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Skin hyperemia induced by local heating : why is it blunted on repeat stimulations ?

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ABSTRACT

Background: In human skin, local heating produces local vasodilatation, a response termed thermal hyperemia. Thermal hyperemia is largely mediated by nitric oxide (NO). It is blunted on repeat stimulations applied to the same skin spot, a phenomenon termed desensitization. As this phenomenon could reflect a desensitization in the vasodilator effects of NO, we investigated whether a prior exposure to exogenous NO would result in an attenuated vasodilatory response to a subsequent thermal challenge. **Methods:** Thirteen healthy young men were studied. Skin blood flow (SkBF) was measured on forearm skin with laser Doppler imaging. Exposure to exogenous NO was carried out by iontophoresis of sodium nitroprusside (SNP), a donor of NO. A local thermal stimulus (temperature step from 34 to 41°C maintained for 30 minutes) was applied with temperature-controlled chambers. We tested the influence of a previous transient exposure to exogenous NO on : 1) thermal hyperemia and 2) the response to a second identical exposure to exogeneous NO. **Results:** Thermal hyperemia (plateau SkBF at 30 minutes minus SkBF at 34°C) obtained on a site preexposed to exogenous NO two hours before was lower than obtained on a site preexposed to iontophoretic current only (mean±SD 395±139 perfusion units [PU] vs 540±79 PU ; $p < 0.01$). When repeated on the same skin site two hours after the first one, exposure to exogenous NO led to a blunted vasodilatory response (298±121 PU vs 394±92 PU), although this difference was not statistically significant ($p \approx 0.09$). **Conclusion:** In forearm human skin, prior exposure to exogenous NO partially inhibits thermal hyperemia. These data support that desensitization of thermal hyperemia depends on a downregulation of the NO-cGMP pathway, possibly downstream from the endogenous production of NO.

INTRODUCTION

The regulation of skin blood flow (SkBF) is extremely complex, and its mechanisms remain incompletely defined. SkBF varies constantly, influenced by many stimuli, a main one being changes in temperature. Indeed, local heating of the skin produces local vasodilatation, and thus an increase in SkBF, a termed *thermal hyperemia* [11,9,13,5].

When triggered by a step change in local temperature, thermal hyperemia is biphasic, with the time-course of SkBF showing an early peak, followed by a decrease to a nadir, and then a progressively rise to a plateau (late phase)[9,13]. The early peak occurs within minutes of the onset of heating. It largely depends on an axon reflex triggered by C-fiber nociceptors [11]. Accordingly, it is inhibited by local anesthesia [13,8]. The plateau is fully developed after 20-30 minutes of heating. Its mechanism is non-neural, and involves the local release of nitric oxide (NO) by the microvascular endothelium [9,13]. For this reason, thermal hyperemia has been used as a marker of endothelial integrity in several studies, especially in humans in whom SkBF can easily be measured non invasively with laser Doppler flowmetry [1,19,18,3,12,16].

Recent work from this laboratory has shown that the late phase of thermal hyperemia becomes blunted when repeatedly triggered on the same skin site. We have used the term *desensitization* to designate this phenomenon [4,7]. Desensitization has been consistently observed with different laser instrumentations and heating systems, at least in men [7]. Its mechanism remains unknown. Because the late phase of thermal hyperemia is mediated by NO, one general hypothesis would be that desensitization is related to the effects of NO, produced by the first stimulation, on the NO-dependant signaling pathways, effects that would persist until the second stimulation. For example, the NO produced during the first stimulation could cause a long-lasting down-regulation of endothelial NO synthase (eNOS) activity, as observed in cultured endothelial cells following transient exposure to a NO-donor [17].

If such was the case, we would expect that prior exposure of a cutaneous site to exogenous NO would result in an attenuated vasodilatory response of this site to a subsequent thermal challenge.

The objective of this present work was to test this hypothesis.

METHODS

Subjects

Thirteen healthy male subjects were included. They were aged between 18 and 30 years, were non-smokers and did not take any drug on a regular basis. Exclusion criteria were a flu-like state, or intake of any drug in the 15 days preceding the examination. Men only were included in order to avoid having to deal, in women, with the vascular effects of estrogens and progesterone, which may vary, according to the phase of menstrual cycle and method of contraception. The volunteers were fully informed about the protocol and gave their written consent. The study conformed with the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee of the Medical Faculty of Lausanne, Switzerland.

Measurements

SkBF was measured with a laser Doppler imager (LDI; software version 5.01; Moor Instruments, Axminster, UK) on the volar face of the forearm, and in perfusion units (PU), following the principles of laser Doppler flowmetry. With LDI, it is worth noting that, in contrast with standard laser Doppler flowmetry, the laser beam travels to the measurement site through air rather than through a light guide. Thus, any distortion of SkBF by mechanical contact of a probe with the skin is avoided. The distance between the laser aperture and the skin was set at 41 cm.

Thermal Hyperemia

The setup for carrying out thermal hyperemia has already been described by us in detail [4, 7]. In brief, a stainless steel, temperature-controlled, ring-shaped chamber with inner diameter of 8 mm was affixed to the skin with a double-sided tape. The central well was filled to the rim with medical-grade pure sterile water and overlaid with a transparent glass coverslip. The skin underneath the coverslip and water was thus accessible to laser light. The LDI device was programmed to repetitively scan the area comprising the chamber every 60 seconds, with each scan being accomplished in 50 seconds. The chamber was connected to an analog temperature controller with an adjustable set point. Temperature was set at 34°C until a stable blood-flow reading was obtained (5 minutes), and then it was raised to 41°C in 60 seconds and maintained at this

level for the next 30 minutes. The signal for temperature control came from a sensor incorporated into the chamber walls.

Our installation allowed to simultaneously record in parallel the thermal hyperemias elicited by two chambers affixed to the skin within 2-3cm of each other.

Iontophoresis of SNP

As was the case in our previous study [4], the sensitivity of skin microcirculation to the vasodilatory effects of the NO was evaluated from the response of SkBF to the noninvasive application of sodium nitroprusside (SNP, a donor of NO), using iontophoresis, a technique whereby a ionized molecule is forced through the epidermis by application of an electrical current. A custom-made neoprene ring-shaped chamber fitted with a copper electrode was affixed to the skin with double-sided tape and connected to a current source (MIC 1-e iontophoresis controller; Moor Instruments). The chamber was filled with a 1% solution of SNP in distilled water and overlaid with a glass coverslip to allow the measurement of blood flow with LDI in the exposed skin. The LDI device was programmed to repetitively scan the area comprising the chamber every 60 seconds, with each scan being accomplished in 50 seconds. Eight pulses of 8 microA, 60 seconds each, were administered, with interspersed current-free intervals of 60 seconds. Thus, the last pulse terminated 15 minutes after the onset of the first pulse. SkBF was recorded once per minute during this interval and for the ensuing 5 minutes. The absence of a non-specific vasodilatory response, induced by the iontophoretic current itself, was verified by the simultaneous iontophoresis of NaCl 0,9 % on the nearby site.

As in the case of thermal hyperemia, two responses to SNP iontophoresis were recorded in parallel.

Study protocol

The experiments took place in a quiet environment, with room temperature strictly maintained between 22 and 25 °C. Subjects reported to the laboratory for a single visit, at 13:30 pm. They had been instructed to abstain from caffeine-containing beverages from the previous evening on, and to take a meal before 11:30 am. on the study day. They were examined in the supine position with the arm supported by a vacuum cushion. Blood pressure was measured

serially during the course of the study, using an automated oscillometric device (Stabil-O-Graph, IEM, Germany). The principle of the experiment was to test for the influence on thermal hyperemia of a previous exposure to exogenous NO (Part 1 of the protocol).

In addition, we wished to evaluate whether such an exposure might be associated with a subsequent inhibition of the NO-cGMP signaling pathway at a level downstream from the production of NO by eNOS. Therefore, Part 2 of the protocol was designed to test for a possible impact of a first SNP iontophoresis on a second one, carried out later on the same skin site.

The two parts of the protocol were practically implemented as follows. On each arm, two sites free of visible vein were selected and marked with permanent ink to avoid any confusion. Sites A and B were chosen within 2 -3 cm of each other on the volar face of the dominant forearm. Sites C and D were similarly chosen, on the volar face of the non-dominant forearm. The following sequence was applied:

- 1.** Two iontophoresis chambers were positioned on sites A and B, in the vicinity of which skin temperature was measured with a thermocouple. At time zero (T₀), iontophoresis of SNP was applied on site A, while site B was iontophoretically exposed to NaCl 0.9% (i.e a control to test for the possible vasomotor effect of electrical current alone).
- 2.** Then, the iontophoresis chambers were positioned on sites C and D on the other arm, skin temperature was measured again, and iontophoresis was also carried out, starting 45 minutes after T₀ (T₀+45). Site C received SNP and site D received NaCl 0.9%.
- 3.** Two hours after T₀ (T₂), two temperature-controlled chambers were positioned on sites A and B, in the vicinity of which baseline skin temperature was measured. Thermal hyperemia were carried out as described above. So, site A had been pre-treated by SNP two hours before being thermally stimulated and site B had not, and thus served as a control. SkBF before chamber insertion was not assessed in this protocol, but in preliminary experiments, SkBF was not measurably influenced by the minor heating from "normal" skin temperature (31+33°C) to 34°C.

4. At T2+45, iontophoresis of SNP and NaCl 0.9% were repeated on sites C and D, respectively.

The entire protocol lasted 4 hours. The time interval of 2 hours between two stimulations on the same site was chosen based on our previous observations, which indicated that desensitization of thermal hyperemia occurred within this time frame [4, 7]

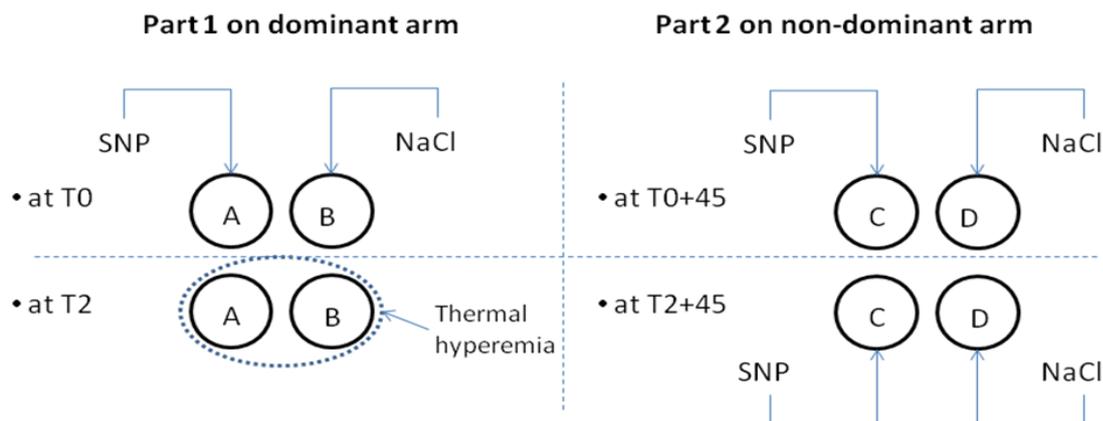


Figure 1. Schematic outline of protocol. SNP: sodium nitroprussiate iontophoresis. NaCl: iontophoresis of NaCl. T0: onset of protocol. T0+45: 45 minutes after T0. T2: 2 hours after T0. For further explanations, see text.

Data analysis

Data are presented as the mean \pm SD of the 13 subjects (Part 1) and the 12 subjects (Part 2, which could not be carried out in one subject due to a technical difficulty). The responses of SkBF to local heating were quantified as the early peak minus the baseline, and the plateau minus the baseline. The baseline SkBF was the mean of 5 values recorded in the 5 minutes which immediately preceded the onset of heating. The early peak SkBF was the maximal value attained within the 5 minutes immediately following the onset of heating. The plateau SkBF was the average of 5 values recorded between 25 and 30 minutes after the onset of heating. The response to SNP iontophoresis was quantified as the average SkBF measured in the 5 minutes immediately following the end of iontophoresis, with the baseline value also subtracted.

Statistical analysis was carried out with ANOVA for repeated measures, using the JMP version 5.0.1.2 software (SAS Institute, Heideberg, Germany). The alpha level of all tests was set at 0.05.

RESULTS

Part 1

In this part of the protocol, we wanted to test for a possible effect of a prior exposure of the skin microcirculation to exogenous NO on its subsequent sensitivity to a thermal stimulus. Sites A and B were chosen on the volar face of the dominant arm, then we proceeded as shown in Figure 1. So, at the time of thermal hyperemia, site A had been pre-exposed to exogenous NO and site B had not. The upper part of Table 1 shows that room temperature, skin temperature and blood pressure were stable over the time of the experiment. The baseline SkBF were comparable among the two sites and the two times in this part of protocol. Figure 2 shows the mean time courses of thermal hyperemia recorded at T2 on sites A and B. As expected, the observed patterns were consistently biphasic with an early peak within 5 minutes of the heating onset and a late plateau after 25-30 minutes. The major difference between both responses is a lower plateau on site B. As shown in Table 1, the mean peak and plateau responses on site B were lower by respectively 16% ($p < 0.05$) and 27% ($p < 0.01$) relative to site A.

Table 1. Hemodynamic and skin blood flow parameters regarding part 1 of the experiment (iontophoresis at T0 followed by thermal hyperemia at T2)

	T0		T2	
Temperature (°C)				
Room	24.3	± 0.5	24.7	± 0.4
Skin	33.4	± 0.8	33.4	± 0.6
Blood pressure (mmHg)				
Systolic	117	± 12	120	± 8
Diastolic	64	± 8	68	± 8
Mean	82	± 9	85	± 7
Skin blood flow (PU)				
baseline	66	± 19	64	± 27
iontophoresis, plateau response				
NaCl			50	± 104
SNP	387	± 150		
Thermal Hyperemia				
Early peak			379	± 92*
Plateau			395	± 139**
			453	± 98
			540	± 79

The symbols A and B designate specific sites on the volar face of the dominant arm, and T0, T2 refer to specific times (in hours) in the protocol. PU, perfusion units. Iontophoresis applied on T0 and thermal hyperemia on T2. Plateau response is expressed as the largest skin blood flow following iontophoresis minus corresponding baseline flow. Response to local heating (41°C) is expressed as the pic blood flow after 2 minutes of heating minus the corresponding baseline flow and the plateau blood flow after 25 minutes of heating minus the corresponding baseline flow. Data are means ± SD of the 13 subjects. * $p < 0.05$, early peak on site A vs site B. ** $p < 0.01$, plateau on site A vs site B. All other pairwise comparisons were nonsignificant ($p > 0.5$).

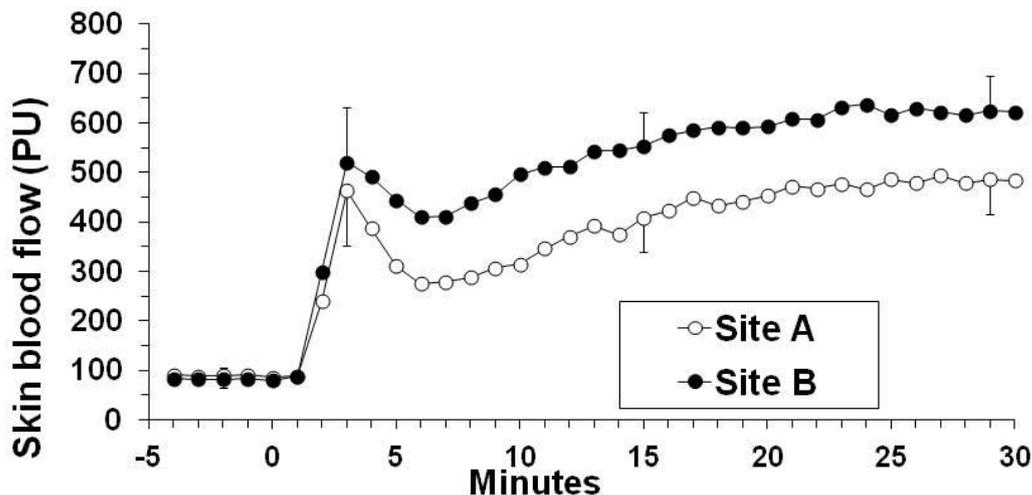


Figure 2. Thermal hyperemia recorded on two different skin sites. Site A but not B, was prestimulated by iontophoresis of SNP at onset of protocol. Thermal hyperemia carried out two hours later (T2). PU, perfusion unit. Data points are means of 13 subjects. Error bars are representative SD. Statistical symbols are specifically omitted in the figure, since statistical analysis was not carried out on the complete time courses of skin blood flow, but only on the derived values shown in Table 1.

Part 2

In this part of protocol, we wanted to test for a possible effect of a prior exposure of the skin microvasculature to exogenous NO on its subsequent sensitivity to a second identical exposure. Sites C and D were chosen on the volar face of the non-dominant arm, then we proceeded as shown in Figure 1. As in Part 1, room temperature, skin temperature and blood pressure were stable over the time of the experiment (upper part of Table 2), and baseline SkBF were comparable among the two sites and the two experimental times. Figure 3 shows the mean time courses of SkBF responses recorded at T0 and T2 on sites C and D. Site C was exposed to exogenous NO at T0 and reexposed at T2, while site B was a control site, exposed to iontophoretic current only at both times. Thus, the main comparison of interest is between the responses on site C at T0 and T2. Figure 3 shows an attenuation of these responses from T0 to T2, by an average

of roughly 25%. However, as shown in Table 2, this difference did not reach statistical significance ($p \approx 0.09$). Returning to Figure 3, there was almost no response to current alone on site D at T0. At T2 by contrast, this response was, on average, massively larger (Figure 3 and Table 2, $p < 0.001$ vs T0), indicating sensitization to the vasodilatory effect of current alone in this protocol. Observation of individual results shows that this sensitization occurred in half the subjects ($n=6$), but not in the other half ($n=6$). Post hoc separate analyses of these two subgroups disclosed the same trend in both towards a lower response to SNP on site C at T2, compared to T0.

Table 2. Hemodynamic and skin blood flow parameters regarding part 2 of the experiment (iontophoresis at T0 followed by a second iontophoresis at T2)

	T0 + 45		T2 + 45		
Temperature (°C)					
Room	24.6 ± 0.3		24.8 ± 0.3		
Skin	33.4 ± 0.6		33.3 ± 0.4		
Blood pressure (mmHg)					
Systolic	116 ± 8		116 ± 8		
Diastolic	64 ± 7		66 ± 6		
Mean	81 ± 6		83 ± 6		
Skin blood flow (PU)					
baseline		Site C 69 ± 16	Site D 74 ± 21	Site C 81 ± 24	Site D 83 ± 24
iontophoresis, plateau response					
NaCl			48 ± 67		171 ± 232*
SNP		394 ± 92		298 ± 121 ^(p=0.09)	

The symbols C and D designate specific sites on the volar face of the non dominant arm, and T0,T2 refer to specific times (in hours) in the protocol. PU, perfusion units. Plateau response is expressed as the largest skin blood flow following iontophoresis minus corresponding baseline flow. Data are means±SD of the 12 subjects. * $p < 0.05$, NaCl at T2+45 vs at T0+45. $p=0.09$, SNP at T2+45 vs at T0+45. All other pairwise comparisons were nonsignificant ($p > 0.5$).

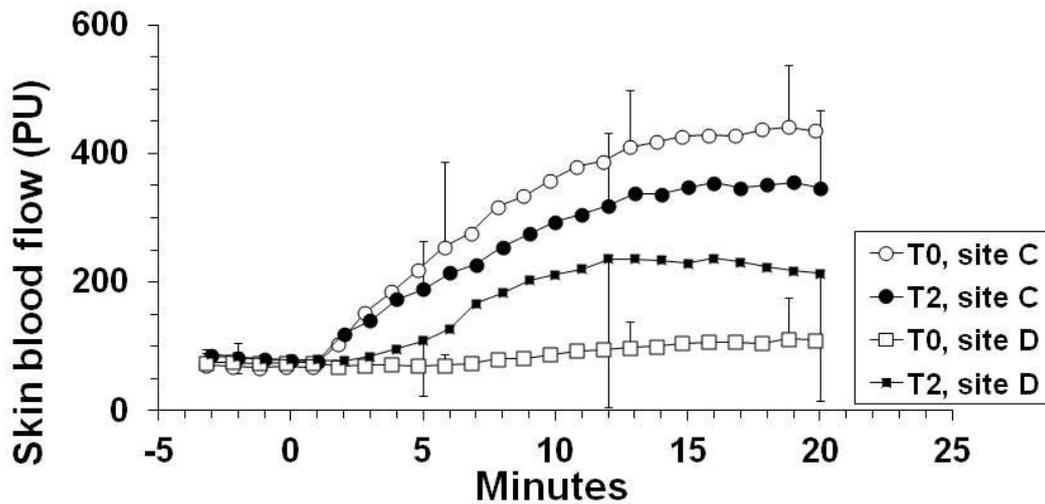


Figure 3. Responses to iontophoresis recorded on two different skin sites. Site C and D were prestimulated by a first iontophoresis of SNP on site C and of NaCl on site D 45 minutes after onset of protocol (T0+45). A second iontophoresis of SNP on sites C and of NaCl on site D was carried out two hours later (T2+45). PU, perfusion unit. Data points are means of 12 subjects. Error bars are representative SD. Statistical symbols are specifically omitted in the figure, since statistical analysis was not carried out on the complete time courses of skin blood flow, but only on the derived values shown in Table 2.

DISCUSSION

The major new finding of this study is the following: a previous exposure of the skin microcirculation to exogenous NO attenuates its subsequent sensitivity to the vasodilator effects of a local thermal stimulus (thermal hyperemia). Recent studies from this laboratory demonstrated that the NO-dependent part of thermal hyperemia was blunted on a repeat stimulation, if carried out 2, although not 4 hours later [4,7]. This desensitization was consistently observed, using a variety of equipment for laser Doppler flowmetry and skin heating [7]. Our studies gave however little insight into potential mechanisms. We had obtained one hind, finding that transient thermal stimulation of the skin partially inhibited the vasodilatory effects of a NO donor applied later on the same spot [4]. Thus, local heating equally affected the responses to two different stimuli,

suggesting that it acted on a pathway shared between them, namely the NO-dependent activation of guanylyl-cyclase (GC). It remained however unclear whether this action of heating was directly due to the induced production of NO (i.e NO-induced downregulation of the NO-cGMP pathway) or to another consequence of raised temperature. A necessary condition for the viability of the former hypothesis would be to demonstrate that exposure of the skin microcirculation to NO at constant temperature diminishes the subsequent response to local heating. The present study provides such a demonstration (Figure 2 and Table 1). One might argue that the impact of SNP administration at T0 on thermal hyperemia at T2 could be due to the iontophoretic current rather than to the drug itself, but we can exclude this interpretation because the experimental design included the control site B exposed to current alone.

The impact of NO, either produced endogenously, during a first thermal hyperemia [4,7], or exogenously administered (this study), can theoretically occur at four different, non exclusive levels: 1. attenuation in activity of endogenous NO production – i.e a negative feedback control of NOS activity, 2. inactivation of NO by free radicals, before it reaches its target, 3. decreased cGMP production – i.e. negative feedback control of GC activity, 4. enhanced degradation of cGMP. These possibilities have been evoked in various combinations to explain the well known pharmacological phenomenon of tolerance to organic nitrates [15]. There is good evidence that mechanisms operating at level 1 [17], 2 [15], and 4 [14] exist, at least in specific circumstances, while those at level 3 are less well supported by data.

The purpose of Part 2 was to gain some insight into the levels implicated in the desensitization of skin microcirculation to the vasodilatory effects of NO. If the negative feedback of NO on its own-production by eNOS was predominant (level 1), we would expect no attenuation of response on repeat stimulation with exogenous NO. Our data do not point into that direction, because on the average the second iontophoresis of SNP produced less vasodilation than did the first one (Figure 3, Table 2), suggesting intervention of mechanisms at levels 2 -4, without further possibility for discrimination. This interpretation must however be done with some caution, first because statistical significance was not reached for this effect ($p = 0.09$), second and most importantly because, at T2, the observed vasodilation induced by SNP iontophoresis was, at least in half the subjects, a

composite effect related both to NO and to current administration. The sensitization of skin to vasodilation induced by electrical current, especially if anodal as was the case here, is a well established phenomenon [6]. To better establish whether desensitization to exogenous NO actually exists in human skin, a method of local administration devoid of this confounding effect should be used, such as intradermal microdialysis of the agent [9,10,13].

CONCLUSION

The present study proves for the first time that prior exposure to exogenous NO partially inhibits thermal hyperemia in forearm human skin. These data support that desensitization of thermal hyperemia, as previously described by us, depends on a down-regulation of the NO-cGMP pathway, possibly downstream from the endogenous production of NO.

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REFERENCES

1. Arora S, Smakowski P, Frykberg RG, Simeone LR, Freeman R, LoGerfo FW, et al. Differences in foot and forearm skin microcirculation in diabetic patients with and without neuropathy. *Diabetes care*. 1998;21(8):1339-44. Epub 1998/08/14.
2. Boegli Y, Gremion G, Golay S, Kubli S, Liaudet L, Leyvraz PF, et al. Endurance training enhances vasodilation induced by nitric oxide in human skin. *The Journal of investigative dermatology*. 2003;121(5):1197-204. Epub 2004/01/08.

3. Charkoudian N, Eisenach JH, Atkinson JL, Fealey RD, Joyner MJ. Effects of chronic sympathectomy on locally mediated cutaneous vasodilation in humans. *J Appl Physiol.* 2002;92(2):685-90. Epub 2002/01/18.
4. Ciplak M, Pasche A, Heim A, Haerberli C, Waeber B, Liaudet L, et al. The vasodilatory response of skin microcirculation to local heating is subject to desensitization. *Microcirculation.* 2009;16(3):265-75. Epub 2009/02/03.
5. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends in pharmacological sciences.* 2006;27(9):503-8. Epub 2006/08/01.
6. Durand S, Fromy B, Bouye P, Saumet JL, Abraham P. Vasodilatation in response to repeated anodal current application in the human skin relies on aspirin-sensitive mechanisms. *The Journal of physiology.* 2002;540(Pt 1):261-9. Epub 2002/04/03.
7. Frantz J, Engelberger R, Liaudet L, Mazzolai L, Waeber B, Feihl F. Desensitization Of Thermal Hyperemia In The Skin Is Reproducible. *Microcirculation.* 2011. Epub 2011/08/09.
8. Golay S, Haerberli C, Delachaux A, Liaudet L, Kucera P, Waeber B, et al. Local heating of human skin causes hyperemia without mediation by muscarinic cholinergic receptors or prostanoids. *J Appl Physiol.* 2004;97(5):1781-6. Epub 2004/07/13.
9. Kellogg DL, Jr., Liu Y, Kosiba IF, O'Donnell D. Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol.* 1999;86(4):1185-90. Epub 1999/04/08.
10. Kellogg DL, Jr., Zhao JL, Wu Y. Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *American journal of physiology Heart and circulatory physiology.* 2008;295(1):H123-9. Epub 2008/05/13.
11. Magerl W, Treede RD. Heat-evoked vasodilatation in human hairy skin: axon reflexes due to low-level activity of nociceptive afferents. *The Journal of physiology.* 1996;497 (Pt 3):837-48. Epub 1996/12/15.

12. Medow MS, Minson CT, Stewart JM. Decreased microvascular nitric oxide-dependent vasodilation in postural tachycardia syndrome. *Circulation*. 2005;112(17):2611-8. Epub 2005/10/19.
13. Minson CT, Berry LT, Joyner MJ. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol*. 2001;91(4):1619-26. Epub 2001/09/25.
14. Mullershausen F, Lange A, Mergia E, Friebe A, Koesling D. Desensitization of NO/cGMP signaling in smooth muscle: blood vessels versus airways. *Molecular pharmacology*. 2006;69(6):1969-74. Epub 2006/03/03.
15. Munzel T, Daiber A, Gori T. Nitrate therapy: new aspects concerning molecular action and tolerance. *Circulation*. 2011;123(19):2132-44. Epub 2011/05/18.
16. Salvat-Melis M, Carpentier PH, Minson CT, Boignard A, McCord GR, Paris A, et al. Digital thermal hyperaemia impairment does not relate to skin fibrosis or macrovascular disease in systemic sclerosis. *Rheumatology (Oxford)*. 2006;45(12):1490-6. Epub 2006/05/18.
17. Sheehy AM, Burson MA, Black SM. Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. *The American journal of physiology*. 1998;274(5 Pt 1):L833-41. Epub 1998/06/05.
18. Sokolnicki LA, Roberts SK, Wilkins BW, Basu A, Charkoudian N. Contribution of nitric oxide to cutaneous microvascular dilation in individuals with type 2 diabetes mellitus. *American journal of physiology Endocrinology and metabolism*. 2007;292(1):E314-8. Epub 2006/09/07.
19. Veves A, Akbari CM, Primavera J, Donaghue VM, Zacharoulis D, Chrzan JS, et al. Endothelial dysfunction and the expression of endothelial nitric oxide synthetase in diabetic neuropathy, vascular disease, and foot ulceration. *Diabetes*. 1998;47(3):457-63. Epub 1998/03/31.