

# Maximum Skin Hyperaemia Induced by Local Heating: Possible Mechanisms

Kim M. Gooding<sup>a</sup> Michael M. Hannemann<sup>a</sup> John E. Tooke<sup>a</sup>  
Geraldine F. Clough<sup>b</sup> Angela C. Shore<sup>a</sup>

<sup>a</sup>Institute of Biomedical and Clinical Sciences, Peninsula Medical School, Exeter, and <sup>b</sup>Division of Infection, Inflammation and Repair, School of Medicine, University of Southampton, Southampton, UK

## Key Words

Maximum hyperaemia · Local heating · Nitric oxide · Prostaglandins · Histamine · Axon reflex

## Abstract

**Background:** Maximum skin hyperaemia (MH) induced by heating skin to  $\geq 42^\circ\text{C}$  is impaired in individuals at risk of diabetes and cardiovascular disease. Interpretation of these findings is hampered by the lack of clarity of the mechanisms involved in the attainment of MH. **Methods:** MH was achieved by local heating of skin to  $42\text{--}43^\circ\text{C}$  for 30 min, and assessed by laser Doppler fluximetry. Using double-blind, randomized, placebo-controlled crossover study designs, the roles of prostaglandins were investigated by inhibiting their production with aspirin and histamine, with the  $H_1$  receptor antagonist cetirizine. The nitric oxide (NO) pathway was blocked by the NO synthase inhibitor,  $N^G$ -nitro-*L*-arginine methyl ester (*L*-NAME), and enhanced by sildenafil (prevents breakdown of cGMP). **Results:** MH was not altered by aspirin, cetirizine or sildenafil, but was reduced by *L*-NAME: median placebo 4.48 V (25th, 75th centiles: 3.71, 4.70) versus *L*-NAME 3.25 V (3.10, 3.80) ( $p = 0.008$ , Wilcoxon signed rank test). Inhibition of NO production (*L*-NAME) resulted in a more rapid reduction in hyperaemia

after heating ( $p = 0.011$ ), whereas hyperaemia was prolonged in the presence of sildenafil ( $p = 0.003$ ). The increase in skin blood flow was largely confined to the directly heated area, suggesting that the role of heat-induced activation of the axon reflex was small. **Conclusion:** NO, but not prostaglandins, histamine or an axon reflex, contributes to the increase in blood flow on heating and NO is also a component of the resolution of MH after heating.

Copyright © 2006 S. Karger AG, Basel

## Introduction

It is increasingly being recognized that subjects at risk of, or exhibiting, vascular disease, for example subjects with an increased risk of coronary heart disease, exhibit microvascular abnormalities demonstrable in the skin microcirculation [1]. An impaired skin maximum hyperaemic (MH) response to local heating has been observed in type 1 [2, 3], type 2 [4], and MODY3 diabetic subjects [5] who are highly prone to both macro- and microcirculation complications. This reduced vasodilatory capacity predates diabetes as it is present in those at risk of developing type 2 diabetes mellitus, for example in subjects with fasting hyperglycaemia [6], in women with previous

gestational diabetes [7], and in 3-month-old infants of low birth weight [8]. Impaired MH is also associated with increased cardiovascular risk and with end organ damage [9].

In view of these observations, the skin microvasculature represents a useful model for exploring microvascular function when direct examination of the relevant vascular bed is difficult or impossible, or as in the case of diabetes, where a specific microangiopathy is part of the disease complex. With the advent of commercially available equipment, identical protocols as those used above are being adopted by many groups worldwide. The key to interpreting the findings of this impaired MH response in a disease state is an understanding of the factors/mechanisms involved in inducing MH in health. There are numerous control mechanisms/factors present in the vascular system, including nitric oxide (NO), prostaglandins, histamine and the nervous system [10]. To date, the attainment of MH by local heating was attributed to the axon reflex and NO, and roles for prostaglandins or muscarinic receptors have been sought but could not be demonstrated [11–13]. Mechanisms involved in the reduction in hyperaemia on cessation of heating have not been investigated. Therefore, the present study sought to further elucidate the mechanisms involved in the attainment of MH and also to investigate the role of NO in the sustainability of the hyperaemic response after heating. To dissect the relative contribution of potential mechanisms, double-blind, randomized, placebo-controlled crossover studies in healthy individuals were conducted to examine the effects of prostaglandins, NO and histamine on the attainment of MH by local heating of a 0.76-cm<sup>2</sup> area of skin. The effect of local heating on blood flow in the surrounding tissue was also assessed to investigate whether the heating stimulus produced an axon reflex-mediated flare. The sustainability of the hyperaemic response following the removal of the heat stimulus (resolution of MH) was investigated by attenuating the NO pathway by inhibiting NO synthase (NOS) and by enhancing the NO pathway by prolonging the action of cGMP with sildenafil.

## Subjects and Methods

### *Subjects*

Healthy subjects were recruited from the local community by advertisements. Subjects who were receiving medication (other than the oral contraceptive pill), had a history or evidence of diabetes, hypertension, venous, arterial or Raynaud's disease were excluded. Ethics approval was granted by the Local Exeter and North

Devon Medical Research Ethics Committee, and written informed consent was obtained from all subjects. The studies conformed to the principles outlined in the declaration of Helsinki.

### *Assessment of MH*

Subjects were supine, in a temperature-controlled microvascular laboratory (21.5–22.5°C) throughout the study. The area of skin under investigation was initially warmed to 36°C using warm air. A plastic collar, into which was fitted a small brass heater (0.76 cm<sup>2</sup>; Moor Instruments, Axminster, UK), was then attached to the dorsal surface of the foot using a double-sided adhesive disc and the skin was heated to maintain skin temperature at 42–43°C throughout the 30-min heating period (it takes approximately 2–5 min for skin to reach the required temperature). Skin temperature was assessed by a thermocouple underlying the heater probe. At the end of the heating phase, MH was either assessed using single point laser Doppler fluximetry (LDF) or laser Doppler perfusion imaging (LDPI). In protocol 1 (prostaglandins) and 2 (histamine action via H<sub>1</sub> receptor), single point LDF (PI2, Perimed, Sweden) was used to assess skin hyperaemia on the dorsum of the foot. The laser Doppler probe was fitted into the non-centric hole of the small brass heater, and the heater was rotated within the adherent collar to obtain eight equally spaced, 30-second measurements of directly heated skin. The mean of these eight measurements was used as the MH. In protocols 4 and 5, forearm skin hyperaemia was assessed by LDPI (Pim II, Perimed) every minute for 30 min following the removal of the heater. The first reading taken after the heater removal represented MH. The sustainability of the MH following the removal of the heat stimulus (the resolution of MH) was represented by the skin hyperaemia at 30 min after the removal of the heater, expressed as a percentage of the MH. LDPI was used for protocols 4 and 5 for two reasons: firstly, to remove any potential artefacts caused by perturbing skin blood flow whilst rotating the heater head during assessment of the skin hyperaemia with LDF. Although such artefacts are without effect on MH (since no further increase in perfusion can occur), it is important to minimize them during measurements of sub-maximal values. Secondly, the use of LDPI enabled more rapid measurements of perfusion.

MH is traditionally assessed on the dorsal surface of the foot. However, the irregular surface and underlying tissues of the foot made it very difficult to insert microdialysis probes in parallel at similar depths in the skin of the dorsal surface of the foot. Thus, the ventral aspect of the forearm was used for protocol 4. For comparison, protocol 5, which was also investigating NO-mediated effects, was also performed on the ventral aspect of the forearm.

The day-to-day intra-subject coefficient of variation of MH in 3 subjects assessed on 5 separate occasions was 6.6%, and it was 7.1% in 1 male subject assessed over a 2-year period using LDF (mean  $\pm$  standard deviation: 2.11  $\pm$  0.15 AU V). Day-to-day intra-subject coefficient of variation was 12.5% in 1 subject assessed on 5 separate occasions (3.12  $\pm$  0.39 AU V), and 4.9% in 1 subject assessed over a 5-month period (2.67  $\pm$  0.13 AU V) using LDPI. The difference in absolute values for MH obtained by LDF and LDPI reflect the different arbitrary units of the instruments rather than a different absolute value. The day-to-day intra-subject coefficient of variation of the resolution of MH (the percent of MH remaining 30 min after heating) in 1 subject assessed on 5 separate occasions was 18.9% (41  $\pm$  8%).

Historically, the MH technique, as described above, is rarely associated with either a flare response or any sensation of pain or

discomfort either during the heating phase or following the removal of the heater. After heating the skin, a clear red circle (0.76 cm<sup>2</sup>, the size of the brass heater) is evident with no other visible hyperaemia in surrounding areas.

*Protocol 1: The Effect of Prostaglandins on the Induction of MH*

The role of prostaglandins in the induction of MH was investigated by inhibition of cyclooxygenase, a crucial enzyme in the prostaglandin biosynthetic pathway, prior to the commencement of, and throughout the period of heating. Ten healthy subjects (7 males), with a median age of 30 years (25th, 75th centiles: 24.5, 32.3), were randomized to receive a single dose of either soluble aspirin (600 mg) or placebo in orange juice with a minimum of 2 weeks washout in between. Local heating of the skin commenced 30 min after treatment, a time which has been demonstrated to provide a maximal 86% inhibition of bradykinin-induced production of prostaglandins [14]. The position of the skin site under investigation on the first visit was accurately recorded to ensure that the same area of skin was examined on the second visit.

*Protocol 2: The Effect of H<sub>1</sub> Receptor Inhibition on the Induction of MH*

Cetirizine, a selective H<sub>1</sub> receptor inhibitor, was used to explore the role of histamine via the H<sub>1</sub> receptor in the induction and maintenance of MH. Ten healthy subjects (7 males), with a mean age of 30 years (25th, 75th centiles: 27.3, 35.3), were randomized to receive a single dose of cetirizine (10 mg) or matching placebo on 2 occasions separated by at least 4 weeks, in a double-blind, randomized, placebo-controlled crossover design. The tablets were administered 6 h prior to the commencement of local heating as this corresponds to the time of maximal inhibition of histamine-induced wheal and flare by cetirizine [15–17]. The skin site under investigation on the first visit was accurately recorded to ensure that the same area of skin was examined on the second visit.

*Protocol 3: Examining the Potential Activation of an Axon Reflex during the Assessment of MH*

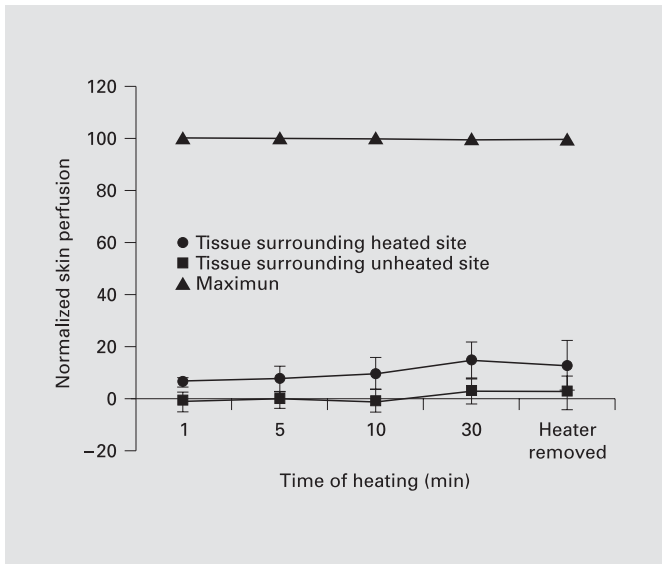
To explore whether heating activated the axon reflex, as judged by a flare response, skin perfusion in the skin surrounding the heater (i.e. not directly heated) was assessed during local heating on the dorsum of the foot and under control conditions (a heater head probe was attached on the skin for 30 min but not heated). Prior to attaching the heater probe for the control assessments, the probe was attached to an adjacent area of skin to enable the probe to adjust to the subject's skin temperature, thereby limiting any potential cooling effect of the brass probe. The heated and control site assessments were performed in 6 healthy subjects (2 males), with a mean age of 33 years (25th, 75th centiles: 30, 40), in a randomized order, with a 30-min rest period between the assessments. To enable the visualization of the areas surrounding the heater, a transparent collar was manufactured, thus allowing the quantification of skin perfusion in the skin underlying the collar. Skin perfusion scans of the entire area were performed at baseline, at approximately 1, 5 and 10 min into the heating period, at 30 min of heating, and following the removal of the heater using the LDPI. To aid the comparison of the effect of the heater on the surrounding skin, perfusion was normalized to each subject's MH response to enable the data to be combined.

*Protocol 4: The Effect of Inhibiting NO Production on the Induction of MH*

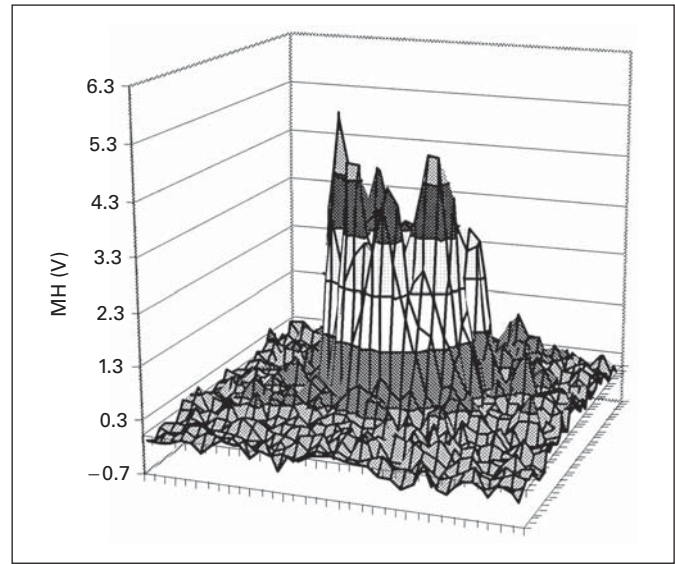
The role of NO was investigated using N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), an NOS competitive inhibitor, delivered by microdialysis for 30 min before and throughout the heating and resolution periods. Nine healthy female subjects, with a mean age of 33 years (25th, 75th centiles: 25, 42), were recruited for this double-blind placebo-controlled randomized study. Linear flow microdialysis probes (cuprophane membranes) (Focus 90H Hemophan Hollow Fiber Dialyzer, National Medical Care, Rockleigh, N.J., USA), with a 5-kDa molecular mass cut-off, internal diameter of 200 µm, and reinforced internally with a length of stainless steel wire (AISI 302, Goodfellow Cambridge Ltd., Huntingdon, UK), were used. A pair of probes were introduced into the dermis of the volar surface of the forearm, using 23-gauge guide needles under local anaesthesia (EMLA, Astra, Kings Langley, UK). The microdialysis probes were inserted into the volar aspect of the forearm as it provided a more accessible skin microvascular bed for uniform insertion of the microdialysis probes than the dorsum of the foot, thus minimizing any variations in the depth of penetration of the probes and maximizing delivery. One of the probes was randomized to act as the treatment probe (L-NAME), while the other acted as its paired control (saline). The probes ran parallel to the skin surface and to each other, at a depth of approximately 0.6 mm, and 5 mm apart. A 22-mm length of each probe was available for microdialysis exchange with the skin interstitial space, with 10 mm of this length within the heated area. Previous data suggest that a 5-mm space between the two probes minimizes the likelihood that the L-NAME-perfused probe is influencing the data obtained from the placebo-perfused probe [18, 19]. Perfusion of the microdialysis probes commenced 90 min after their insertion, at which time the effects of the EMLA anaesthesia had reversed [19]. The perfusates were 5 mM L-NAME (Clinalfa, Switzerland) and a control solution of 0.9% saline (MacoPharma, France), delivered at a flow rate of 5 µl·min<sup>-1</sup>. This concentration of L-NAME (5 mM) has previously been shown to significantly attenuate acetylcholine, histamine, bradykinin and substance P-induced vasodilation, an attenuation which could not be further enhanced by increasing the concentration of L-NAME [18–20]. Perfusion was continued for 30 min prior to commencement of skin heating. Skin heating was effected by the technique described above, with the heater placed directly overlying the microdialysis probes. The probes were continually perfused during both heating and resolution phases. Perfusion imaging (LDPI) was used in this protocol in order to minimize the potential damage to the probes caused by repeated rotation of the heater head for LDF used in protocols 1 and 2, during the resolution phase. Skin hyperaemia scans were analysed offline in a blinded fashion using LDISoft4.0 software (Perimed, Jarfalla, Sweden). Skin hyperaemia was measured in a 10 × 4-mm area of heated skin overlying each microdialysis probe.

*Protocol 5: The Effect of Enhancing the NO Pathway on the Induction of MH*

Sildenafil, a phosphodiesterase 5 inhibitor, was used to prevent the breakdown of cGMP, a downstream signalling molecule of the NO pathway, and thus prolong its action. Eleven healthy male subjects, with a mean age of 28 years (25th, 75th centiles: 21, 36), were randomized to a double-blind, placebo-controlled crossover study of a single dose of sildenafil (100 mg oral; Pfizer, Sandwich, UK) or matching placebo with a washout period of at least a week. Skin



**Fig. 1.** Normalized skin perfusion in the tissue surrounding the heater probe relative to skin MH (mean MH equals 100) of the heated and control sites in 6 healthy subjects. Markers represent the mean, and the error bars represent standard deviation.



**Fig. 2.** Example of a spectral skin perfusion graph of the heated site and surrounding skin following 30 min of heating and heater removal in 1 subject. The position of the circular brass heater corresponded directly to the markedly elevated values of perfusion.

heating commenced 1 h after tablet administration. To ensure that this study protocol was comparable with protocol 4, both of which were designed to investigate the involvement of NO in the MH response, skin heating was performed on the volar aspect of the forearm. The position of the skin site under investigation on the first visit was accurately recorded to ensure that the same area of skin was examined on the second visit.

#### Statistical Analysis

All volunteers participating in the double-blind, placebo-controlled studies acted as their own controls. Data are presented as median (25th, 75th centiles). Active therapy was compared with placebo data using the non-parametric paired test, Wilcoxon signed rank. Protocols 1 and 2 had 90% power at the 5% level to detect a 0.13 V difference (7–9% change) in MH using LDF. Protocols 4 and 5 had 90% power at the 5% level to detect a 0.43 V (10%) and 0.39 V (9%) difference in MH using LDPI.

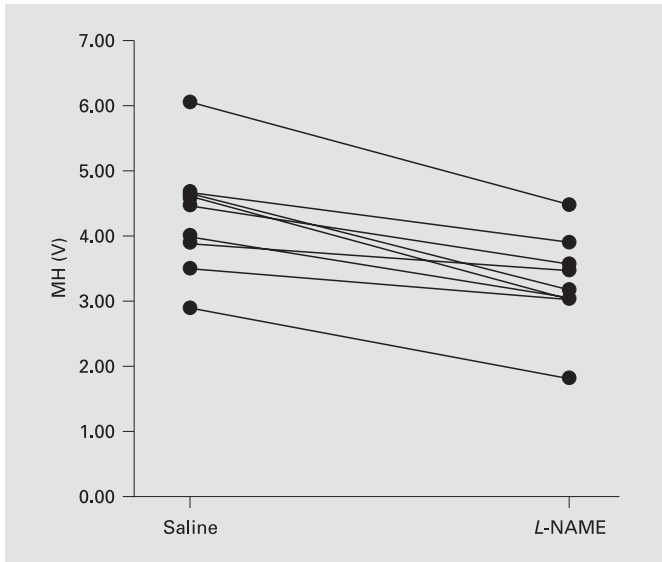
## Results

Inhibition of prostaglandin synthesis did not alter MH compared with placebo: placebo median MH 1.46 V (25th, 75th centiles: 1.09, 1.88), active therapy MH 1.50 V (1.31, 1.80) ( $p = 0.920$ , Wilcoxon signed rank test). In addition,  $H_1$  receptor inhibition did not alter MH: placebo 1.87 V (1.46, 1.99) compared with active therapy 1.90 V (1.55, 2.03) ( $p = 0.880$ ).

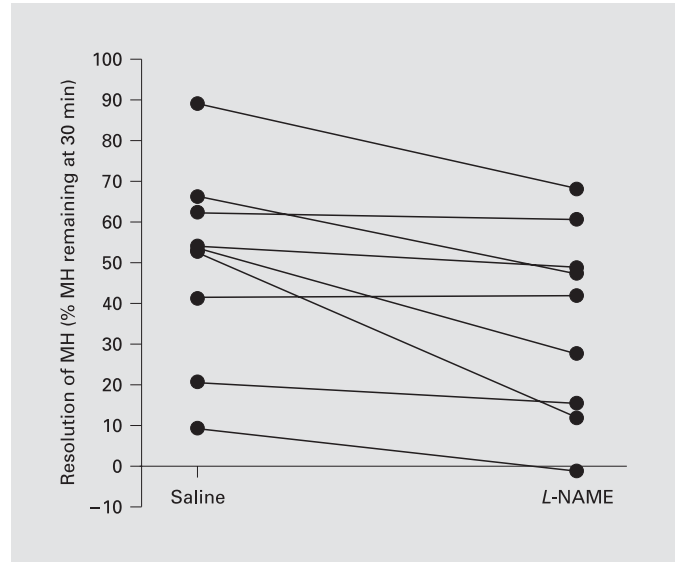
There was an increase in skin perfusion in the area surrounding the heater (fig. 1, 2). However, this increase was very small compared with the MH elicited under the heater probe. Indeed, only 1.9% of the surrounding tissue exceeded 80% of the maximum at 30 min (ranging from 0.1 to 0.8% of the surrounding area during the heating period).

MH was significantly attenuated (28%) by the inhibition of NOS: saline site 4.48 V (3.71, 4.70) compared with *L-NAME* site 3.25 V (3.10, 3.80) ( $p = 0.008$ ) (fig. 3). The inhibition of NOS enhanced the resolution of MH as demonstrated by the significant reduction in the percentage of MH remaining at 30 min after heating with *L-NAME*: placebo median percentage of MH remaining 30 min after heating was 54% (31, 65) compared with active therapy 42% (14, 55) ( $p = 0.011$ ) (fig. 4).

Sildenafil did not alter MH: placebo 4.43 V (3.24, 5.03) compared with sildenafil 4.17 V (3.57, 5.72) ( $p = 0.533$ ). The enhancement of the NO pathway significantly attenuated the resolution of MH: placebo percentage of MH remaining 30 min after heating 29% (25, 44) compared with active therapy 77% (56, 91) ( $p = 0.003$ ) (fig. 5).



**Fig. 3.** MH induced by local heating of the skin to 42°C for 30 min following NOS inhibition by *L*-NAME or placebo administration delivered to the site of heating by microdialysis in 9 healthy women. MH was significantly attenuated following NOS inhibition by *L*-NAME ( $p = 0.008$ , Wilcoxon signed rank test).

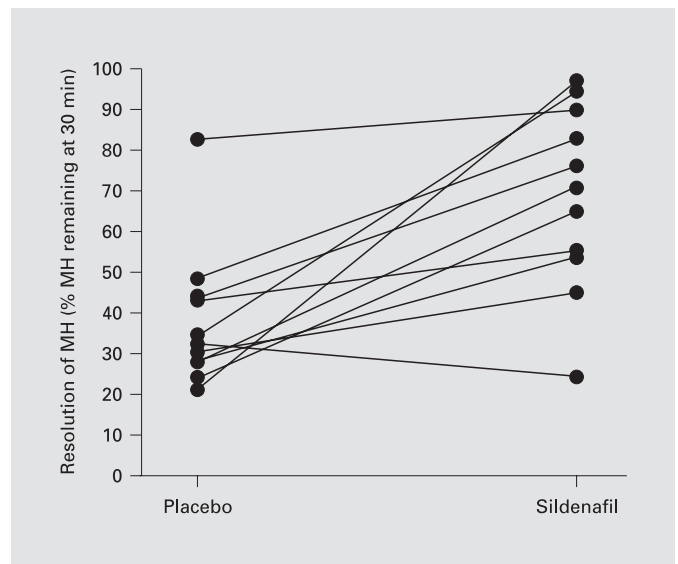


**Fig. 4.** Resolution of MH following the administration of *L*-NAME and saline by microdialysis in 8 healthy women. In the presence of *L*-NAME, there was a significantly lower percentage of MH remaining at 30 min compared with the saline site (control) ( $p = 0.011$ , Wilcoxon signed rank test).

## Discussion

This study has shown that NO, but neither prostaglandins nor histamine action via the H<sub>1</sub> receptor, is involved in the attainment of skin MH in response to locally heating a 0.76-cm<sup>2</sup> area of skin to 42–43°C for 30 min in healthy subjects. It is also unlikely that any axon reflex response is contributing to MH. In addition, it has been shown that NO is involved in the sustainability (the resolution) of MH following the removal of the heat stimulus.

The role of NO in skin MH was investigated by blocking its production prior to the start of and throughout heating with the use of the NOS inhibitor, *L*-NAME, and also by enhancement of the action of NO by preventing the breakdown of cGMP, a downstream signalling molecule of the NO pathway, again prior to the start of and throughout heating using sildenafil. The attenuation of MH by *L*-NAME clearly demonstrates that NO is a contributor to skin MH in response to local heating. This is in agreement with previous hyperaemic studies that heated to a similar temperature range (39–41°C) [12, 13], with one of the studies presenting submaximal hyperaemic responses (87% of maximum, with maximum assessed as hyperaemic response to 50 mM sodium nitroprusside)



**Fig. 5.** Resolution of MH following the administration of sildenafil and placebo in 11 healthy men. After the administration of sildenafil, there was a significantly higher percentage of MH remaining at 30 min compared with placebo administration ( $p = 0.003$ , Wilcoxon signed rank test).

[13]. Interestingly, the magnitude of the involvement of NO differs between the current maximal study and the previous hyperaemic studies, which show that NO is a major contributor to the skin hyperaemic response to local heating. One potential but unlikely explanation for this discrepancy could be the efficiency of NOS inhibition. Previous studies [12, 13] delivered either the same or a higher concentration of *L*-NAME at a slower flow rate ( $2 \mu\text{l}\cdot\text{min}^{-1}$ ) than the current study. Whilst these differences may account for some of the discrepancies, our findings that further inhibition of substance P or calcitonin gene-related peptide vasodilatation was not evident despite increasing *L*-NAME concentration from 5 to 10 and 20 mM [19] suggest this is not a likely explanation. In keeping with this conclusion, Kellogg et al. [12] demonstrated that increasing the dose of *L*-NAME from 5 to 20 mM did not further reduce the skin hyperaemic response to local heating. Thus, it is likely that NOS inhibition was maximal at 5 mM and that increasing the concentration of *L*-NAME would not have altered the findings of the current study.

Another potential reason for the discrepancies between this study and previous studies is a gender effect. Previous work has predominantly used male subjects, e.g., Kellogg et al. [12] only used 1 female subject, and 33% of the study population of Minson et al. [13] was female, whereas in the current *L*-NAME study, all the subjects were female. Gender has previously been shown to have a differential effect on NO both in humans and animals [21, 22], but in those studies, NO activity was shown to be higher in females, which is in the opposite direction from what the current observations may suggest. However, the observation that the endothelium-derived hyperpolarizing factor plays a more important role in female rats and NO in male rats does support the proposal for gender difference being a potential explanation for the discrepancies between the current and previous studies [23].

In contrast, sildenafil did not modulate skin MH, providing support for our previous observations that the response to local heating to  $42^{\circ}\text{C}$  is indeed maximal.

The inhibition and enhancement of the NO pathway also modulated the resolution (sustainability) of skin MH after heating. The inhibition of the NO pathway resulted in a more rapid recovery to baseline flow, as indicated by the observation that the *L*-NAME site had a significantly lower percentage of maximal blood flow remaining 30 min after the cessation of heating compared with control. The administration of sildenafil significantly attenuated the resolution of MH, resulting in a maintained

blood flow response following the removal of the heat stimulus, which is represented by a higher percentage of MH remaining 30 min after heating compared with placebo visit. Thus, NO is also involved in the sustainability of skin MH after heating. This finding warrants further investigation as it may provide a novel and non-invasive way of assessing NO activity in the skin microcirculation, which may aid our understanding of vascular health and pathophysiology.

The interpretation of the findings with sildenafil are complex as a number of additional pathways result in the production of cGMP, for example carbon monoxide (CO). CO can be produced in endothelial cells and then act on the smooth muscle cells as a vasodilator. The magnitude of its role and the quantity of CO in the skin microcirculation is not clear. Thus, it is possible that sildenafil is potentiating the effect of an additional cGMP pathway, e.g., CO and not NO in the current study. Nonetheless, it is known that soluble guanylate cyclase, an enzyme that produces cGMP, has a much greater affinity for NO than it does for CO [24]. In support of sildenafil mediating its effect via NO in the vascular system, it has been observed that the administration of the NOS inhibitor,  $\text{N}^{\text{G}}$ -monomethyl-*L*-arginine, abolished sildenafil augmentation of the forearm blood flow response to acetylcholine in young healthy men [25]. A further confounder in the interpretation of the findings with systemically administered sildenafil is its potential to reduce blood pressure. However, observations from this laboratory have observed no such decrease in blood pressure in either groups of young, healthy men or in type 2 diabetic men [unpubl. observations], which is in agreement with Dundar et al. [26]. Thus, it is unlikely that changes in systemic blood pressure influenced the findings of the current study.

Numerous studies have been carried out investigating the potential neurogenic mechanisms involved in the MH response to local heating. Previous studies have shown that the sympathetic system is not a significant contributor to the skin hyperaemic response to local heating to  $39\text{--}42^{\circ}\text{C}$  [27, 28]. However, it has been proposed that the activation of the axon reflex via the C fibre nociceptors with the release of the neuropeptides, substance P, and calcitonin gene-related peptide is the pathway responsible for the induction of skin MH in response to local heating [29]. Arguments against this proposal include the observations that acute and chronic capsaicin administration to stimulate (acute treatment) and deplete (chronic) substance P and calcitonin gene-related peptide release did not modulate MH in response to sustained local heat-

ing [30]. Evidence that an axon reflex is activated during local heating includes the observation of a flare response at an average temperature threshold of 39.4–39.6°C following short duration local heating (13.3-cm<sup>2</sup> area of skin) [30]. In addition, during prolonged (20–40 min) local heating (0.7-cm<sup>2</sup> area of skin) to 39–40°C, a bimodal response has been observed, with the initial response (first 5 min) attributed to an axon reflex and the secondary plateau phase ascribed to NO. The initial peak was shown to be attenuated by blocking the C fibre nociceptors using EMLA cream, but this had no effect on the NO-mediated secondary plateau phase [13]. The finding that only the initial peak and not the final hyperaemic response following sustained local heating is modulated by blocking the axon reflex argues against the proposal that the axon reflex is responsible for the hyperaemic response to prolonged local heating. It has been observed by others that sustained heating to 42°C, necessary for the induction of MH, is associated with a brief sensation of pain in a few subjects, and that in these cases, the hyperaemic response to local heating was not affected by inhibition of NO production by *L*-NAME. It was proposed by the authors that this response was mediated by the release of neuropeptides [12, 13] to evoke an axon reflex in these particular subjects. Historically, the MH technique used in our own laboratory is not associated with either a flare response or a sensation of pain, and results presented in this paper demonstrate that there is only a small increase in perfusion in skin surrounding the heater, which may possibly be neurogenic in nature or be a result of heat dissipation from the heated area, but which is unlikely to be responsible for generating MH.

Prostaglandins are likely candidates to contribute to the skin hyperaemic response to local heating as they have previously been shown to be involved in the regulation of the skin microcirculation; for example, they may contribute to the skin microvascular response to acetylcholine [31, 32]. However, in the current study, aspirin did not modulate MH, demonstrating that prostaglandins do not significantly contribute to skin MH. This is in agreement with a previous hyperaemic study that heated to a similar temperature (41°C) in healthy male subjects [11].

In addition, it was shown that histamine action via the H<sub>1</sub> receptor was not involved in the induction of MH. H<sub>1</sub> receptor inhibition has previously been shown to attenuate the skin hyperaemic response to whole body heating [33], and to practically eliminate the wheal and flare response to histamine [15–17, 34, 35]. Despite this effect, it is not possible to completely rule out histamine as a potential contributor to skin MH as its effects may be

mediated by the H<sub>2</sub> receptor. This is supported by the fact that both H<sub>1</sub> and H<sub>2</sub> receptors have been shown to mediate the effects of histamine in isolated human subcutaneous resistance arteries [36] and the skin vasodilatory response to the iontophoretic administration of histamine [37].

Although this study has shown that NO contributes to the induction of MH, a large proportion, approximately two thirds, of the MH response is unaccounted for by this pathway alone. A potential mediator that may also be involved is the endothelium-dependent hyperpolarizing factor, whose effect is functionally defined as an agonist-induced, endothelium-dependent relaxation that is not blocked by the inhibitors of NOS or cyclooxygenase [38]. Thus, the endothelium-dependent hyperpolarizing factor may be a potential contributor to skin MH in response to local heating. Further research is needed to clarify whether this or additional mediators/mechanisms are involved in the induction of skin MH.

In summary, this work has shown that NO is a significant but small contributor to skin MH in response to local heating of a small area of skin, but that prostaglandins and histamine action via the H<sub>1</sub> receptor are not involved in this response. It is also unlikely that any axon reflex response is contributing to MH. In addition, it has shown that NO is involved in sustaining the hyperaemic response after heating, such that removal of NO leads to a faster fall in blood flow and that by prolonging the action of cGMP sustains the blood flow at a higher level. This finding warrants further examination as it may provide a novel and non-invasive method of investigating NO activity in the skin microcirculation. These mechanistic observations may provide a platform for understanding the failings in microvascular function observed in diabetes, pre-diabetes and other vascular pathologies.

### Acknowledgements

K.M.G. was supported by the local diabetes charity DIRECT and the Wellcome Trust (grant number 032627/Z/96/A/MP/JF). M.M.H. was supported by a research fellowship grant from Pfizer. G.F.C. was supported by the Wellcome Trust (grant number 057474/99).

## References

- 1 Ijzerman RG, de Jongh RT, Beijl MAM, van Weissenbruch MM, Delamarre-van de Waal HA, Serne EH, Stehouwer CDA: Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. *Eur J Clin Invest* 2003;33: 536–542.
- 2 Khan F, Elhadd TA, Greene SA, Belch JJ: Impaired skin microvascular function in children, adolescents, and young adults with type 1 diabetes. *Diabetes Care* 2000;23:215–220.
- 3 Shore AC, Price KJ, Sandeman DD, Green EM, Tripp JH, Tooke JE: Impaired microvascular hyperaemic response in children with diabetes mellitus. *Diabet Med* 1991;8:619–623.
- 4 Sandeman DD, Pym CA, Green EM, Seamark C, Shore AC, Tooke JE: Microvascular vasodilatation in the feet of newly diagnosed non-insulin dependent diabetic patients. *BMJ* 1991; 302:1122–1123.
- 5 Lee BC, Appleton M, Shore AC, Tooke JE, Hattersley AT: Impaired maximum microvascular hyperaemia in patients with MODY 3 (hepatocyte nuclear factor-1alpha gene mutations). *Diabet Med* 1999;16:731–735.
- 6 Jaap AJ, Hammersley MS, Shore AC, Tooke JE: Reduced microvascular vasodilatory function in subjects at risk of developing type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1994;37:214–216.
- 7 Hannemann MM, Liddell WG, Shore AC, Clark PM, Tooke JE: Vascular function in women with previous gestational diabetes mellitus. *J Vasc Res* 2001;39:311–319.
- 8 Goh KL, Shore AC, Quinn M, Tooke JE: Impaired microvascular vasodilatory function in 3-month-old infants of low birth weight. *Diabetes Care* 2001;24:1102–1107.
- 9 Strain WD, Chaturvedi N, Leggetter S, Nihoyannopoulos P, Rajkumar C, Bulpitt CJ, Shore AC: Ethnic differences in skin microvascular function and their relation to cardiac target-organ damage. *J Hypertens* 2005;23:133–140.
- 10 Shore AC: Vascular biology and physiology; in Lowe GD, Tooke JE (eds): *A Textbook of Vascular Medicine*. Bath, Arnold, 1996, pp 7–42.
- 11 Golay S, Haerberli C, Delacahux A, Liaudet L, Kucera P, Waeber B, Feihl F: Local heating of human skin causes hyperaemia without mediation by muscarinic cholinergic receptors or prostanoids. *J Appl Physiol* 2004;97:1781–1786.
- 12 Kellogg DL, Liu Y, Kosiba IF, O'Donnell D: Role of nitric oxide in vascular effects of local warming of the skin in humans. *J Appl Physiol* 1999;86:1185–1190.
- 13 Minson CT, Berry LT, Joyner MJ: Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 2001; 91:1619–1626.
- 14 Heavey DJ, Barrow SE, Hickling NE, Ritter JM: Aspirin causes short-lived inhibition of bradykinin-stimulated prostacyclin production in man. *Nature* 1985;318:186–188.
- 15 Clough GF, Bennett AR, Church MK: Effects of H<sub>1</sub> antagonists on the cutaneous vascular response to histamine and bradykinin: a study using scanning laser Doppler imaging. *Br J Dermatol* 1998;138:806–814.
- 16 Denham KJ, Boutsouki P, Clough GF, Church MK: Comparison of the effects of desloratadine and levocetirizine on histamine-induced wheal, flare, and itch in human skin. *Inflamm Res* 2003;52:424–427.
- 17 Simons FE, Murray HE, Simons KJ: Quantitation of H<sub>1</sub>-receptor antagonists in skin and serum. *J Allergy Clin Immunol* 1995;95:759–764.
- 18 Boutsouki P, Georgiou S, Clough GF: Recovery of nitric oxide from acetylcholine-mediated vasodilatation in human skin in vivo. *Microcirculation* 2004;11:249–259.
- 19 Clough GF: Role of nitric oxide in the regulation of microvascular perfusion in human skin in vivo. *J Physiol* 1999;516:549–557.
- 20 Klede M, Clough GF, Lischetzki G, Schmelz M: The effect of the nitric oxide synthase inhibitor N-nitro-L-arginine-methyl ester on neuropeptide-induced vasodilation and protein extravasation in human skin. *J Vasc Res* 2003;40:105–114.
- 21 Sato A, Miura H, Liu Y, Somberg LB, Otterson MF, Demeure MJ, Schulte WJ, Eberhardt LM, Loberiza FR, Sakuma I, Gutterman DD: Effect of gender on endothelium-dependent dilation to bradykinin in human adipose microvessels. *Am J Physiol* 2002;283:H845–H852.
- 22 Taylor TA, Garipey CE, Pollock DM, Pollock JS: Gender differences in ET and NOS systems in ET-B receptor-deficient rats: effect of a high salt diet. *Hypertension* 2003;41:657–662.
- 23 Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ, Ahluwalia A: Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. *Circulation* 2005;111:796–803.
- 24 Furchgott RF, Jothianandan D: Endothelium-dependent and -independent vasodilation involving cyclic GMP relaxation induced by nitric oxide, carbon dioxide and light. *Blood Vessels* 1991;28:52–61.
- 25 Kimura M, Higashi Y, Hara K, Noma K, Sasaki S, Nakagawa K, Goto C, Oshima T, Yoshizumi M, Chayama K: PDE5 inhibitor sildenafil citrate augments endothelium-dependent vasodilation in smokers. *Hypertension* 2003; 41:1106–1110.
- 26 Dunder M, Kocak I, Dunder SO, Erol H: Evaluation of side effects of sildenafil in a group of young healthy volunteers. *Int Urol Nephrol* 2001;32:705–708.
- 27 Charkoudian N, Eisenach JH, Atkinson JLD, Fealey RD, Joyner MJ: Effects of chronic sympathectomy on locally mediated cutaneous vasodilation in humans. *J Appl Physiol* 2002;92: 685–690.
- 28 Pergola PE, Kellogg DL, Johnson JM, Kosiba WA, Solomon DE: Role of sympathetic nerves in the vascular effects of local temperature in human forearm skin. *Am J Physiol* 1993;265: H785–H792.
- 29 Magrerl W, Treede RD: Heat-evoked vasodilatation in human hairy skin: axon reflexes due to low-level activity of nociceptive afferents. *J Physiol* 1996;497:837–848.
- 30 Charkoudian N, Fromy B, Saumet JL: Reflex control of the cutaneous circulation after acute and chronic local capsaicin. *J Appl Physiol* 2001;90:1860–1864.
- 31 Khan F, Davidson NC, Littleford RC, Litchfield SJ, Struthers AD, Belch JJ: Cutaneous vascular responses to acetylcholine are mediated by a prostanoid-dependent mechanism in man. *Vasc Med* 2000;2:82–86.
- 32 Noon JP, Walker BR, Hand MF, Webb DJ: Studies with iontophoretic administration of drugs to human dermal vessels in vivo: cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. *Br J Clin Pharmacol* 1998;45:545–550.
- 33 Wong BJ, Wilkins BW, Minson CT: H<sub>1</sub> but not H<sub>2</sub> histamine receptor activation contributes to the rise in skin blood flow during whole body heating. *J Physiol* 2004;560:941–948.
- 34 Clough GF, Boutsouki P, Church MK: Comparison of the effects of levocetirizine and loratadine on histamine-induced wheal, flare, and itch in human skin. *Allergy* 2001;56:985–988.
- 35 Frossard N, Melac M, Benabdesselam O, Pauli G: Consistency of the efficacy of cetirizine and ebastine on skin reactivity. *Ann Allergy Asthma Immunol* 1998;80:61–65.
- 36 Van de Voorde J, Delaey C, Depypere H, Vanheel B: Mechanisms involved in the vasorelaxing influence of histamine on isolated human subcutaneous resistance arteries. *Eur J Pharmacol* 1998;349:61–66.
- 37 Grossmann M, Jamieson MJ, Kirch W: Histamine response and local cooling in the human skin: involvement of H<sub>1</sub>- and H<sub>2</sub>-receptors. *Br J Clin Pharmacol* 1999;48:216–222.
- 38 Campbell WB, Harder DR: Prologue: EDHF – what is it? *Am J Physiol* 2001;280:H2413–H2416.