Neurokinin-1 receptor desensitization to consecutive microdialysis infusions of substance P in human skin

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The neuropeptide substance P is known to be localized in nerve terminals in human skin and substance P-induced vasodilatation is believed to be partially dependent on nitric oxide (NO) and H1 histamine receptor activation. Unlike other neuropeptides investigated in human skin, substance P-induced vasodilatation has been shown to decline during continuous infusion, possibly suggestive of an internalization of neurokinin-1 (NK_1) receptors, which are highly specific to substance P. However, questions remain regarding these mechanisms in human skin. Fifteen subjects participated in this series of studies designed to investigate the effect of consecutive infusions and possible mechanisms of substance P-induced vasodilatation in human skin. Two concentrations of substance P (10 μM and 20 μM) were tested via intradermal microdialysis in two groups of subjects. Site 1 served as a control and received substance P only. Site 2 received substance P combined with 10 mM L-NAME to inhibit NO synthase. Site 3 received substance P combined with 500 μM pyrilamine, an H1 receptor antagonist. Site 4 received substance P combined with 10 mM L-NAME plus 500 μM pyrilamine. Red blood cell (RBC) flux was measured via laser-Doppler flowmetry to provide an index of skin blood flow. Cutaneous vascular conductance was calculated as RBC flux/mean arterial pressure and was normalized to maximal vasodilatation via 28 mM sodium nitroprusside. Substance P was perfused through each microdialysis fibre at a rate of 4 μl min^{-1} for 15 min. The subsequent increase in skin blood flow was allowed to return to baseline ($\sim$45–60 min) and a stable 5 min plateau was used as a new baseline (post-infusion baseline). A second dose of substance P was then delivered to the skin and skin blood flow was monitored for 45–60 min. Substance P produced a dose-dependent increase in skin blood flow with the concentrations of substance *P* tested, which was significantly attenuated in the presence of L-NAME and the combination of L-NAME plus pyrilamine. However, substance P-induced vasodilatation was unaffected in the presence of pyrilamine. There was no significant difference between the L-NAME-only sites and the L-NAME plus pyrilamine sites. Importantly, the second dose of substance P did not produce a significant increase in skin blood flow compared to the initial baseline or the post-infusion baseline. These data suggest substance P-induced vasodilatation delivered via microdialysis contains an NO component but does not contain an H1 receptor activation component at the doses tested. Additionally, these data provide evidence for NK_1 receptor desensitization as there was no observable increase in skin blood flow following a second administration of substance P. This may provide a useful model for studying the role of substance P in the control of skin blood flow in humans.

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Vasodilatation in human skin has been shown to involve a complex interaction between locally produced vasodilator substances such as nitric oxide (NO), and neurally released substances such as neuropeptides, including vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P. Substance P is thought to play an important role in several vasodilatory pathways. For example, substance P has been implicated in the wheal and flare reaction as well as in axon reflex-mediated vasodilatation to local heating. substance P is an undecapeptide and binds with high affinity to the neurokinin-1 (NK_1) receptor (Quartara & Maggi, 1997). Vasodilatation to substance P has been shown to be

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dependent on an intact endothelium and on the NK_1 receptor located on the endothelium (Jansen *et al.* 1991; Stjärne *et al.* 1994; Holzer, 1998).

Interestingly, studies in human skin have shown that substance P-induced vasodilatation begins to decline during a 30 min infusion period (Weidner *et al.* 2000; Klede *et al.* 2003). It has been postulated that the decrease in skin blood flow during substance P administration is due to internalization of the NK_1 receptor upon binding of substance P (Quartara & Maggi, 1997; Weidner *et al.* 2000; Klede *et al.* 2003). Internalization and desensitization of NK_1 receptors has been observed in epithelial cells (Garland *et al.* 1994), neurones (Grady *et al.* 1996; McConalogue *et al.* 1998; Mann *et al.* 1999; Jenkinson *et al.* 2000) and endothelial cells (Bowden *et al.* 1994). However, it remains unclear if NK_1 receptors in the cutaneous vasculature internalize or become desensitized as the above studies did not determine the effects of a second infusion of substance P on skin blood flow (Weidner *et al.* 2000; Klede *et al.* 2003). If there is indeed NK_1 receptor internalization or desensitization in the cutaneous vasculature, this could potentially be a useful model for studying the mechanisms of skin blood flow in which substance P is thought to be involved, such as reactive hyperaemia, local heating (i.e. thermal hyperaemia), and active vasodilatation. Thus, it is of importance to further investigate the possibility of NK_1 receptor desensitization in the cutaneous circulation.

It has been suggested that substance P-induced vasodilatation is mediated, in part, by NO and histamine (Barnes *et al.* 1986; Boolell & Tooke, 1990; Petersen *et al.* 1994, 1997; Huttunen *et al.* 1996; Weidner *et al.* 2000; Klede *et al.* 2003). Klede *et al.* (2003) have recently demonstrated an NO component to substance P-induced vasodilatation using two low concentrations of substance P (10^{-7} and 10^{-6} M). However, the contribution of H1 receptor activation to substance P-induced vasodilatation remains equivocal. Using the microdialysis technique, Weidner *et al.* (2000) were unable to attenuate substance P-mediated vasodilatation with an H1 receptor antagonist and were only able to detect measurable increases in skin histamine with a high concentration of substance P (10^{-5} M). The H1 isoform of the histamine receptor has been located on endothelial cells and vasodilatation to H1 receptor activation has been shown to be secondary to the production of local vasodilators such as NO (Hill, 1990; Clough, 1999; Brown & Roberts, 2001). Additionally, our laboratory (Wilkins *et al.* 2004) has recently demonstrated that the majority of VIP-mediated vasodilatation in human skin can be attenuated in the presence of a NO synthase inhibitor and H1 receptor antagonist. Therefore, it is possible that by blocking both H1 receptors and NO synthase, a greater role for H1 receptor activation in the infusion of substance P may be unmasked. However, no studies to date have examined the combined effect of an NO synthase inhibitor and H1 receptor antagonist on substance P-induced vasodilatation in human skin. Furthermore, if NK_1 receptors internalize or become desensitized upon binding substance P, it is unclear if this receptor internalization or desensitization is affected by NO or H1 receptors.

Based on the above studies, our understanding of the specific mechanisms underlying substance P-induced vasodilatation in human skin remains unclear. The purpose of the present investigation was to investigate mechanisms of substance P-induced vasodilatation in human skin using the microdialysis technique. Specifically, we sought to provide more direct evidence of NK_1 receptor desensitization in the cutaneous vasculature by administering two consecutive doses of substance P via the microdialysis technique. The concentrations of substance P used in this study were chosen to elevate skin blood flow to levels similar to those observed during reactive hyperaemia, thermal hyperaemia and active vasodilatation in order to test the following hypotheses. First, the cutaneous vasodilatation following a second infusion of substance P would be abolished in all sites and would provide evidence for NK_1 receptor desensitization in the cutaneous vasculature. Second, substance P-induced vasodilatation would be attenuated in the presence of an NO synthase inhibitor and H1 antagonist.

Methods

Subjects

All methods were approved by the Institutional Review Board of the University of Oregon and conformed to the standards as set forth in the *Declaration of Helsinki*. All subjects gave written informed consent prior to participation. Eleven men (22 ± 1 years) and four women (21 ± 1 years) participated in this series of studies. All subjects were healthy, normotensive, and non-smokers. All studies were performed in a thermoneutral laboratory with subjects in the supine position and the experimental arm at heart level.

Instrumentation

Subjects' blood pressure was measured via automated brachial auscultation (Cardiocap, Datex-Ohmeda, Tewksbury, MA, USA) every 5 min for the duration of the protocol to ensure changes in skin blood flow were not influenced by changes in perfusion pressure.

To investigate mechanisms of substance P-induced vasodilatation in human skin, subjects were instrumented with four microdialysis fibres (MD 2000, Bioanalytical Systems, West Lafayette, IN, USA) on the ventral surface of the forearm. Briefly, a 25 gauge needle was placed in the dermal layer of the skin in the absence of anaesthaesia. The microdialysis fibre was then threaded

through the lumen of the needle. The needle and microdialysis fibre were then pulled through the skin leaving the membrane of the microdialysis fibre in the dermal layer of the skin and completely removing the needle. The membranes of the microdialysis fibres had a 10 mm long membrane with a 20 kDa molecular weight cutoff with a functional cutoff of approximately 5 kDa. Placement of the microdialysis fibres results in a minor trauma hyperaemia that subsides in approximately 60–90 min. During the trauma resolution period, each microdialysis fibre was perfused with sterile lactated Ringer solution (Abbot Laboratories, North Chicago, IL, USA) at a rate of 2 μl min^{-1} with a microinfusion pump (CMA 102, CMA Microdialysis, Stockholm, Sweden).

Red blood cell (RBC) flux was monitored via laser-Doppler flowmetry (Moor LAB, Moor Instruments, Devon, UK) to obtain an index of skin blood flow. Integrated laser-Doppler flow probes were placed on the surface of the skin directly above each microdialysis membrane.

Drugs

Substance P (Sigma, St Louis, MO, USA) was dissolved in sterile lactated Ringer solution to concentrations of 10 and 20 μM. Extensive pilot work was performed in order to determine that these concentrations of substance P elicited consistent and reproducible increases in skin blood flow similar to those observed during reactive hyperaemia, thermal hyperaemia and whole body heat stress. A 10 mM solution of N^G-nitro-L-arginine methyl ester (L-NAME; Calbiochem, San Diego, CA, USA) dissolved in sterile lactated Ringer solution was used to inhibit NO synthase. It has been shown previously that this concentration of L-NAME adequately inhibits NO in human skin (Kellogg *et al.* 1999; Minson *et al.* 2001). Pyrilamine (500 μM; Sigma) was used as an H1 histamine receptor antagonist. Our laboratory has demonstrated that this concentration of pyrilamine is adequate to attenuate the rise in skin blood flow to exogenous histamine and VIP as well as during whole body heat stress (Wilkins *et al.* 2004; Wong *et al.* 2004). Maximal skin blood flow was achieved by perfusing each microdialysis fibre with 28 mM sodium nitroprusside (Abbott Laboratories, North Chicago, IL, USA), which has been shown previously to elicit maximal vasodilatation in human skin (Kellogg *et al.* 1999; Minson *et al.* 2001).

Protocol

Figure 1 is a schematic diagram depicting the general experimental design. Subjects were randomly assigned to receive 10 or 20 μM substance P. For each concentration of substance P investigated, each microdialysis site was randomly assigned to receive one of four treatments. Site 1 served as a control and received a given concentration of substance P only. Site 2 received the same concentration of substance P combined with L-NAME. This site was used to determine the contribution of NO to substance P-induced vasodilatation. Site 3 received substance P combined with pyrilamine. This site was used to investigate the contribution of H1 receptor activation to substance P-induced vasodilatation. Site 4 received substance P combined with L-NAME and pyrilamine and was used to investigate any possible interaction between

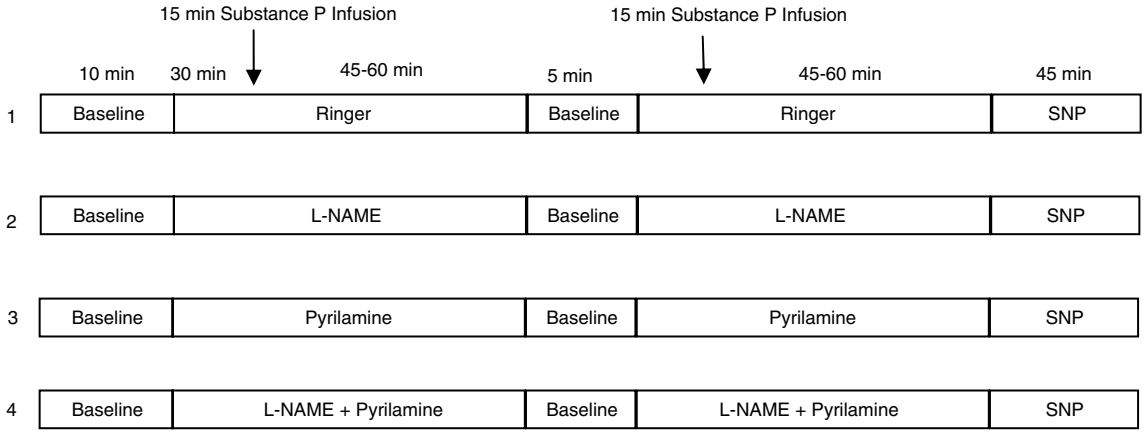

Figure 1. Schematic drawing of the general experimental protocol
Numbers on left side refer to microdialysis sites. Site 1 served as a control and received a dose of substance P with Ringer solution only. Site 2 received substance P in the presence of L-NAME to inhibit NO synthase. Site 3 received substance P in the presence of pyrilamine to antagonize H1 histamine receptors. Site 4 received substance P in the presence of L-NAME plus pyrilamine. Concentrations of substance P used were 10 and 20 μM infused for 15 min at a rate of 4 μl min^{-1}. NO, nitric oxide; L-NAME, N^G-nitro-L-arginine methyl ester; SNP, sodium nitroprusside.

NO and H1 receptor activation and substance P-induced vasodilatation.

Following the trauma resolution period, baseline data were collected for a period of 5–10 min. Sites 2–4 were then perfused with the respective drug treatments for a period of 30–45 min to inhibit NO synthase and to antagonize H1 histamine receptors. Substance P was perfused through each microdialysis fibre at a rate of 4 μl min^{-1} for 15 min. Based on the infusion rate and time, a dose of approximately 600 pmol and 1200 pmol of substance P were delivered to the microdialysis membrane for the 10 μM and 20 μM substance P concentrations, respectively. Substantial pilot work was performed to determine this infusion rate and the time period that elicited the most consistent skin blood flow responses to substance P. Sites 2–4 were perfused with substance P combined with the respective drug treatments during the 15 min substance P stimulation period. At the end of the 15 min infusion period, the microdialysis fibres were switched back to their respective drug treatments and the perfusion pump rate was returned to 2 μl min^{-1}.

The subsequent increase in skin blood flow following administration of substance P to the skin was allowed to return to baseline levels (~45–60 min). After establishing baseline skin blood flow for a period of 5–10 min, a second dose of substance P was perfused through the microdialysis fibres at 4 μl min^{-1} for 15 min. The second dose of substance P was administered in order to investigate the possibility of NK_1 receptor desensitization upon the binding of the first dose of substance P. At the end of the 15 min stimulation period, each site was returned to its respective drug treatment at an infusion rate of 2 μl min^{-1} and skin blood flow was monitored for an additional 45–60 min. Sodium nitroprusside was then perfused through each site to achieve maximal vasodilatation.

Follow-up protocol

The purpose of the follow-up protocol was to examine further the duration of the observed NK_1 receptor desensitization up to 3 h after the first substance P infusion. Two subjects were equipped with four microdialysis fibres and laser-Doppler flow probes as described above. A 10 μM dose of substance P was perfused through the microdialysis sites as described above. Following the first substance P stimulation period, a second dose of substance P was perfused through the microdialysis fibres at four different time points. These time points were 1.5, 2, 2.5 and 3 h after the first substance P infusion. Skin blood flow was monitored for an additional 30 min following the second substance P infusion. Following the infusion of substance P at 3 h and the 30 min post-infusion monitoring period, maximal vasodilatation was achieved in all sites via 28 mM SNP infusion.

Data acquisition and analysis

Data were digitized and stored at 20 Hz on a personal computer and analysed off-line using signal processing software (Windaq, Dataq Instruments, Akron, OH, USA). All skin blood flow values were analysed by averaging laser-Doppler flux values over a stable 5–10 min period. Cutaneous vascular conductance (CVC) was calculated as RBC flux divided by mean arterial pressure and normalized to maximal vasodilatation via perfusion with sodium nitroprusside. All data are presented as a percentage of maximal CVC (%CVC_{max}).

Cutaneous vascular conductance at the plateau in skin blood flow following substance P infusion was analysed for each concentration of substance P and for each drug treatment. To determine if substance P produced a dose-dependent increase in skin blood flow across the three concentrations used in this study, the increase in skin blood flow for each concentration of substance P in control sites was compared using a one-way ANOVA. In order to determine the contribution of NO and H1 histamine receptor activation to substance P-induced vasodilatation, CVC values at plateau in skin blood flow following substance P administration in each microdialysis site were compared using a one-way repeated measures ANOVA for each concentration of substance P tested. Initial baseline skin blood flow was compared to baseline following the first infusion of substance P (termed 'post-infusion baseline') using paired *t* tests for each concentration of substance P tested and for each drug treatment. For a given concentration of substance P and drug treatment, first and second plateau values following substance P infusion were compared using paired *t* tests. A one-way repeated measures ANOVA was used to compare initial baseline CVC, plateau in CVC to the first substance P infusion, post-infusion baseline CVC and plateau in CVC to the second substance P infusion for a given concentration of substance P and drug treatment. For all ANOVA statistical analyses, Holm-Sidak *post hoc* analysis was used to determine where significant differences occurred. A P value < 0.05 was considered statistically significant and all data are presented as mean ± S.E.M.

Results

Figure 2 is a representative skin blood flow tracing to the first and second 20 μM substance P infusions. Depicted are initial baseline, plateau in CVC to the first substance P infusion, post-infusion baseline, and plateau in CVC to the second substance P infusion. Baseline skin blood flow was unaffected by L-NAME, pyrilamine, or L-NAME plus pyrilamine.

The skin blood flow response to the first substance P infusion in control sites resulted in a dose-dependent increase in CVC for the two concentrations of

substance P examined in this study. Average CVC response to 10 and 20 μM substance P was 39 ± 2 and $50 \pm 2\%CVC_{max}$, respectively ($P < 0.05$ for all conditions; Fig. 3*A*).

Figure 3*A–D* shows group data for the plateau in CVC to the first and second substance P infusions for all three concentrations of substance P used and in all treatment sites. For statistical comparison, the CVC response to each concentration of substance P and drug treatment was compared to its respective initial and post-infusion baseline. For the clarity of the graphical presentation, the initial baseline for all three concentrations of substance P under each treatment condition has been grouped as there was no significant difference between initial baseline values. Similarly, post-infusion baseline CVC data have been grouped.

In control sites (Fig. 3*A*), there was no significant difference between initial and post-infusion baseline in either 10 μM or 20 μM substance P sites. The increase in CVC to the first infusion of substance P was significantly greater than initial baseline, post-infusion baseline and plateau to the second substance P infusion ($P < 0.01$ for all conditions). There was no significant difference between post-infusion baseline and plateau in CVC to the second infusion of substance P.

In sites treated with L-NAME (Fig. 3*B*), there was no significant difference between initial and post-infusion baseline in 10 and 20 μM substance P sites. The increase in CVC to the first infusion of 10 μM ($18 \pm 3\%CVC_{max}$) and 20 μM ($26 \pm 5\%CVC_{max}$; $P < 0.01$ for all conditions) substance P was significantly reduced compared to control and pyrilamine sites. The increase in CVC to the first infusion was significantly greater than initial baseline, post-infusion baseline, and plateau in CVC to the second substance P infusion. There was no significant difference between initial baseline, post-infusion baseline, and plateau in CVC to the second infusion of substance P. The plateau in CVC to the second substance P infusion was significantly attenuated compared to the second plateau in control and pyrilamine sites for both 10 and 20 μM substance P ($P < 0.01$ for all conditions).

Pyrilamine (Fig. 3*C*) had no effect on the plateau in CVC to the first substance P infusion compared to control sites. There was no significant difference between initial and post-infusion baseline in 10 μM and 20 μM substance P sites. The increase in CVC to the first infusion was significantly greater than initial baseline, post-infusion baseline, and plateau in CVC to the second infusion ($P < 0.01$ for all conditions). There was no significant difference between initial baseline, post-infusion baseline, and plateau in CVC to the second infusion.

L-NAME plus pyrilamine (Fig. 3*D*) significantly reduced the first CVC response to 10 μM ($19 \pm 2\%CVC_{max}$) and 20 μM ($25 \pm 3\%CVC_{max}$) substance P compared to control and pyrilamine sites ($P < 0.01$ for all conditions) but there was no difference compared to L-NAME sites. The increase in CVC to the first infusion was significantly greater than initial baseline, post-infusion baseline, and plateau in CVC to the second infusion ($P < 0.01$ for all conditions). There was no significant difference between initial baseline, post-infusion baseline, and plateau in CVC to the second substance P infusion. The plateau in CVC to the second substance P infusion was significantly attenuated compared to the second plateau in control and pyrilamine sites for both 10 and 20 μM substance P ($P < 0.01$ for all conditions).

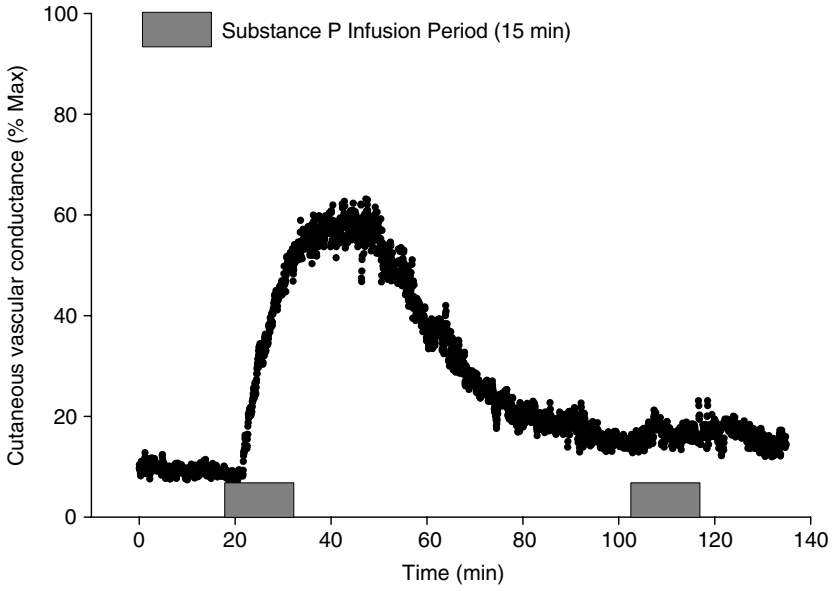

Figure 2. Representative tracing of the skin blood flow response to the first and second substance P infusions
Tracing is from a 20 μM control site from one subject. Note the lack of an increase in cutaneous vascular conductance response (CVC) in response to a second 20 μM substance P infusion. Grey box indicates substance P infusion period.

Follow-up protocol

The initial baseline skin blood flow for the two subjects in the follow-up protocol averaged $9 \pm 3\%CVC_{max}$. The plateau in CVC to the first 10 μM substance P infusion averaged $43 \pm 4\%CVC_{max}$. After 1.5 h, the post-infusion baseline averaged $11 \pm 3\%CVC_{max}$. The plateau in CVC to the second substance P infusion averaged $13 \pm 2\%CVC_{max}$. Two hours after the first substance P infusion, the post-infusion baseline averaged $10 \pm 2\%CVC_{max}$ and the plateau in CVC to the second substance P infusion averaged $13 \pm 3\%CVC_{max}$. After 2.5 h, the post-infusion baseline averaged $8 \pm 2\%CVC_{max}$. The plateau in CVC to the second substance P infusion averaged $11 \pm 2\%CVC_{max}$. Three hours after the first substance P infusion, the post-infusion baseline averaged $8 \pm 2\%CVC_{max}$. The plateau in CVC to the second substance P infusion averaged $21 \pm 3\%CVC_{max}$, which was significantly greater than the initial and post-infusion baseline ($P < 0.01$). However, the plateau in CVC to the second substance P infusion was significantly less than the plateau in CVC to the first substance P infusion ($P < 0.01$). These data suggest that the NK_1 receptors remain desensitized for up to 2.5 h and begin to regain sensitivity approximately 3 h after the first substance P infusion period.

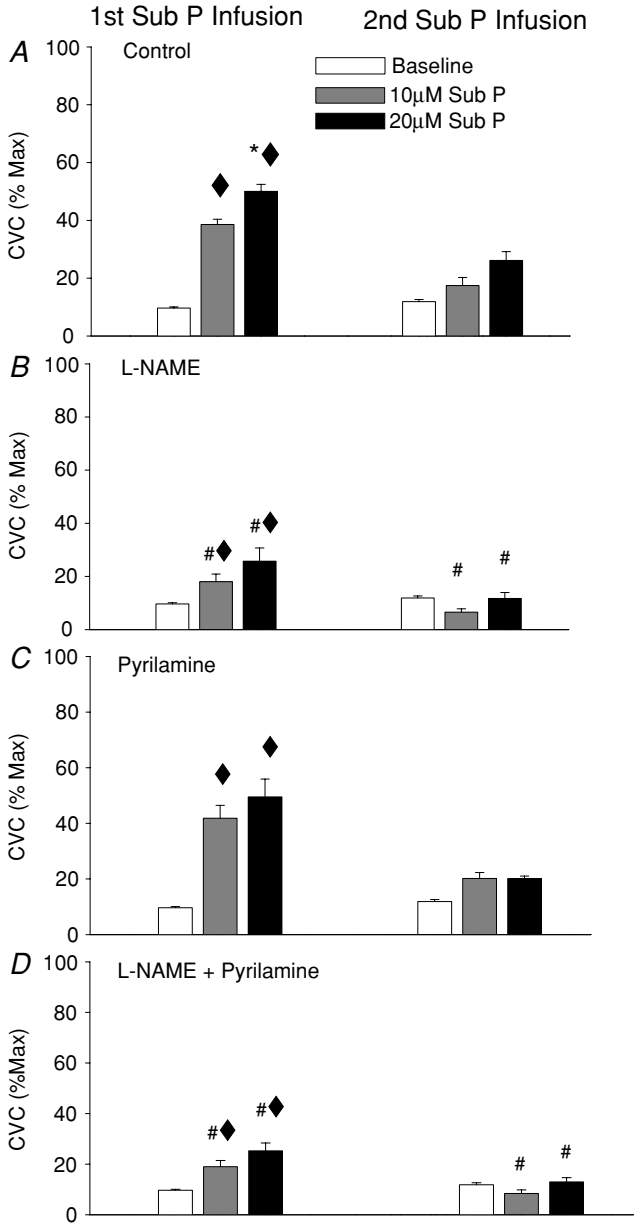

Figure 3. CVC response to the first and second infusions of 10 and 20 μM substance P in L-NAME and pyrilamine
A, control site; *B*, L-NAME site; *C*, pyrilamine site; *D*, L-NAME plus pyrilamine site.*Significantly greater than 10 μM plateau. ♦ Significantly greater than both baselines and plateau to 2nd substance P infusion. # Significantly attenuated compared to control and pyrilamine sites.

Discussion

The novel finding from this study is that NK_1 receptors in the cutaneous vasculature become desensitized following consecutive infusions of substance P for up to 2.5 h after skin blood flow returns to baseline following an initial infusion. This was evidenced by a greatly attenuated, and, in most cases, abolished, skin blood flow response to a second infusion of substance P (Fig. 3*A–D*). Additionally, we found substance P-induced vasodilatation at the concentrations tested contains an NO but not an H1 receptor component, and that H1 receptor activation does not account for any portion of the NO component (Fig. 3*B–D*).

Substance P is known to bind preferentially to the NK_1 receptor and this receptor is known to be a G protein-coupled receptor, where the intracellular carboxyl terminus interacts with G proteins (Regoli *et al.* 1994; Khawaja, 1996). The NK_1 receptor works through the phospholipase C–inositol triphosphate (IP_3) second messenger system to increase intracellular Ca^{2+} concentration and has been shown to undergo a process of endocytosis when bound with a ligand (Krause *et al.* 1990; Regoli *et al.* 1994; Mann *et al.* 1999). This process involves phosphorylation of NK_1 receptors by G protein receptor kinases followed by endocytosis via endosomes (McConalogue *et al.* 1998; Roush *et al.* 1999). In endosomes, ligand and receptor dissociate from each other, the NK_1 receptor is dephosphorylated, and is ultimately recycled back to the cell surface (Grady *et al.* 1995, 1996, 1997; Mukherjee *et al.* 1997). The process of NK_1 receptor endocytosis and recycling has been shown to occur in a number of tissues, including neurones (Grady *et al.* 1996; McConalogue *et al.* 1998; Mann *et al.* 1999; Jenkinson *et al.* 2000), epithelial cells (Garland *et al.* 1994), and endothelial cells (Bowden *et al.* 1994). However, no study has provided clear evidence of this phenomenon in human skin *in vivo*.

Previous studies in human skin have reported a progressive decrease in substance P-induced vasodilatation after the initial rise during a single 30 min substance P infusion and have suggested that this decrease in skin blood flow is due to internalization of NK_1 receptors (Weidner *et al.* 2000; Klede *et al.* 2003). However, the proposed concept of NK_1 receptor internalization in the cutaneous vasculature can only be taken as speculation as the above studies did not determine the skin blood flow response to a subsequent substance P infusion and did not perform receptor labelling studies.

It has been suggested that endocytosis, or internalization, of the NK_1 receptor is not required to render the receptors desensitized (Garland *et al.* 1996). Rather, the process of desensitization is due to phosphorylation of the NK_1 receptors while the process of endocytosis and recycling is required for resensitization of the receptors (Garland *et al.* 1996). Along these lines, the NK_1 receptor has been shown to have multiple sites for phosphorylation by G protein receptor kinases and protein kinase C (PKC) and it has been shown, *in vitro,* that sequential cleavage of the carboxyl terminus attenuates receptor desensitization but does not affect its functional properties, suggesting that the carboxyl terminus may be the site of phosphorylation required for receptor desensitization (Sasakawa *et al.* 1994; Garland *et al.* 1996).

In the majority of subjects we observed an attenuated CVC response to the second dose of substance P. However, in a few subjects there was a slight increase in skin blood flow to the second dose. In these few subjects, the increase in CVC to the second dose may be related to differential processes of desensitization and internalization. Recently, Bennett *et al.* (2005) have shown that NK_1 receptor resensitization occurs prior to NK_1 receptor recycling to the cell surface. In this study, Bennett *et al.* (2005) suggest that resensitization of the receptors occurs at the level of the G proteins and that there may be two different NK_1 receptors, one type which undergoes internalization and another type which does not undergo internalization upon ligand binding. In this scenario, receptors that undergo internalization would need to be recycled back to the cell surface in order to be resensitized. However, receptors that remain at the cell surface regain sensitivity due to an increased ability to couple to G proteins and may partially explain the slight increase in CVC in a few subjects to the second dose of substance P.

Despite our observation of an attenuated/abolished CVC response to a second infusion of substance P, the data from our study suggest that there is NK_1 receptor desensitization but do not allow us to distinguish the molecular mechanism(s) involved. For this reason, we have chosen to use the term NK_1 receptor *desensitization* as opposed to *internalization.* Thus, findings from the present study provide evidence suggesting NK_1 receptor *desensitization* upon binding substance P in the cutaneous vasculature as evidenced by a significantly attenuated/abolished plateau in skin blood flow in response to a second infusion of substance P for all concentrations and drug treatments tested (Fig. 3).

The observations that NK_1 receptors work via the phospholipase C–IP_3 second messenger system and have phosphorylation sites for PKC raise the question as to which second messenger system mediates receptor desensitization and which mediates the cutaneous vasodilatation, or if the pathways are redundant. As the phosphorylation sites on the NK_1 receptor are intracellular and the IP_3 pathway increases intracellular Ca^{2+} concentration (Sasakawa *et al.* 1994; Garland *et al.* 1996), a possible scenario is that the PKC pathway is involved in receptor desensitization while the phospholipase C–IP_3 pathway is involved in the cutaneous vasodilatation. In this scenario, the binding of substance P to the NK_1 receptor would activate G proteins and, subsequently, PKC, which would phosphorylate the receptor and render it desensitized. Furthermore, binding of substance P would activate phospholipase C and increase IP_3, which would subsequently increase intracellular Ca^{2+} levels, and possibly increase the production of NO, which seems plausible based on the data from this study. However, we cannot make conclusions on this issue directly based on the data from the present study and more direct research is needed to fully elucidate the second messenger pathways involved in receptor desensitization and cutaneous vasodilatation.

Data from our follow-up protocol suggest NK_1 receptors in the cutaneous vasculature remain desensitized for up to 2.5 h after the initial infusion of substance P, as we did not observe a significant increase in skin blood flow to a subsequent dose of substance P for up to 2.5 h after the initial dose. However, 3 h after the first substance P infusion, we observed a significant increase in skin blood flow compared to both the initial and post-infusion baseline. Although this substance P-induced vasodilatation was significantly less than the vasodilatation to the first infusion, these data suggest that the NK_1 receptors begin to regain sensitivity approximately 3 h after the initial infusion. A study by Jenkinson *et al.* (2000) using rat myenteric neurones has suggested that the recycling of NK_1 receptors requires approximately 120–180 min. The findings from our follow-up protocol are consistent with the report by Jenkinson *et al.* (2000) and it appears that NK_1 receptor recycling, and subsequent resensitization, in the cutaneous vasculature requires a similar amount of time to that reported for rat myenteric neurones.

A secondary goal of our study was to investigate further the contribution of NO and H1 receptor activation to

substance P-induced vasodilatation using concentrations of substance P that elicited substantial increases in skin blood flow. Furthermore, we sought to determine if there was an interaction between NO and/or H1 receptor activation to substance P infusion or to the observed NK_1 receptor desensitization. Our results demonstrate that the plateau in CVC to the first microdialysis infusion of substance P produces a dose-dependent increase in skin blood flow and that this vasodilatation contains a substantial NO component, but does not contain an H1 receptor activation component at the concentrations used in this study. Furthermore, we found H1 receptor activation does not account for any portion of the NO component to substance P-induced vasodilatation in our study. An interesting finding from this study was the observation that the plateau in CVC to the second substance P infusion observed in some subjects could be further reduced in the presence of an NO synthase inhibitor compared to both control and pyrilamine sites (Fig. 3), suggesting a possible interaction between NK_1 receptors and NO. However, there appeared to be no additional effect of an H1 receptor antagonist on NK_1 receptor desensitization (Fig. 3).

Our findings of an NO component to substance P-induced vasodilatation are in accord with the findings of Klede *et al.* (2003) who observed an attenuated vasodilatation to two low concentrations of substance P (10^{-7} M and 10^{-6} M) delivered via microdialysis in the presence of an NO synthase inhibitor. We extended these observations by examining NO-dependent vasodilatation using two sequential infusions of higher concentrations of substance P. Despite our observations and those of Klede *et al.* (2003), it is unclear as to the source of NO and precisely how substance P results in a NO-dependent vasodilatation. It is possible that the source of NO is secondary to an increase in flow. That is, after substance P binds to the NK_1 receptor on the endothelial cell, the ensuing vasodilatation increases shear stress across the endothelium thereby increasing NO production. However, this scenario seems unlikely as reactive hyperaemia, a condition which increases endothelial shear stress, is unaltered by NO synthase inhibition and does not result in an increase in NO concentration in human skin (Wong *et al.* 2003; Zhao *et al.* 2004). Alternatively, it could be speculated that NO could be produced secondary to histamine release and H1 receptor activation. In human skin it has been shown that substance P increases the concentration of histamine and, secondly, that histamine is associated with an increase in NO production in human skin (Petersen *et al.* 1994, 1997; Huttunen *et al.* 1996; Clough, 1999). Thus, it is possible that the NO component of substance P-induced vasodilatation is due to mast cell degranulation and subsequent H1 receptor activation. Again, this scenario seems unlikely in our study as we did not observe an H1 receptor component to substance P-induced vasodilatation. Lastly, it is possible that the binding of substance P to the NK_1 receptor directly stimulates the production of NO. Clearly, further investigation is warranted in order to determine the source(s) of NO and how NO may interact with NK_1 receptors.

The observation that our H1 receptor antagonist was without effect on substance P-induced vasodilatation is consistent with the findings of Weidner *et al.* (2000) who were also unable to attenuate the vasodilatation induced by vasoactive concentrations of substance P with the H1 receptor antagonist, cetirizine. However, we cannot rule out the possibility that substance P did indeed increase the concentration of histamine in the skin as previous studies have reported an increase in the concentration of histamine following an intradermal injection of substance P (Petersen *et al.* 1994, 1997; Huttunen *et al.* 1996). Further, an attenuated substance P-induced vasodilatation to an intradermal injection of substance P with the H1 antagonist chlorpheniramine has been observed (Boolell & Tooke, 1990). Weidner *et al.* (2000) observed that only the highest concentration of substance P (10^{-5} M) resulted in a significant increase in intradermal histamine levels, which was not reduced by an H1 antagonist.

The reason for the discordant findings regarding the contribution of histamine and H1 receptors is unclear. One possibility may be due to technical differences. In the study of Weidner *et al.* (2000), plasmapheresis microdialysis fibres with a molecular weight cutoff of 3000 kDa were used compared to microdialysis fibres with a 20 kDa cutoff (functional cutoff ~5 kDa). Although the concentrations of substance P used in the two studies were similar, the difference in membrane size may have resulted in a difference in the number of moles of substance P delivered to the vasculature thus partially explaining the equivocal findings. Furthermore, Petersen *et al.* (1997) reported that at least a 100 nM concentration of histamine was required to significantly increase skin blood flow. Thus, histamine may not be released in sufficient concentrations to contribute to the vasodilatation in response to the concentrations of substance P used in our study, which were chosen to increase skin blood flow to levels observed during reactive hyperaemia, thermal hyperaemia and active vasodilatation, and could thus explain the lack of an apparent H1 receptor component to substance P-induced vasodilatation.

In conclusion, we have presented comprehensive data describing mechanisms of substance P-mediated vasodilatation in human skin. An important novel finding from this study was the observation of an attenuated, and in many cases abolished, skin blood flow response to a second infusion of substance P, suggestive of NK_1 receptor desensitization upon binding substance P. This finding of

NK_1 receptor desensitization could provide a useful model for studying mechanisms of skin blood flow in which substance P is thought to be involved, including reactive hyperaemia, thermal hyperaemia and cutaneous active vasodilatation. Furthermore, the cutaneous vascular response to exogenous substance P produces dose-dependent vasodilatation and contains a clear NO component. However, substance P-mediated vasodilatation when delivered via microdialysis does not appear to work through H1 histamine receptor activation at the concentrations used in our study and, thus, there appears to be no interaction between NO and H1 receptor activation.

References

Barnes PH, Brown MJ, Dollery CT, Fuller RW, Heavey DJ & Ind PW (1986). Histamine is released from skin by substance P but does not act as the final vasodilator in the axon reflex. *Br J Pharmacol* **88**, 741–745.

Bennett VJ, Perrine SA & Simmons MA (2005). Neurokinin-1 receptor resensitization precedes receptor recycling. *J Pharmacol Exp Ther* **313**, 1347–1354.

Boolell M & Tooke JE (1990). The skin hyperemic response to local injection of substance P and capsaicin in diabetes mellitus. *Diabetic Med* **7**, 898–901.

Bowden JJ, Garland AM, Baluk P, Lefevre P, Grady EF, Vigna SR, Bunnett SW & McDonald DM (1994). Direct observation of substance P-induced internalization of NK_1 receptors at sites of inflammation. *Proc Natl Acad Sci U S A* **91**, 8964–8968.

Brown NJ & Roberts LJ (2001). Histamine, bradykinin, and their antagonists. In *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 10th edn, ed. Hardman JG & Limbird LE, pp. 645–667. McGraw-Hill, New York.

Clough GF (1999). Role of nitric oxide in the regulation of microvascular perfusion in human skin in vivo. *J Physiol* **516**, 549–557.

Garland AM, Grady EF, Lovett M, Vigna SR, Frucht MM, Krause JE & Bunnett NW (1996). Mechanisms of desensitization and resensitization of G protein-coupled neurokinin$_1$ and neurokinin$_2$ receptors. *Mol Pharmacol* **49**, 438–446.

Garland AM, Grady EF, Payan DG, Vigna SR & Bunnett NW (1994). Agonist-induced internalization of the substance P (NK_1) receptor expressed in epithelial cells. *Biochem J* **303**, 177–186.

Grady EF, Bohm SK & Bunnett NW (1997). Turning off the signal: mechanisms that attenuate signaling by G-protein-coupled receptors. *Am J Physiol* **273**, G586–G601.

Grady EF, Gamp PD, Jones E, Baluk P, McDonald DM, Payan DG & Bunnett NW (1996). Endocytosis and recycling of neurokinin 1 receptors in enteric neurons. *Neuroscience* **79**, 1239–1254.

Grady EF, Garland AM, Gamp PD, Lovett M, Payan DG & Bunnett NW (1995). Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol Biol Cell* **6**, 509–524.

Hill SJ (1990). Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacol Rev* **42**, 45–82.

Holzer P (1998). Neurogenic vasodilatation and plasma leakage in the skin. *Gen Pharmacol* **30**, 5–11.

Huttunen M, Harvima IT, Ackermann L, Harvima RJ, Naukkarinen A & Horsmanheimo M (1996). Neuropeptide- and capsaicin-induced histamine release in skin monitored with the microdialysis technique. *Acta Derm Venereol* **76**, 205–209.

Jansen I, Alafaci C, McCulloch J, Uddman R & Edvinsson L (1991). Tachykinins (substance P, neurokinin A, neuropeptide K, and neurokinin B) in the cerebral circulation. Vasomotor responses in vitro and in situ. *J Cereb Blood Flow Metab* **11**, 567–575.

Jenkinson KM, Mann PT, Southwell BR & Furness JB (2000). Independent endocytosis of the NK_1 and NK_3 tachykinin receptors in neurons of the rat myenteric plexus. *Neuroscience* **100**, 191–199.

Kellogg DL Jr, Liu Y, Kosiba IF & O'Donnell D (1999). Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol* **86**, 1185–1190.

Khawaja AM & Rogers DF (1996). Tachykinins: receptor to effector. *Int J Biochem Cell Biol* **28**, 721–738.

Klede M, Clough G, Lischetzki G & Schmelz M (2003). 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* **40**, 105–114.

Krause JE, Hershey AD, Dykema PE & Takeda Y (1990). Molecular biological studies on the diversity of chemical signaling in tachykinin peptidergic neurons. *Ann NY Acad Sci U S A* **81**, 3005–3009.

McConalogue K, Corvera CU, Gamp PD, Grady EF & Bunnett NW (1998). Desensitization of the neurokinin-1 receptor (NK1-R) in neurons. Effects of substance P on the distribution of NK1-R, Gaq/11, G-protein receptor kinase-2/3, and β-arrestin-1/2. *Mol Biol Cell* **9**, 2305–2324.

Mann PT, Southwell BR & Furness JB (1999). Internalisation of the neurokinin 1 receptor in rat myenteric neurons. *Neuroscience* **91**, 353–362.

Minson CT, Berry LT & Joyner MJ (2001). Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* **91**, 1619–1626.

Mukherjee S, Gnosh RN & Maxfield FR (1997). Endocytosis. *Physiol Rev* **77**, 759–803.

Petersen LJ, Church MK & Skov PS (1997). Histamine is released in the wheal but not the flare following challenge of human skin in vivo: a microdialysis study. *Clin Exp Allergy* **27**, 284–295.

Petersen LJ, Poulsen LK, Søndergaard J & Skov PS (1994). The use of cutaneous microdialysis to measure substance P-induced histamine release in intact human skin in vivo. *J Allergy Clin Immunol* **94**, 773–783.

Quartara L & Maggi CA (1997). The tachykinin NK_1 receptor. Part I: ligands and mechanisms of cellular activation. *Neuropeptides* **31**, 537–563.

Regoli D, Boudon A & Fauchere JL (1994). Receptors and antagonists for substance P and related peptides. *Pharmacol Rev* **46**, 551–599.

Roush ED, Warabi K & Kwatra MM (1999). Characterization of differences between rapid agonist-dependent phosphorylation and phorbol ester-mediated phosphorylation of human substance P receptor in intact cells. *Mol Pharmacol* **55**, 855–862.

Sasakawa N, Sharif M & Hanley MR (1994). Attenuation of agonist-induced desensitization of the rat substance P receptor by progressive truncation of the C-terminus. *FEBS Lett* **347**, 181–184.

Stjärne P, Rinder J & Delay-Goyet P (1994). Effects of NK1 receptor antagonists on vasodilation induced by chemical and electrical activation of sensory C-fibre afferents in different organs. *Acta Physiol Scand* **152**, 153–161.

Weidner C, Klede M, Rukwied R, Lischetzki G, Neisius U, Skov PS, Petersen LJ & Schmelz M (2000). Acute effects of substance P and calcitonin gene-related peptide in human skin – a microdialysis study. *J Invest Dermatol* **115**, 1015–1020.

Wilkins BW, Chung LH, Tublitz NJ, Wong BJ & Minson CT (2004). Mechanisms of vasoactive intestinal peptide-mediated vasodilation in human skin. *J Appl Physiol* **97**, 1291–1298.

Wong BJ, Wilkins BW, Holowatz LA & Minson CT (2003). Nitric oxide synthase does not alter the reactive hyperemic response in the cutaneous circulation. *J Appl Physiol* **95**, 504–510.

Wong BJ, Wilkins BW & Minson CT (2004). H1 but not H2 histamine receptor activation contributes to the rise in skin blood flow during whole body heating in humans. *J Physiol* **560**, 941–948.

Zhao JL, Pergola PE, Roman LJ & Kellogg DL Jr (2004). Bioactive nitric oxide concentration does not increase during reactive hyperemia in human skin. *J Appl Physiol* **96**, 628–632.

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