Tempol improves cutaneous thermal hyperemia through increasing nitric oxide bioavailability in young smokers

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Abstract

We recently found that young cigarette smokers display cutaneous vascular dysfunction relative to non-smokers, which is partially due to reduced nitric oxide (NO) synthase (NOS)-dependent vasodilation. In this study, we tested the hypothesis that reducing oxidative stress improves NO bioavailability, enhancing cutaneous vascular function in young smokers. Ten healthy young male smokers, who had smoked for 6.3±0.7 years with an average daily consumption of 9.1±0.7 cigarettes, were tested. Cutaneous vascular conductance (CVC) during local heating to 42ºC at a rate of 0.1ºC/sec was evaluated as laser-Doppler flux divided by mean arterial blood pressure and normalized to maximal CVC, induced by local heating to 44ºC plus sodium nitroprusside administration. We evaluated plateau CVC during local heating, which is known to be highly dependent on NO, at four intradermal microdialysis sites: 1) Ringer’s (control), 2) 10μM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), a superoxide dismutase mimetic, 3) 10mM Nω-Nitro-L-arginine (L-NNA), a non-specific NOS inhibitor, and 4) a combination of 10μM Tempol and 10mM L-NNA. Tempol increased the plateau CVC compared with the Ringer’s site (90.0±2.3 vs. 77.6±3.9%max, P=0.028). Plateau CVC at the combination site (56.8±4.5%max) was lower than the Ringer’s site (P<0.001), and was not different from the L-NNA site (55.1±4.6%max) (P=0.978), indicating the Tempol effect was exclusively NO-dependent. These data suggest that in young smokers, reducing oxidative stress improves cutaneous thermal hyperemia to local heating by enhancing NO production.

Key words: tobacco, reactive oxygen species, free radicals, microcirculation, skin,
Almost 6 million people die from tobacco use and exposure each year (53), and the majority of tobacco-related deaths are due to cardiovascular disease (11). Indeed, chronic exposure to cigarette smoking changes the structure and function of human conduit arteries (45). Oxidative stress is suspected to be a major contributor to chronic cigarette smoking-induced vascular alterations, as reducing oxidative stress with antioxidants (e.g., vitamin C) in smokers improves conduit artery vascular function, as evaluated by non-invasive flow-mediated dilation (FMD) (41, 43, 50) or by intra-arterial administration of the endothelium-dependent vasodilators, such as acetylcholine (ACh) (17-19) and bradykinin (17). Furthermore, antioxidant-induced improvements in conduit artery vascular function in smokers are not observed when administered in conjunction with nitric oxide (NO) synthase (NOS) inhibition (31), suggesting that oxidative stress impairs conduit artery function by reducing NO bioavailability.

In addition to human conduit artery function, chronic cigarette smoking impairs function of the human microcirculation, such as the skin (9, 10, 14, 42). Given that microvascular dysfunction is a crucial step in the complications that lead to cardiovascular disease (1, 34, 38), exploring the mechanistic underpinnings of impaired microvascular function in smokers is important; however, few investigators have studied this issue. We recently reported that young smokers have an impaired cutaneous vasodilatory response to administration of ACh compared with non-smokers, which was partially due to attenuated NOS-dependent vasodilation (14). Given that oxidative stress reduces NO bioavailability in the conduit arteries of smokers (31), reducing oxidative stress may also improve NOS-dependent vasodilation in the cutaneous microcirculation.
of smokers, thereby enhancing vascular function. However, this has not been directly tested.

Using the above information as background, we hypothesized that Tempol (a superoxide dismutase mimetic) would improve cutaneous vascular function through enhancing NOS-dependent vasodilation in young smokers. As a test of cutaneous vascular function, we evaluated cutaneous thermal hyperemia to local heating to 42ºC at a rate of 0.1ºC/sec. This test was selected as plateau vasodilation during local heating is predominantly (~50-70%) mediated by NO (3, 5, 13, 20, 32, 39, 40, 49).

Materials and Methods

Subjects. This study was approved by the Institutional Review Board at The University of Oregon and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written informed consent was obtained from all subjects prior to their participation in the study. Smokers were defined as having smoked for at least 1 year with an average daily cigarette consumption ≥ 6. We recruited ten healthy young (19-26 years of age) smokers who had no history of hypertension, heart disease, diabetes, or autonomic disorders. This is important since advanced age (22, 26, 35, 40), hypertension (46), and disease status (e.g., postural tachycardia syndrome) (47, 48) are known to independently impair skin microvascular function. All subjects were not currently taking prescription medications. All subjects abstained from taking over-the-counter medications (including non-steroidal anti-inflammatory agents and vitamins), alcohol, and caffeine for at least 24h before the study. They also refrained from heavy exercise the night before the study, and cigarette smoking for at least 12 h before the
study to avoid any acute effects of cigarette smoking on skin blood flow regulation (9, 28, 52).

Instrumentation: Upon arrival at the laboratory, subjects voided their bladder and body weight and height were measured. Subjects were placed in a semi-recumbent position and instrumented with four microdialysis fibers (MD2000, Bioanalytical Systems, West Lafayette, IN, USA) (30KDa cutoff, 10mm membrane) on the ventral side of the forearm in the dermal layer of the skin. A 25-gauge needle was first inserted into the unanesthetized skin using aseptic technique with at least 4.0cm between each site. The entry and exit points were about ~2.5cm apart. The microdialysis fiber was then threaded through the lumen of the needle, after which the needle was withdrawn leaving the fiber in place. Microdialysis fibers were secured with tape. Lactated Ringer’s solution was perfused through each microdialysis fiber at a rate of 2.0µl/min (CMA 1025 microdialysis pump, CMA Microdialysis AB, Kista, Sweden) until the start of drug infusions (see below).

Experimental protocol: Once the trauma caused by microdialysis fiber placement had dissipated (~60-90min), the experimental protocol began. Microdialysis fibers were randomly assigned to receive 1) Ringer’s (control), 2) 10µM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, EMD Millipore Chemicals, Billerica, MA, USA) to reduce superoxide (O$_2^-$), 3) 10mM No-Nitro-L-arginine (L-NNA; Sigma-Aldrich Co., St. Louis, MO, USA) to non-selectively inhibit NOS and thus NO production, and 4) 10µM Tempol plus 10 mM L-NNA. Drug concentrations were selected as the minimum dose required for maximal effects, as reported in previous studies (36, 37). All pharmacological agents were dissolved in lactated Ringer’s solution. All drugs were
infused continuously at a rate of 2.0µl/min (CMA 102 Microdialysis Pump; CMA Microdialysis AB, Kista, Sweden) until the end of local heating to 42°C. To ensure adequate drug effects, all pharmacological agents were perfused for at least 75min before the start of local heating.

Following 75+min of drug infusion, baseline was recorded for at least 10min while skin temperature was held constant at 33°C (Skin Heater/Temperature Monitor SHO2, Moor Instruments, Devon, UK). Thereafter, local heating of the skin to 42°C at a rate of 0.1°C/sec was applied to all skin sites to induce cutaneous vasodilation. Once skin vasodilation reached a plateau (25-35min after initiation of heating), local skin temperature was further elevated to 44°C at a rate of 0.1°C/sec with an administration of 56mM sodium nitroprusside (SNP; Nitropress, Ciba Pharmaceuticals, East Hanover, NJ, USA) at a rate of 2.0µl/min to achieve maximal vasodilation.

Measurements: Arterial blood pressure was measured via automated brachial oscillation (Dinamap ProCare 100, GE Medical Systems, Tampa, FL, USA) throughout the protocol. Mean arterial blood pressure (MAP) was calculated as diastolic arterial blood pressure plus one-third pulse pressure. To obtain an index of skin blood flow, cutaneous red blood cell flux was measured with a single-point laser-Doppler flowmetry probe seated in the center of the local heater over each microdialysis fiber. Cutaneous vascular conductance (CVC) was evaluated as cutaneous red blood cell flux divided by MAP. All CVC data were expressed as percentage of maximal CVC to minimize the effect of site-to-site heterogeneity in the level of skin blood flow (38). Data were recorded and stored on a computer using Windaq data acquisition software (Dataq Instruments, Akron, OH). Figure 1 displays the CVC response to local heating, averaged
across all subjects, which was characterized as follows. Baseline CVC was determined by taking an average CVC at least over 3 min before heating. Upon initiation of local heating, CVC rapidly increased and exhibited an initial peak. Then following a brief nadir, CVC gradually increased and reached a stable plateau. The initial peak and nadir CVC were determined by taking averaged CVC over 30 sec, and the plateau and maximal CVC were determined from averaged CVC over at least 2 min. We evaluated the difference in plateau CVC between the Tempol and Tempol plus L-NNA sites as an index of NOS-dependent vasodilation with Tempol. Similarly, the difference in plateau CVC between the Ringer’s and L-NNA sites was evaluated as an index of NOS-dependent vasodilation without Tempol.

Statistical analyses: A two-way repeated measures analysis of variance (ANOVA) was conducted with factors of drug (Ringer’s, Tempol, L-NNA, and combination of Tempol and L-NNA) and phase of response (baseline, initial peak, nadir, plateau, and maximal periods) for absolute (mV/MAP • 100) and relative (%max) CVC. We employed two-way ANOVA rather than one-way ANOVA to consider a potential interaction between drug and phase of response. When a significant main effect or interaction was detected, significant differences between paired variables across drug sites were determined by Tukey’s honestly significant difference post hoc test. Two-tailed paired t-test was used to compare the difference in plateau CVC between the Tempol and Tempol plus L-NNA sites with the difference in plateau CVC between the Ringer’s and L-NNA sites. The level of significance was set at 0.05. Values are presented as mean± standard error (SE).


**Results**

*Characteristics of subjects.* The subjects were 22.5±0.7 years of age, with an average body mass index of 23.8±0.8kg·m$^{-2}$. They had smoked for 6.3±0.7 years with an average daily consumption of 9.1±0.7 cigarettes. Their systolic, diastolic, and mean arterial pressures were 112.9±2.7mmHg, 63.9±1.8mmHg, and 80.2±1.7mmHg, respectively. Note that their body mass indices and arterial blood pressures were within healthy ranges.

*Cutaneous variables.* There was an interaction between drug and phase of response on CVC represented as both absolute or %max value (both P<0.001). Plateau CVC at the Tempol site was greater than that at the Ringer’s site (Figure 1). Plateau CVC at the L-NNA site was reduced relative to the Ringer’s site (Figure 1). Plateau CVC at the site which received combined Tempol and L-NNA was lower compared with the Ringer’s site, and was not different from the value at the L-NNA site (P=0.978) (Figure 1). The difference in plateau CVC between the Tempol and Tempol plus L-NNA sites tended to be higher compared with the difference in plateau CVC between the Ringer’s and L-NNA sites (31.5±4.1 vs. 19.2±6.7%max, P=0.163).

Baseline CVC at the Ringer’s site did not differ from that at the Tempol (P=0.999), L-NNA (P=0.948), and Tempol+L-NNA (P=0.999) sites (Figure 1). Initial peak CVC at the Ringer’s site was not different from that at the Tempol (P=0.455), L-NNA (P=0.095), and Tempol+L-NNA (P=0.250) sites (Figure 1). Absolute maximal CVC (unit, mV/MAP·100) at the Ringer’s site (246±25) was not different from the Tempol (314±52, P=0.180), L-NNA (277±34, P=0.790), and Tempol+L-NNA (292±22, P=0.514) sites. Based on these data, we calculated the minimum sample sizes...
required to produce a significant level of 0.05 with 80% power, which demonstrated we
would need 18 subjects for the difference in baseline CVC between the Ringer’s and L-
NNA sites to be significant, 28 subjects for the difference in initial peak CVC between
the Ringer’s and Tempol sites to be significant, 28 subjects for the difference in absolute
maximal CVC between the Ringer’s and Tempol sites to be significant, and 23 subjects
for the difference in absolute maximal CVC between the Ringer’s and Tempol+L-NNA
sites to be significant. Relative to the Ringer’s site, nadir CVC at the Tempol site was
higher, while that at the L-NNA and combination sites were lower (Figure 1)

Discussion

We are the first to investigate how Tempol, a superoxide dismutase mimetic,
affects the cutaneous vascular response to local heating in young smokers. We also
employed Nω-Nitro-L-arginine (L-NNA, NOS inhibitor) to evaluate whether Tempol-
induced improvements in microvascular function was through improved NO
bioavailability. Our main findings were that 1) Tempol enhanced the plateau phase of
cutaneous vasodilation to local heating to 42°C, 2) the plateau at the combination site
(Tempol and L-NNA) was lower than the Ringer’s site, but was comparable to the L-
NNA site. These results suggest that in young smokers, reducing oxidative stress in the
microvasculature improves cutaneous thermal hyperemia to local heating through NO-
dependent mechanisms.

Oxidative stress. Accumulating evidence supports the concept that smokers have
impaired cutaneous vascular function compared with non-smokers (9, 10, 14, 42). In line
with this, plateau CVC in the young smokers of the present study was attenuated
compared with that in the young non-smokers of previous studies in which the same heating protocol was employed (Table 1) (3, 5, 16, 21, 33, 49, 54). In the present study, we found that Tempol significantly improved plateau CVC compared with the Ringer’s site (Figure 1), up to a similar level as the plateau in those same studies in non-smokers (~90%), suggesting that oxidative stress is a major factor contributing to the impaired cutaneous vascular function in young smokers.

On the other hand, in healthy non-smokers, antioxidants such as vitamin C do not affect vascular function, as evaluated by FMD or ACh-induced vasodilation in forearm conduit arteries (17, 19, 41, 43), as well as by the cutaneous vasodilatory response during whole-body heating at rest (23, 25). More relevant to the present study, Medow et al. (36) showed in healthy young non-smokers that Tempol did not affect plateau CVC during the same local heating protocol as was used in the present study. The lack of an effect of antioxidants in healthy, non-smoking subjects is not surprising, as healthy humans are expected not to have significant oxidative stress. Medow et al. (36) further showed that Tempol restored plateau CVC when oxidative stress was induced in healthy non-smokers by infusing angiotensin II, thus suggesting an antioxidative role of Tempol during local heating. However, it should be considered that Tempol may have non-antioxidative effects. For example, Tempol-mediated opening of ATP-sensitive potassium (K\textsubscript{ATP}) channels has been reported in rats with systemic MAP changes (8) and opening of calcium-activated potassium (K\textsubscript{Ca}) channels has been reported in mesenteric arterial smooth muscle of rats (55). However, these effects are unlikely to have occurred in the present study focusing on human skin blood flow regulation in smokers as no NO-independent Tempol effects were observed, as discussed below.
**NOS pathway.** An impaired NOS-dependent cutaneous vasodilation in smokers is suggested by our previous study, which employed ACh administration (14), and by the fact that, in the present study, NOS inhibition reduced plateau CVC during local heating to a lesser extent than what has previously been reported in healthy young non-smokers (19 vs. 33-72 %max) (3, 5, 13, 39, 40). Given that oxidative stress generally reduces NO bioavailability, it is plausible that in the present study, plateau CVC was improved with Tempol administration through restoring NOS-dependent vasodilation. This notion is strongly supported by our observation that there was no difference in plateau CVC between the L-NNA and Tempol+L-NNA sites (Figure 1). Also, the difference in plateau CVC between the Tempol and Tempol+L-NNA sites (an index of NOS-dependent vasodilation with Tempol) tended to be higher as compared to the difference in plateau CVC between the Ringer’s and L-NNA sites (an index of NOS-dependent vasodilation without Tempol) (31.5±4.1 vs. 19.2±6.7%max, P=0.163).

**Possible mechanism(s) for how Tempol improves NO bioavailability.** Cigarette smoking causes oxidative stress as a direct effect of the compounds within cigarette smoke itself (44). For example, the semiquinone radical in the cigarette tar yields O$_2$•\(^{-}\) (44). Additionally, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces O$_2$•\(^{-}\), is directly activated by both nicotine, as shown in rat pial arterioles (12), and by stable thiol-reactive agent, as indicated in bovine, human and rat pulmonary arteries (29). O$_2$•\(^{-}\) easily binds with NO to produce peroxynitrite (ONOO\(^{-}\)), thus reducing NO bioavailability. Additionally, ONOO\(^{-}\) depletes tetrahydrobiopterin (BH$_4$), an essential cofactor for endothelial NOS (eNOS). This results in an increase in uncoupled eNOS, which then procures O$_2$•\(^{-}\) instead of NO (51), further reducing NO bioavailability. By
removing O$_2^-$, preventing it from binding with NO, and reducing uncoupled eNOS,

Tempol leads to higher NO bioavailability and thus improved plateau CVC. Moreover, the reaction of O$_2^-$ and Tempol results in the production of hydrogen peroxide (H$_2$O$_2$), which may be another mechanism by which Tempol improves plateau CVC. For example, scavenging of H$_2$O$_2$ with ebselen attenuates plateau CVC during local heating (36). Additionally, H$_2$O$_2$ can activate K$_{Ca}$ channels, as shown in vascular smooth muscle of pig coronary arteries (2). Vasodilation via K$_{Ca}$ channels contributes substantially to the plateau (5).

**Limitations.** Tempol scavenges O$_2^-$, but not other reactive oxygen species. Other reactive oxygen species, such as H$_2$O$_2$ and hypochlorite (HOCl) may reduce NO bioavailability, as reported in porcine aortic endothelial cells (30), thus contributing to attenuated plateau CVC during local heating in the skin of young smokers.

Only male subjects were included in this study. Thus, our conclusions cannot be applied to females. The female sex hormones may be cardio-protective against the effects of chronic smoking, as reflected by the fact that carotid and femoral artery wall thickness is greater (15) and conduit artery FMD is lower (6) in male smokers, but not in female smokers compared with former or never smokers. Furthermore, the female sex hormones enhance cutaneous thermal hyperemia to local heating (4, 7). Therefore, the effects of chronic cigarette smoking on cutaneous thermal hyperemia may be different between males and females and/or may be modulated by the levels of the female sex hormones. Further studies are warranted to address these issues.

Absolute maximal CVC at the Tempol and Tempol+L-NNA sites tended to be higher relative to that at the Ringer’s site, though it was not significant due to limited
Reduced maximal cutaneous vasodilatory capacity has been reported in young (14) and older (10) smokers relative to non-smoking counterparts. Our results suggest this may be due to oxidative stress, but further studies are required to flush that out.

For this study we chose not to study a subset of non-smokers, based on the number of studies showing no benefit of antioxidant administration on vascular responses in healthy, young non-smokers. Although doing so would have allowed us to make comparisons between smokers and non-smokers, we decided to specifically focus this study on investigating whether Tempol would improve cutaneous vascular function in young smokers.

**Perspectives.** Microvascular dysfunction may be a crucial step in the complications leading to cardiovascular disease, and can be detected in the early stages of disease progression in the cutaneous circulation (24, 27, 38). The present study shows that impaired cutaneous microvascular function in young smokers is caused by oxidative stress in a similar fashion as is observed in aging (25). As such, chronic cigarette smoking has been suggested to cause a premature aging effect. Based on our results, we speculate that, reducing oxidative stress in young smokers may potentially reduce the premature aging effect of chronic cigarette smoking on microvascular function, which in turn, may prevent or delay smoking-related cardiovascular disease and mortality.

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Disclosures

None.

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**Figure Legend**

**Figure 1:** Averaged time-course changes in cutaneous vascular conductance during local heating. Baseline, initial peak, nadir, and plateau cutaneous vascular conductance obtained from individuals were averaged and compared across the four drug sites. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a superoxide dismutase mimetic; L-NNA (N\(^{\gamma}\)-nitro-L-arginine), a non-specific nitric oxide synthase inhibitor; Time 0 indicates initiation of local heating.
Figure 1

![Graph showing cutaneous vascular conductance over time into heating for different conditions with statistical annotations for initial peak, nadir, plateau, baseline, and significance levels (P-values). The legend includes symbols for Ringer's, Tempol, L-NNA, and Tempol+L-NNA.](image)

- Initial peak: N=10, P=0.028
- Nadir: P<0.001
- Plateau: P<0.001
- Baseline: P=0.002
- Tempol: P=0.032
- L-NNA: P<0.001
- Tempol+L-NNA: P<0.001
Table 1  Comparison of averaged plateau cutaneous vascular conductance (CVC) during local heating at the control site between young smokers and non-smokers

<table>
<thead>
<tr>
<th>Subject group</th>
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<th>Plateau CVC (%)</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>Young smokers</td>
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<td></td>
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<tr>
<td>Young non-smokers</td>
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<td>86-90</td>
<td>at a rate of 0.5 ºC/10sec</td>
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<td></td>
<td>Brunt &amp; Minson (2012)</td>
<td>84-88</td>
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<tr>
<td></td>
<td>Kellogg et al. (2008)</td>
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<td>local heating to 41.5ºC at a rate of 0.6ºC/min</td>
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<tr>
<td></td>
<td>Stewart et al. (2008)</td>
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All of the above studies employed local heating to 42 ºC at a rate of 0.1ºC/sec unless otherwise indicated.