Articles in PresS. Am J Physiol Heart Circ Physiol (March 28, 2014). doi:10.1152/ajpheart.00886.2013

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

Naoto Fujii[1], Vienna E. Brunt[1], Christopher T. Minson[1]

[1]The University of Oregon, Department of Human Physiology, Eugene, OR, United States

Running head: oxidative stress & cutaneous local heating in young smokers

Corresponding Author information:

Christopher. T. Minson, Ph.D

University of Oregon,

Department of Human Physiology,

1240 University of Oregon, Esslinger Hall, Eugene, OR, 97403-1240

E-mail: minson@uoregon.edu

Telephone: 541 346-4311

Fax: 541 346-2841

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,

Introduction

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

88 study to avoid any acute effects of cigarette smoking on skin blood flow regulation (9, 28,
89 52).

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97 threaded through the lumen of the needle, after which the needle was withdrawn leaving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

102 *Experimental protocol:* Once the trauma caused by microdialysis fiber placement
103 had dissipated (~60-90min), the experimental protocol began. Microdialysis fibers were
104 randomly assigned to receive 1) Ringer's (control), 2) 10μM 4-hydroxy-2,2,6,6-
105 tetramethylpiperidine-1-oxyl (Tempol, EMD Millipore Chemicals, Billerica, MA, USA)
106 to reduce superoxide ($O_2^{\bullet-}$), 3) 10mM Nω-Nitro-L-arginine (L-NNA; Sigma-Aldrich Co.,
107 St. Louis, MO, USA) to non-selectively inhibit NOS and thus NO production, and 4)
108 10μM Tempol plus 10 mM L-NNA. Drug concentrations were selected as the minimum
109 dose required for maximal effects, as reported in previous studies (36, 37). All
110 pharmacological agents were dissolved in lactated Ringer's solution. All drugs were

111 infused continuously at a rate of 2.0µl/min (CMA 102 Microdialysis Pump; CMA
112 Microdialysis AB, Kista, Sweden) until the end of local heating to 42ºC. To ensure
113 adequate drug effects, all pharmacological agents were perfused for at least 75min before
114 the start of local heating.
115 Following 75+min of drug infusion, baseline was recorded for at least 10min
116 while skin temperature was held constant at 33ºC (Skin Heater/Temperature Monitor
117 SHO2, Moor Instruments, Devon, UK). Thereafter, local heating of the skin to 42ºC at a
118 rate of 0.1ºC/sec was applied to all skin sites to induce cutaneous vasodilation. Once skin
119 vasodilation reached a plateau (25-35min after initiation of heating), local skin
120 temperature was further elevated to 44ºC at a rate of 0.1ºC/sec with an administration of
121 56mM sodium nitroprusside (SNP; Nitropress, Ciba Pharmaceuticals, East Hanover, NJ,
122 USA) at a rate of 2.0µl/min to achieve maximal vasodilation.
123 *Measurements:* Arterial blood pressure was measured via automated brachial
124 oscillation (Dinamap ProCare 100, GE Medical Systems, Tampa, FL,USA) throughout
125 the protocol. Mean arterial blood pressure (MAP) was calculated as diastolic arterial
126 blood pressure plus one-third pulse pressure. To obtain an index of skin blood flow,
127 cutaneous red blood cell flux was measured with a single-point laser-Doppler flowmetry
128 probe seated in the center of the local heater over each microdialysis fiber. Cutaneous
129 vascular conductance (CVC) was evaluated as cutaneous red blood cell flux divided by
130 MAP. All CVC data were expressed as percentage of maximal CVC to minimize the
131 effect of site-to-site heterogeneity in the level of skin blood flow (38). Data were
132 recorded and stored on a computer using Windaq data acquisition software (Dataq
133 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 3min 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 30sec, 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).

157 **Results**

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

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

173 Baseline CVC at the Ringer's site did not differ from that at the Tempol
174 ($P=0.999$), L-NNA ($P=0.948$), and Tempol+L-NNA ($P=0.999$) sites (Figure 1). Initial
175 peak CVC at the Ringer's site was not different from that at the Tempol ($P=0.455$), L-
176 NNA ($P=0.095$), and Tempol+L-NNA ($P=0.250$) sites (Figure 1). Absolute maximal
177 CVC (unit, mV/MAP・100) at the Ringer's site (246±25) was not different from the
178 Tempol (314±52, $P=0.180$), L-NNA (277±34, $P=0.790$), and Tempol+L-NNA (292±
179 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 42ºC, 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_{ATP}) channels has been reported in rats with systemic MAP changes (8) and opening of calcium-activated potassium (K_{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.

226 *NOS pathway*. An impaired NOS-dependent cutaneous vasodilation in smokers is
227 suggested by our previous study, which employed ACh administration (14), and by the
228 fact that, in the present study, NOS inhibition reduced plateau CVC during local heating
229 to a lesser extent than what has previously been reported in healthy young non-smokers
230 (19 vs. 33-72 %max) (3, 5, 13, 39, 40). Given that oxidative stress generally reduces NO
231 bioavailability, it is plausible that in the present study, plateau CVC was improved with
232 Tempol administration through restoring NOS-dependent vasodilation. This notion is
233 strongly supported by our observation that there was no difference in plateau CVC
234 between the L-NNA and Tempol+L-NNA sites (Figure 1). Also, the difference in
235 plateau CVC between the Tempol and Tempol+L-NNA sites (an index of NOS-
236 dependent vasodilation with Tempol) tended to be higher as compared to the difference
237 in plateau CVC between the Ringer's and L-NNA sites (an index of NOS-dependent
238 vasodilation without Tempol) (31.5±4.1 vs. 19.2±6.7%max, P=0.163).
239 *Possible mechanism(s) for how Tempol improves NO bioavailability*. Cigarette
240 smoking causes oxidative stress as a direct effect of the compounds within cigarette
241 smoke itself (44). For example, the semiquinone radical in the cigarette tar yields $O_2^{\bullet-}$
242 (44). Additionally, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,
243 which produces O2•-, is directly activated by both nicotine, as shown in rat pial arterioles
244 (12), and by stable thiol-reactive agent, as indicated in bovine, human and rat pulmonary
245 arteries (29). $O_2^{\bullet-}$ easily binds with NO to produce peroxynitrite ($ONOO^-$), thus reducing
246 NO bioavailability. Additionally, $ONOO^-$ depletes tetrahydrobiopterin (BH_4), an essential
247 cofactor for endothelial NOS (eNOS). This results in an increase in uncoupled eNOS,
248 which then procures $O_2^{\bullet-}$ instead of NO (51), further reducing NO bioavailability. By

249 removing $O_2^{\bullet-}$, preventing it from binding with NO, and reducing uncoupled eNOS,
250 Tempol leads to higher NO bioavailability and thus improved plateau CVC. Moreover,
251 the reaction of $O_2^{\bullet-}$ and Tempol results in the production of hydrogen peroxide (H_2O_2),
252 which may be another mechanism by which Tempol improves plateau CVC. For
253 example, scavenging of H_2O_2 with ebselen attenuates plateau CVC during local heating
254 (36). Additionally, H_2O_2 can activate K_{Ca} channels, as shown in vascular smooth muscle
255 of pig coronary arteries (2). Vasodilation via K_{Ca} channels contributes substantially to the
256 plateau (5).

257 *Limitations.* Tempol scavenges $O_2^{\bullet-}$, but not other reactive oxygen species. Other
258 reactive oxygen species, such as H_2O_2 and hypochlorite (HOCl) may reduce NO
259 bioavailability, as reported in porcine aortic endothelial cells (30), thus contributing to
260 attenuated plateau CVC during local heating in the skin of young smokers.

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

270 Absolute maximal CVC at the Tempol and Tempol+L-NNA sites tended to be
271 higher relative to that at the Ringer's site, though it was not significant due to limited

sample size. 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.

Acknowledgements

We sincerely acknowledge the volunteer subjects in the present study.

Grants

This study was supported by the National Institutes of Health Grant HL081671. N. Fujii is the recipient of a research fellowship from the Uehara Memorial Foundation.

Disclosures

None.

References

1. **Antonios TF, Rattray FM, Singer DR, Markandu ND, Mortimer PS, and MacGregor GA**. Rarefaction of skin capillaries in normotensive offspring of individuals with essential hypertension. *Heart* 89: 175-178, 2003.
2. **Barlow RS, and White RE**. Hydrogen peroxide relaxes porcine coronary arteries by stimulating BKCa channel activity. *Am J Physiol* 275: H1283-1289, 1998.
3. **Bruning RS, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL, and Holowatz LA**. Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin. *J Appl Physiol* 112: 2019-2026, 2012.
4. **Brunt VE, Miner JA, Meendering JR, Kaplan PF, and Minson CT**. 17-beta estradiol and progesterone independently augment cutaneous thermal hyperemia but not reactive hyperemia. *Microcirculation* 18: 347-355, 2011.
5. **Brunt VE, and Minson CT**. KCa channels and epoxyeicosatrienoic acids: major contributors to thermal hyperaemia in human skin. *J Physiol* 590: 3523-3534, 2012.
6. **Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, and Deanfield JE**. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* 88: 2149-2155, 1993.
7. **Charkoudian N, Stephens DP, Pirkle KC, Kosiba WA, and Johnson JM**. Influence of female reproductive hormones on local thermal control of skin blood flow. *J Appl Physiol* 87: 1719-1723, 1999.
8. **Chen X, Patel K, Connors SG, Mendonca M, Welch WJ, and Wilcox CS**. Acute antihypertensive action of Tempol in the spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol* 293: H3246-3253, 2007.
9. **Dalla Vecchia L, Palombo C, Ciardetti M, Porta A, Milani O, Kozakova M, Lucini D, and Pagani M**. Contrasting effects of acute and chronic cigarette smoking on skin microcirculation in young healthy subjects. *J Hypertens* 22: 129-135, 2004.
10. **Edvinsson ML, Andersson SE, Xu CB, and Edvinsson L**. Cigarette smoking leads to reduced relaxant responses of the cutaneous microcirculation. *Vasc Health Risk Manag* 4: 699-704, 2008.

11. **Ezzati M, and Lopez AD**. Estimates of global mortality attributable to smoking in 2000. *Lancet* 362: 847-852, 2003.
12. **Fang Q, Sun H, Arrick DM, and Mayhan WG**. Inhibition of NADPH oxidase improves impaired reactivity of pial arterioles during chronic exposure to nicotine. *J Appl Physiol (1985)* 100: 631-636, 2006.
13. **Fieger SM, and Wong BJ**. Adenosine receptor inhibition with theophylline attenuates the skin blood flow response to local heating in humans. *Exp Physiol* 95: 946-954, 2010.
14. **Fujii N, Reinke MC, Brunt VE, and Minson CT**. Impaired acetylcholine-induced cutaneous vasodilation in young smokers: roles of nitric oxide and prostanoids. *Am J Physiol Heart Circ Physiol* 304: H667-673, 2013.
15. **Gariepy J, Denarie N, Chironi G, Salomon J, Levenson J, and Simon A**. Gender difference in the influence of smoking on arterial wall thickness. *Atherosclerosis* 153: 139-145, 2000.
16. **Greaney JL, Dupont JJ, Lennon-Edwards SL, Sanders PW, Edwards DG, and Farquhar WB**. Dietary sodium loading impairs microvascular function independent of blood pressure in humans: role of oxidative stress. *J Physiol* 590: 5519-5528, 2012.
17. **Guthikonda S, Sinkey C, Barenz T, and Haynes WG**. Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers. *Circulation* 107: 416-421, 2003.
18. **Heitzer T, Herttuala SY, Wild E, Luoma J, and Drexler H**. Effect of vitamin E on endothelial vasodilator function in patients with hypercholesterolemia, chronic smoking or both. *J Am Coll Cardiol* 33: 499-505, 1999.
19. **Heitzer T, Just H, and Munzel T**. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* 94: 6-9, 1996.
20. **Hodges GJ, Kosiba WA, Zhao K, and Johnson JM**. The involvement of norepinephrine, neuropeptide Y, and nitric oxide in the cutaneous vasodilator response to local heating in humans. *J Appl Physiol* 105: 233-240, 2008.
21. **Hodges GJ, and Sparks PA**. Noradrenaline and neuropeptide Y contribute to initial, but not sustained, vasodilatation to local skin warming in humans. *Exp Physiol* 99: 381-392, 2014.
22. **Holowatz LA, Houghton BL, Wong BJ, Wilkins BW, Harding AW, Kenney WL, and Minson CT**. Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol Heart Circ Physiol* 284: H1662-1667, 2003.
23. **Holowatz LA, and Kenney WL**. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. *Am J Physiol Heart Circ Physiol* 293: H1090-1096, 2007.
24. **Holowatz LA, Thompson-Torgerson CS, and Kenney WL**. The human cutaneous circulation as a model of generalized microvascular function. *J Appl Physiol (1985)* 105: 370-372, 2008.
25. **Holowatz LA, Thompson CS, and Kenney WL**. Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. *Am J Physiol Heart Circ Physiol* 291: H2965-2970, 2006.

26. **Holowatz LA, Thompson CS, Minson CT, and Kenney WL**. Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol* 563: 965-973, 2005.
27. **Ijzerman RG, de Jongh RT, Beijk MA, van Weissenbruch MM, Delemarre-van de Waal HA, Serne EH, and Stehouwer CD**. Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. *Eur J Clin Invest* 33: 536-542, 2003.
28. **Ijzerman RG, Serne EH, van Weissenbruch MM, de Jongh RT, and Stehouwer CD**. Cigarette smoking is associated with an acute impairment of microvascular function in humans. *Clin Sci* 104: 247-252, 2003.
29. **Jaimes EA, DeMaster EG, Tian RX, and Raij L**. Stable compounds of cigarette smoke induce endothelial superoxide anion production via NADPH oxidase activation. *Arterioscler Thromb Vasc Biol* 24: 1031-1036, 2004.
30. **Jaimes EA, Sweeney C, and Raij L**. Effects of the reactive oxygen species hydrogen peroxide and hypochlorite on endothelial nitric oxide production. *Hypertension* 38: 877-883, 2001.
31. **Jitsuiki D, Higashi Y, Goto C, Kimura M, Noma K, Hara K, Nakagawa K, Oshima T, Chayama K, and Yoshizumi M**. Effect of edaravone, a novel free radical scavenger, on endothelium-dependent vasodilation in smokers. *Am J Cardiol* 94: 1070-1073, 2004.
32. **Kellogg DL, Jr., Liu Y, Kosiba IF, and O'Donnell D**. Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol* 86: 1185-1190, 1999.
33. **Kellogg DL, Jr., Zhao JL, and Wu Y**. Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *Am J Physiol Heart Circ Physiol* 295: H123-129, 2008.
34. **Levy BI, Schiffrin EL, Mourad JJ, Agostini D, Vicaut E, Safar ME, and Struijker-Boudier HA**. Impaired tissue perfusion: a pathology common to hypertension, obesity, and diabetes mellitus. *Circulation* 118: 968-976, 2008.
35. **Martin HL, Loomis JL, and Kenney WL**. Maximal skin vascular conductance in subjects aged 5-85 yr. *J Appl Physiol* 79: 297-301, 1995.
36. **Medow MS, Bamji N, Clarke D, Ocon AJ, and Stewart JM**. Reactive Oxygen Species (ROS) from NADPH and Xanthine Oxidase Modulate the Cutaneous Local Heating Response in Healthy Humans. *J Appl Physiol* 111: 20-26, 2011.
37. **Medow MS, Glover JL, and Stewart JM**. Nitric oxide and prostaglandin inhibition during acetylcholine-mediated cutaneous vasodilation in humans. *Microcirculation* 15: 569-579, 2008.
38. **Minson CT**. Thermal provocation to evaluate microvascular reactivity in human skin. *J Appl Physiol* 109: 1239-1246, 2010.
39. **Minson CT, Berry LT, and Joyner MJ**. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 91: 1619-1626, 2001.
40. **Minson CT, Holowatz LA, Wong BJ, Kenney WL, and Wilkins BW**. Decreased nitric oxide- and axon reflex-mediated cutaneous vasodilation with age during local heating. *J Appl Physiol* 93: 1644-1649, 2002.
41. **Motoyama T, Kawano H, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, Ogawa H, and Yasue H**. Endothelium-dependent vasodilation in the

423 brachial artery is impaired in smokers: effect of vitamin C. *Am J Physiol* 273: H1644-
424 1650, 1997.
425 42. **Pellaton C, Kubli S, Feihl F, and Waeber B**. Blunted vasodilatory responses in
426 the cutaneous microcirculation of cigarette smokers. *Am Heart J* 144: 269-274, 2002.
427 43. **Peluffo G, Calcerrada P, Piacenza L, Pizzano N, and Radi R**. Superoxide-
428 mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in
429 vascular endothelium: studies in cultured cells and smokers. *Am J Physiol Heart Circ*
430 *Physiol* 296: H1781-1792, 2009.
431 44. **Pryor WA, and Stone K**. Oxidants in cigarette smoke. Radicals, hydrogen
432 peroxide, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci* 686: 12-27; discussion 27-
433 18, 1993.
434 45. **Rahman MM, and Laher I**. Structural and functional alteration of blood vessels
435 caused by cigarette smoking: An overview of molecular mechanisms. *Curr Vasc*
436 *Pharmacol* 5: 276-292, 2007.
437 46. **Smith CJ, Santhanam L, Bruning RS, Stanhewicz A, Berkowitz DE, and**
438 **Holowatz LA**. Upregulation of inducible nitric oxide synthase contributes to attenuated
439 cutaneous vasodilation in essential hypertensive humans. *Hypertension* 58: 935-942,
440 2011.
441 47. **Stewart J, Kohen A, Brouder D, Rahim F, Adler S, Garrick R, and**
442 **Goligorsky MS**. Noninvasive interrogation of microvasculature for signs of endothelial
443 dysfunction in patients with chronic renal failure. *Am J Physiol Heart Circ Physiol* 287:
444 H2687-2696, 2004.
445 48. **Stewart JM, Ocon AJ, Clarke D, Taneja I, and Medow MS**. Defects in
446 cutaneous angiotensin-converting enzyme 2 and angiotensin-(1-7) production in postural
447 tachycardia syndrome. *Hypertension* 53: 767-774, 2009.
448 49. **Stewart JM, Taneja I, Glover J, and Medow MS**. Angiotensin II type 1
449 receptor blockade corrects cutaneous nitric oxide deficit in postural tachycardia
450 syndrome. *Am J Physiol Heart Circ Physiol* 294: H466-473, 2008.
451 50. **Takase B, Etsuda H, Matsushima Y, Ayaori M, Kusano H, Hamabe A,**
452 **Uehata A, Ohsuzu F, Ishihara M, and Kurita A**. Effect of chronic oral
453 supplementation with vitamins on the endothelial function in chronic smokers. *Angiology*
454 55: 653-660, 2004.
455 51. **Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS,**
456 **Karoui H, Tordo P, and Pritchard KA, Jr.** Superoxide generation by endothelial nitric
457 oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 95: 9220-9225,
458 1998.
459 52. **Warner DO, Joyner MJ, and Charkoudian N**. Nicotine increases initial blood
460 flow responses to local heating of human non-glabrous skin. *J Physiol* 559: 975-984,
461 2004.
462 53. **WHO**. Global status report on noncommunicable diseases 2010
463 Description of the global burden of NCDs, their risk factors and determinants.
464 54. **Wong BJ, and Fieger SM**. Transient receptor potential vanilloid type-1 (TRPV-
465 1) channels contribute to cutaneous thermal hyperaemia in humans. *J Physiol* 588: 4317-
466 4326, 2010.

55. **Xu H, Jackson WF, Fink GD, and Galligan JJ**. Activation of potassium channels by tempol in arterial smooth muscle cells from normotensive and deoxycorticosterone acetate-salt hypertensive rats. *Hypertension* 48: 1080-1087, 2006.

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^G-nitro-L-arginine), a non-specific nitric oxide synthase inhibitor; Time 0 indicates initiation of local heating.

Figure 1

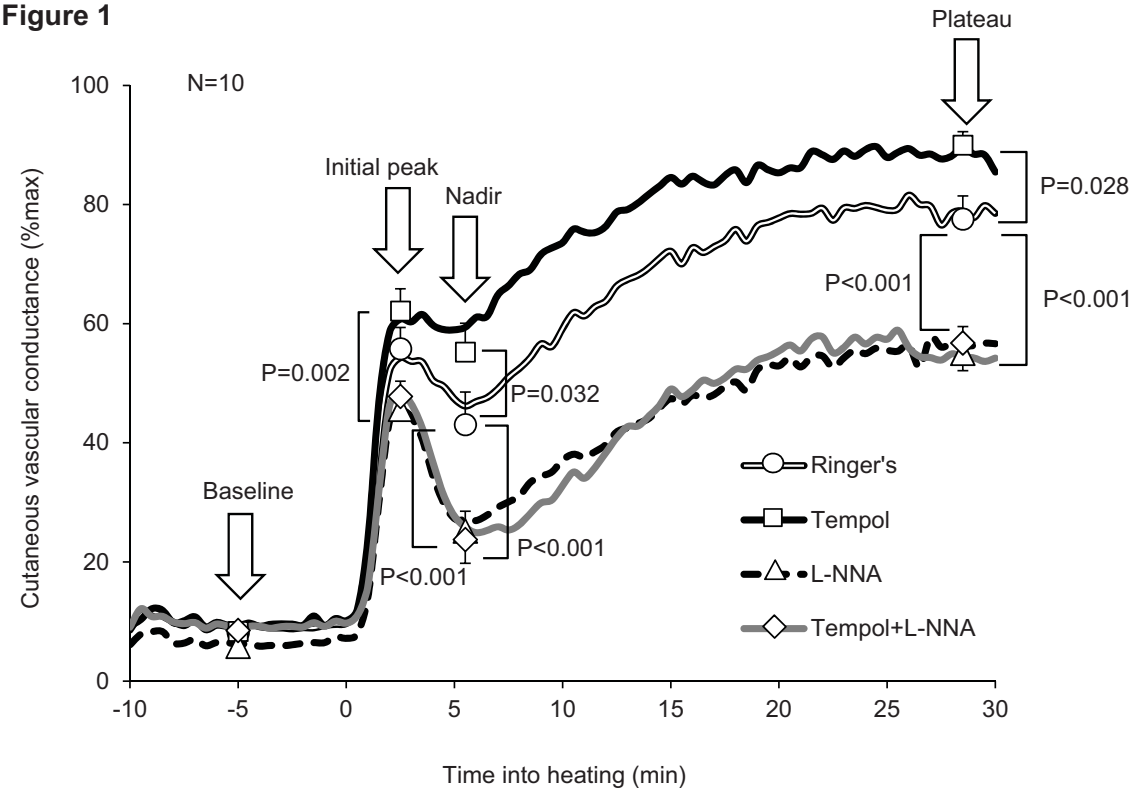
N=10
Plateau
Initial peak
Nadir
Baseline
P=0.028
P<0.001
P<0.001
P=0.002
P=0.032
P<0.001
P<0.001
Ringer's
Tempol
L-NNA
Tempol+L-NNA
Cutaneous vascular conductance (%max)
Time into heating (min)
100
80
60
40
20
0
-10
-5
0
5
10
15
20
25
30

Table 1 Comparison of averaged plateau cutaneous vascular conductance (CVC) during local heating at the control site between young smokers and non-smokers

Subject group	Author (year)	Plateau CVC (%max)	Comment
Young smokers	Present study	77	
Young non-smokers	Hodges & Sparks (2013)	86-90	at a rate of 0.5 ºC/10sec
	Brunt & Minson (2012)	84-88	
	Bruning *et al.* (2012)	95	
	Greaney *et al.* (2012)	93	
	Wong & Fieger (2010)	92	
	Kellogg *et al.* (2008)	85	local heating to 41.5ºC at a rate of 0.6ºC/min
	Stewart *et al.* (2008)	91	

All of the above studies employed local heating to 42 ºC at a rate of 0.1ºC/sec unless otherwise indicated.