Axon Reflex Flare Evoked by Nicotine in Human Skin

Hiroshi Izumi and Keishiro Karita

Department of Physiology, Tohoku University School of Dentistry,
Aoba-ku, Sendai, 980 Japan

Abstract (1) The purpose of the present study is to investigate whether or not the vasodilatation evoked by intradermal (i.d.) injection of nicotine is mediated through axon reflex mechanism, and to examine the involvement of histamine receptors or capsaicin-sensitive C-fibers in nicotine-induced vasodilator response, using a band method and a laser Doppler technique. (2) The vasodilator response, whether it was caused by nicotine or histamine, developed as quickly on the uninjected side as on the injected side of the band, while the wheal reaction was elicited only by histamine and was localized in the injection side of the band. (3) Pretreatment with either a local anesthetic (lidocaine) or 1% capsaicin markedly reduced the nicotine- and histamine-induced blood flow responses, whereas pretreatment with antihistaminergic agent (diphenhydramine) showed inhibitory effect to the blood flow response only to histamine. (4) These data suggest that two types of chemical receptors, i.e. the nicotinic and the histamine-sensitive receptors, exist in capsaicin-sensitive C-fibers to elicit axon reflex vasodilatation in human skin.

Key words: nicotine, axon reflex flare, nicotinic receptor, band method, laser Doppler flowmeter, capsaicin-sensitive C-fibers.

In human skin, stroking or intradermal (i.d.) injection of histamine elicits the familiar triple responses characterized by vasodilatation (redness), increased permeability (wheal) and an axon reflex mediated vasodilatation (flare) [1–5]. Peripheral nerve terminals of capsaicin-sensitive sensory neurons, particularly the C-polymodal nociceptors, have recently been reported to be responsible for mediation of axon reflex vasodilatation [6–8] and antidromic vasodilatation [9–11].

The flare reaction evoked by i.d. injection of neuropeptides such as bradykinin, calcitonin gene-related peptide, substance P, and other tachykinins in human skin is suggested to be initiated by histamine released from cutaneous mast cells, since a histamine H1-receptor antagonist markedly attenuated the responses [5, 7, 12–15], suggesting that histamine is the common mediator of the flare response. This is supported by the parallelism of histamine-releasing activity and flare-inducing.

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activity [7, 16] of these peptides and by the observation that certain primary afferents have histamine H₂-receptors [17]. Thus far, the histamine receptor has been thought to be the only chemical receptor in polymodal nociceptive fibers to mediate axon reflex flare. On the other hand, wheal response is thought to be caused by the direct action of histamine on the capillary permeability at the injected area and afferent sensory nerve do not seem to be deeply involved in wheal response [6, 8].

The i.d. injection of drugs with nicotine-like action has previously been reported to elicit the vasodilatory response in human skin [18, 19]; however, no detailed mechanisms of these effects have been studied. Therefore, the purpose of this study is (1) to investigate whether or not the vasodilatation evoked by i.d. injection of nicotine is mediated through axon reflex mechanism, and (2) to examine the involvement of histaminergic receptors in this vasodilatation, using a band method and a laser Doppler technique.

MATERIALS AND METHODS

The experiments were performed in ten healthy adult volunteers of both sexes, including authors, in the age range 20–50 years after obtained informed consent. None suffered from allergic responses or headache, and none had received any kind of drugs during the preceding 24 h. All experiments were carried out between 10:00 and 16:00 h at an ambient temperature between 20 and 22°C. The test substances were prepared in Ringer solution in appropriate concentration just before each experiment and injected i.d. with a 0.4 mm diameter needle and volumes of about 20 μl in all cases.

Visual observation by band method. The band method employed has been described previously in detail [8]. Briefly, a rubber band (1.6 m in width) was stretched around the middle of the ventral surfaces of forearm, and the band was fastened and left in place during the experiments. The band method can prevent the agents, applied intradermally to either side of the band from diffusing beyond it to the other side [20–22]. This can easily be judged by the following observation. An effective dose of agents for eliciting local vasodilatation is injected into the skin at the site 5 mm distal or proximal to the band. When vasodilatation was induced, it was localized only on the injected side and never reached the other side. In case of wheal response, which is assumed to be caused by the direct action of histamine released by agents on the capillary permeability at the injected area, it was observed only on the injected side and never observed on the other side. In contrast, when agents which would produce vasodilation of the axon reflex mechanism were administered in the same manner as described above, vasodilatation developed as quickly on the uninjected side within several minutes after application of the agents.

The maximal sizes of the flare and wheal were measured by marking the borders on the skin with ink, and then transferring marks onto tracing paper. The areas of the flares and wheals were measured planimetrically. Control experiments

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were usually carried out on the corresponding area of the opposite forearm and the control injections were made with Ringer solution.

**Blood flow changes measured by a laser Doppler flowmeter.** The changes in blood flow in the volar forearm skin were continuously recorded 10 mm apart from the injection site by a laser Doppler flowmeter (Med. Pacific 5000, Seattle, WA, U.S.A.) as described before [5]. The tip of the fiber optic probe was secured in a holder on the forearm and was maintained at a distance of ca. 5 mm from the superficial skin layers. In the present experiments blood flow value has been expressed in terms of the output signal (mV) from the instrument recorded on a pen recorder.

**Pretreatment with antihistamine or local anesthetic.** Diphenhydramine ointment (10 mg/g) or lidocaine jelly (20 mg/ml) was applied onto the volar forearm during 1 h prior to the injection of nicotine as an antihistamine or local anesthetic. Nicotine solution was injected i.d. after the ointment or jelly was cleanly wiped off.

**Pretreatment with capsaicin.** A solution of capsaicin (1% in ethanol) was applied to the flexor area (5 × 5 cm) of one forearm. After approximately 10 min the capsaicin was washed out. Within 20 min of the first application, intense burning pain and a wide spread flare developed. Painting was repeated 4 times at approximately 2 h intervals on the first day and further applications were performed on subsequent days until no local reaction occurred. On average a total 6–7 applications were done. The control areas were treated by only ethanol.

**Statistical analysis.** All numerical data are given as the mean ± SE of the means. Significant changes in data was analyzed with the paired t-test and sign test (for flare response in Table 1). A p-value < 0.05 was considered to be statistically significant.

**Chemicals.** The following drugs were used: nicotine (Eastman Organic Chemicals, U.S.A.), histamine diphosphate (Wako Pure Chemicals, Osaka), synthetic substance P (Peptide Institute, Inc., Osaka), capsaicin (Sigma Chemical Co., St. Louis, Mo., U.S.A.), diphenhydramine ointment (diphenhydramine, 10 mg/g, Kowa Chemicals, Nagoya), lidocaine jelly (lidocaine HCl, 20 mg/ml, Fujisawa Chemicals, Osaka), and Ringer’s solution (Otsuka Chemicals, Tokyo).

### RESULTS

**Flare and wheal responses by visual observation**

As shown in Table 1, i.d. injections of nicotine (10⁻⁵ g/ml) and acetylcholine (ACh) (10⁻³ g/ml) into the skin of the human volar forearm at a site 5 mm either proximal or distal to the band produced the blood flow increase on the uninjected side across the band as well as the injected side. Similar vasodilator response occurred after i.d. injections of histamine (10⁻³ g/ml) and substance P (10⁻² g/ml). Injections of nicotine and ACh did not cause any wheal response on the either side which was almost similar to the control, while injection of histamine and substance P elicited the wheal response only on the injection side which are significantly
Table 1. The area (cm²) of flare and wheal in the injected side and the uninjected side of the band following intradermal injection of nicotine (10⁻¹ g/ml), acetylcholine (10⁻³ g/ml), bethanechol (10⁻³ g/ml), histamine (10⁻⁵ g/ml), substance P (10⁻⁷ g/ml), or 0.9% saline (control) into the skin 0.5 cm apart from the band of normal human forearm skin.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Injected side</th>
<th></th>
<th></th>
<th>Uninjected side</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flare</td>
<td>Wheal</td>
<td></td>
<td>Flare</td>
<td>Wheal</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.21±0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>7.45±1.35*</td>
<td>0.20±0.04</td>
<td>4.89±0.80</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>8.83±0.69*</td>
<td>0.23±0.07</td>
<td>3.84±0.75</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bethanechol</td>
<td>4.14±0.28*</td>
<td>0.28±0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>16.53±1.91*</td>
<td>0.99±0.09**</td>
<td>9.43±1.67*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>10.72±2.34*</td>
<td>1.58±0.29**</td>
<td>6.00±1.04*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The extent of flare or wheal response was expressed as the maximal area of the flare and wheal both in the injected side and the uninjected side of the band. Values shown were expressed as mean±SEM (cm²). The numbers of subjects tested are six. Statistical significances of the difference between the value of the various groups and the corresponding values of the control group are analyzed with the sign test for flare response and paired t-test for wheal response and are indicated by asterisks: *p<0.05, **p<0.01.

greater than their corresponding control injections (p<0.01, N=6). Injection of bethanechol, a muscarinic cholinceptor agonist (10⁻³ g/ml), produced a long-lasting vasodilator response which was localized to the injection site without eliciting the wheal response on either side.

By these visual observation methods, the vasodilator responses to histamine and substance P have approximately similar time-courses; the vasodilator response to histamine and substance P, an area of vasodilation spreading for several centimeters from the point of injection, was maximal in size approximately 3 min after injection and it declined slowly, and the wheal response, an area of edema localized to the site of injection, took longer to develop and was maximal in size 7–10 min after injection. On the other hand, the maximum vasodilator response to nicotine and ACh was obtained within the first 1 min after injection and faded rapidly.

**Continuous measurement of cutaneous blood flow**

The cutaneous blood flow was continuously measured by use of the laser Doppler flowmetry to examine the amplitude and the duration of the blood flow responses in the following experiments. Injection of nicotine (10⁻⁶–10⁻⁴ g/ml) and histamine (10⁻⁷–10⁻⁵ g/ml) caused a blood flow increase in a dose-dependent manner (Fig. 1). The maximal vasodilator response following injection of nicotine (10⁻⁵ g/ml) or histamine (10⁻⁶ g/ml) was obtained at 0.99±0.1 (N=7) or 2.38±0.3 min (N=7), respectively. The duration of the blood flow increase to nicotine or histamine was 3.46±0.27 (N=7) or 8.9±0.8 min (N=7), respectively, at the

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Fig. 1. Vasodilatory responses following i.d. injection of nicotine at doses of (a) $10^{-6}$, (b) $10^{-5}$, and (c) $10^{-4}$ g/ml, and histamine at doses of (d) $10^{-7}$, (e) $10^{-6}$, and (f) $10^{-5}$ g/ml in Ringer solution into the skin of human ventral forearm at a site 10 mm apart from the injection site. The blood flow was measured by a laser Doppler flowmeter as described in MATERIALS AND METHODS. Ordinate: blood flow in human volar forearm expressed as mV.

**Effects of antihistamine and local anesthetic**

In order to examine the involvement of histamine receptor or neural elements on nicotine-induced blood flow increase, diphenhydramine ointment (10 mg/g) or lidocaine jelly (20 mg/ml) was applied onto the volar forearm 1 h prior to i.d. injection of nicotine ($10^{-5}$ g/ml). As can be seen in Fig. 2A and B, nicotine-induced blood flow increase was reduced by $66.6 \pm 3.1\%$ ($p<0.05$, $N=4$) following pretreatment with local anesthetic, lidocaine, but not with histamine H$_1$-receptor antagonist, diphenhydramine, when compared with the normal skin in the amplitude of blood flow increase ($N=4$).
Fig. 2. Effects of pretreatment with (A) local anesthetic lidocaine and (B) antihistamine (diphenhydramine) on nicotine-induced blood flow increase. Lidocaine jelly (20 mg/ml) or diphenhydramine ointment (10 mg/g) was applied into the volar forearm as described in MATERIALS AND METHODS. Intradermal injection of nicotine (10⁻⁵ g/ml) was placed 10 mm apart from the probe of a laser Doppler flowmeter. Ordinate: blood flow in human volar forearm expressed as mV.

**Effects of capsaicin**

Figure 3A shows the effects of capsaicin pretreatment of the skin on nicotine-induced blood flow increase. After the skins were pretreated with painting of 1% capsaicin for 2 or 3 d as described in MATERIALS AND METHODS, nicotine at a dose of 10⁻⁵ g/ml was injected in the capsaicin-pretreated areas (Fig. 3A). In the skin treated with capsaicin, nicotine produced a weaker blood flow increase (23.9 ± 7.0%; p < 0.01, N = 3) than those observed in the normal skin.

**DISCUSSION**

The band method has previously been devised by Wada et al. [20] for investigating axon reflex sweating. Usage of the band method led Wada et al. [21, 22] to easily bear out that the mode of action of the drugs with nicotine-like action on sweating is clearly different from those of mecholyl or pilocarpine; the former is
axon reflex mechanism and the latter is not. We have previously reported a
difference of the occurrence of axon reflex sweating evoked by nicotine among the
primates [23], and have recently reported that this band method can be used to
estimate whether the vasodilator action of histamine and substance P is due to axon
reflex mechanism [8].

In the present study, we have taken advantage of the property of the band
method as a means of investigating whether nicotine-induced vasodilation is
mediated via axon reflex mechanism or not. As shown in Table 1, I.D. injections of
nicotine and ACh produced vasodilator responses on both the injected and the
uninjected side of the band, while I.D. injection of bethanechol caused a long-lasting
vasodilator response only in the injection side. We have previously reported that the
occurrence of a vasodilator response in the uninjected side of the band is due to
activation of axon reflex mechanism, while the vasodilator response only in the
injected side appears to be a consequence of a direct vasodilator action on the blood
vessels [5]. From these, it may be deduced that the vasodilation in response to
nicotine and ACh is due to activation of axon reflex flare mechanism, whereas the
vasodilation associated with bethanechol is a direct effect of muscarinic action on
the blood vessels.

Either local anesthetic (Fig. 2A) or capsaicin (Fig. 3A) attenuates the
nicotine-induced blood flow increase. These observations correspond well to the our
previous report [5] that histamine-induced blood flow increase was reduced by
pretreatment with local anesthetic or capsaicin. There is increasing evidence that
the repeated application of capsaicin into the human skin results in the desensitiza-
tion of the nociceptive C-fibers to chemical stimuli such as substance P [24]. Taken
together with all, it seems likely that the neural elements, particularly capsaicin-
sensitive nociceptive C-fibers, are involved in vasodilatation in response to nicotine.

Besides histamine, several neuropeptides such as somatostatin, vasoactive intestinal peptide, and calcitonin gene-related peptide have been reported to have an ability to induce an axon reflex flare when injected I.D. into human skin [5–7, 25–27]. These neuropeptides-induced flare responses have been suggested to be initiated by histamine release from cutaneous mast cells in the dermis, which in turn triggers stimulation of the histamine-sensitive nociceptive C-fibers. There has been so far no other receptors except histamine receptors in the nociceptive C-fibers to cause axon reflex flare. However, pretreatment with diphenhydramine, histamine $H_1$-receptor antagonist, did not reduce the blood flow response to nicotine (Fig. 2B), but significantly reduced the blood flow response to both histamine and substance P [5]. These results imply that histamine does not mediate the nicotine-induced vasodilator response, and suggest that mechanism by which nicotine elicits the vasodilator response differs from those of histamine and substance P and that the nicotinic receptors as well as histaminergic receptors in the capsaicin-sensitive C-fibers exist to induce the flare reaction. Further support for this assumption is that, whereas histamine and substance P produced the wheal response, nicotine and ACh did not elicit any wheal response (Table 1), being in harmony with observations of Emmelin and Feldberg [28]. The wheal response seen after histamine and substance P is a well-known effect and is considered to be brought about by a direct action of injected histamine or histamine released from mast cells in the dermis on the capillary permeability [6, 8]. Inability of nicotine to cause a wheal response suggests that the vasodilatation associated with nicotine was not a histamine receptor mediated effect. Brown and Gray [29] have previously observed that arterial injection of nicotine into the skin elicited the discharge of impulses in cutaneous sensory nerves, and have suggested the presence of the nicotinic receptors in some part of terminals of the cutaneous sensory nerves.

The occurrence of sympathetic and parasympathetic vasodilator fibers has recently been suggested in the skin of the forehead and foot of the human and in the hind paw skin and the oro-facial area of the cat [30–37]; however, autonomic nervous system does not seem to be involved in the axon reflex flare investigated in the present studies, since this remained apparently undisturbed by sympathectomy [38].

In human skin, there are 3 different phenomena mediated by axon reflex mechanism: flare, sweating, and pilomotion. The latter two phenomena are well known to be mediated via activation of nicotinic receptors in sympathetic post-ganglionic fibers [1–4, 8, 21, 24, 39]. The present study showing that axon reflex flare is also mediated via activation of capsaicin-sensitive sensory C-fibers indicate that cutaneous non-myelinated sensory and autonomic (sympathetic) fibers are involved in axon reflex responses such as flare, sweating, and pilomotion, and that all of these fibers have a nicotinic receptor. The significance and interpretation of the presence of the nicotinic receptor in all axon reflex phenomenon must await further investigation.

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