect NO-dependent mechanisms and may also exert influence on ROS-dependent functions (36).

We used exogenous ANG II; however, endogenous angiotensin was not measured. While many tissues produce ANG II, there are no data from skin. However, data from skeletal muscle microvessels suggest local concentrations on the order of 100 pmol/l (41), this is far less than the lowest dose of ANG II administered in current experiments.

Microdialysis is invasive and alters the interstitial milieu. The work of Anderson et al. suggests that flow responses return to baseline levels within ~1 h (1). In pilot experiments, we measured baseline flows, removed the LDF probes, instrumented the same site with microdialysis catheters, replaced the probes, waited ~1 h, and repeated the LDF measurements with similar results (on average). The present studies utilized our heat-reheat protocol, which yields reproducible results (40, 41). Other investigators however have reported that repeated heating of the skin can affect its responses (6). This discrepancy is likely due to the numerous methodological differences between their study design and ours, which included use of a different anatomic site and a dissimilar source of heat and laser measuring device.

We were also concerned that the values for %CVCmax recorded in these studies during the nadir phase were lower than those reported by other investigators. While this may be due in part to variability among subjects, it may also be related to our use of the lower leg as the site for investigation, compared with the forearm which is more commonly used (14, 17, 24, 25).

One additional limitation of these studies is that they only inform on the vasoregulatory response of skin to the application of local heat. Therefore any speculation regarding the role of ROS in modulation or influencing skin blood flow should be considered within this context and may not be applicable to whole body response to heat.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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