A Possible Role of Nitric Oxide Formation in the Vasodilatation of Rabbit Ear Artery Induced by a Topically Applied Capsaicin Analogue

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ABSTRACT. Effects of topical application of a capsaicin analogue, nonylic acid vanillylamide (NVA, 0.032–10.0 mM) on the arterial diameter in the ear skin were examined in conscious rabbits using a precise dial caliper. In addition, the possibility of nitric oxide (NO) participating in a vasodilatation induced by low concentrations of NVA was tested by an NO synthase inhibitor. At the lowest concentration of NVA (0.032 mM), no significant change in the diameter was observed after external application of the NVA ointment. At concentrations of 0.32 mM or more, NVA produced a significant vasodilator response. However, at higher concentrations of 3.2 and 10.0 mM, NVA induced substantial shrinkage in the arterial diameter and oedema formation, which was not affected by L-NAME (N^G-nitro-L-arginine methyl ester, 3 mg/kg, i.v.), suggesting fluid leakage induced by oedema from the vessels might suppress the vasodilatation. Thus, the concentration-response curve for NVA was bell-shaped. NVA (0.32 mM)-induced vasodilatation was not significantly affected by atropine (1 mg/kg, i.v.) or propranolol (80 µg/kg, i.v.). However, the NVA-induced vasodilatation was completely suppressed by an NO synthase inhibitor, L-NAME (3 mg/kg, i.v.) which had no influence on the resting diameter, but not by an inactive stereoisomer, D-NAME (3 mg/kg, i.v.). These findings suggest that vanilloid receptor activation results in the release of sensory neuropeptides, which in turn stimulate the synthesis of endothelial NO which is responsible for the vasodilatation. — **KEY WORDS:** capsaicin, L-NAME, neuropeptide, nitric oxide, vasodilatation.

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Capsaicin (Fig. 1) is an active substance responsible for the irritating and pungent effects of hot peppers (paprika, Hungarian red pepper, *Capsicum annuum* L.). Nonylic acid vanillylamide (NVA) is a very stable synthetic capsaicin analogue (Fig. 1), which lacks a C=C bond in the aliphatic side chain, and is a constituent of counterirritant preparations on the market. Capsaicin and NVA are both useful for producing counterirritation [11, 20]. It is now known that capsaicin-like molecules affect C-fiber, thin primary afferent neurons that are connected to distinct sensory receptors [2, 5, 10, 11, 16, 17, 26, 28, 29, 54, 55].

Capsaicin has highly specific actions on polymodal nociceptors [16, 18], which are exerted via a specific membrane receptor (vanilloid receptor) whose activation increases membrane cation permeability and which can be distinguished by the competitive antagonist capsazepine [12, 13, 18, 50] or the polyvalent dye ruthenium red [8, 14]. This mechanism of activation accounts for the burning pain sensation that capsaicin induces after topical application to the skin [9]. In addition, chemical or electrical stimulation of sensory nerves in the skin causes an inflammatory reaction comprising arteriolar dilatation, increase in venular permeability and recruitment of leukocytes [10, 28, 42]. This 'neurogenic inflammation' arises from the release of vasoactive peptide transmitters such as substance P and calcitonin gene-related peptide (CGRP) from the peripheral endings of fine afferent nerve fibres [23, 31, 38, 39, 41, 58]. Following activation, however, capsaicin induces sensory

neurons to become inexcitable; consequently, noxious stimuli are no longer effective. The complexity of capsaicin-induced responses of sensitization and desensitization can often lead to confusion in the interpretation of the capsaicin action [29].

Tissue blood flow is regulated by local autoregulatory mechanisms, factors released from endothelial cells, hormones and neural factors. The role of autonomic neurons, notably of sympathetic vasoconstrictor neurons, is well studied [30]. However, even after blockade of

Fig. 1. Chemical structures of capsaicin and nonylic acid vanillylamide (NVA).

cholinergic and noradrenergic transmission, blood vessels can actively be dilated in response to nerve stimulation. The nonadrenergic noncholinergic (NANC) vasodilator neurons have long eluded identification, but there is now good evidence that NANC vasodilatation in many vascular beds is mediated by nerve fibers that utilize peptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) as their transmitters [30]. The possibility remains that the neurotransmitters from the afferent nerve fibers control the vascular effector system via the release of vasoactive messenger molecules from endothelial cells. The major endothelium-derived relaxing factor is nitric oxide (NO), which is synthesized from L-arginine by the activity of a constitutive NO synthase in the endothelial cells [45]. Endothelium-derived NO participates in the vasodilator action of substance P [19, 49, 57] and CGRP [22, 24, 25, 31, 32, 49]. Therefore, in the present study, it was investigated whether NO participates in the capsaicin-induced vasodilator response in the rabbit ear skin. The involvement of NO was examined with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of the constitutive NO synthase [21, 46, 47]. The inactive enantiomer, D-NAME was given in the control experiments. Some of this work has already been presented in preliminary form [51, 52].

MATERIALS AND METHODS

Animals: Japanese White rabbits of either sex with an average weight of 2.5–3.5 kg were used. About a week before each experiment, the rabbit ear skin was shaved and depilated. All experiments were carried out in a quiet room at a thermo-neutral temperature of 22–24°C in which direct air flow was prevented from falling on and affecting the animals who were conscious and comfortably housed in portable animal cages.

Measurement of blood vessel diameter: In the present study, changes in the diameter of the rabbit ear artery were directly measured to reveal the effects of capsaicin analogue on the blood vessel, according to a technique described previously [51–53]. In brief, the normal resting diameter of the ear artery was measured by precise dial calipers (Tajima, Japan). Following this assessment of each animal, various concentrations of NVA in an inert ointment base were applied to a circular area, approximately 7 cm², on the surface of the ear skin and the arterial diameter of the artery was measured during the experimental period. When NVA-induced oedema formation was observed, the blood vessel area (red zone), but not the oedema area (white zone), was measured to obtain the net diameter of the vessels.

The next series of experiments were carried out to examine the pharmacological characteristics of this acute capsaicin response in more detail. Following assessment of the resting diameter, various antagonists were injected into the ear vein before a topical application of suprathreshold concentration of NVA (0.32 mM). Resultant diameter changes were monitored as above with dial calipers.

Measurement of oedema formation: Formation of oedema

in the rabbit ear skin was measured by a scoring test where the extent of oedema formation around artery are represented in increasing order as follows: -, no effect; +, weak; ++, remarkable. In the L-NAME experiment, L-NAME (3 mg/kg, i.v.) was injected into the ear vein 3 min before a topical application of suprathreshold concentration of NVA (0.32 mM). Resultant pathological changes were monitored using the scoring test.

Drugs: The following drugs were used: NVA (Chemie Linz), atropine sulfate (Nakarai Tesque), DL-propranolol hydrochloride (Nakarai Tesque), L-NAME (Wako), D-NAME (Wako). NVA was mixed with an inert ointment base (5% polyethylene + 95% liquid paraffin). Atropine, propranolol, L-NAME and D-NAME were dissolved in saline.

Statistical analysis: All data are presented as mean $\pm$ SEM. Statistical significance evaluation of the results were performed with Student's *t*-test, Welch's test, paired *t*-test or Tukey's multiple test. *P* < 0.05 was considered significant.

RESULTS

Effects of NVA on the vessel diameter: In the first series of experiments, arterial diameters of rabbit ear skin were measured, before and after acute topical application of NVA ointment at varying concentrations. Figure 2 shows time-course changes in average response from the baseline diameters. At the lowest concentration of 0.032 mM, no change in the diameter compared to the matched control ointment base was observed after the exposure to NVA ointment. At concentration of 0.32 mM, NVA produced an initial slight and transient vasoconstrictor response of the artery, followed by a prolonged vasodilator response which

Fig. 2. Percentage change in diameter of ear artery in response to varying concentrations of topical NVA in conscious rabbits. NVA ointment was applied topically to the ear in concentrations of 0 (○), 0.032 (●), 0.32 (■) and 3.2 mM (▲). Baseline diameters were measured with a precise dial caliper before drug application. Data are expressed as percentage of baseline diameter. Each point is the mean $\pm$ SEM (vertical bars) of five animals. *P < 0.05 and **P < 0.01, as compared to control ointment base by paired *t* test. Baseline diameters (mean $\pm$ SEM in mm) were 1.43 $\pm$ 0.07 (n=20).

Table 1. Effect of L-NAME on the NVA-induced oedema formation in the rabbit ear skin

Drug		Degree of oedema	Number (%) of animals showing the responses			
NVA (mM)	L-NAME (mg/kg, i.v.)		Time after application (min)			
			0	15	30	60
COB	PSS	—	3 (100)	3 (100)	3 (100)	3 (100)
1.0	PSS	—	3 (100)	3 (100)	3 (100)	3 (100)
3.2	PSS	—	3 (100)	3 (100)	3 (100)	3 (100)
		+			3 (100)	3 (100)
10.0	PSS	—	3 (100)	3 (100)	3 (100)	3 (100)
		++			3 (100)	3 (100)
COB	3.0	—	3 (100)	3 (100)	3 (100)	3 (100)
1.0	3.0	—	3 (100)	3 (100)	3 (100)	3 (100)
3.2	3.0	—	3 (100)	3 (100)	3 (100)	3 (100)
		+			3 (100)	3 (100)
10.0	3.0	—	3 (100)	3 (100)	3 (100)	3 (100)
		++			3 (100)	3 (100)

COB: Control ointment base. PSS: Physiological salt solution. Symbols: —, No effect; +, Weak; ++, Remarkable.

reached a maximum usually 20–60 min after the exposure to NVA. However, no such vasodilator response was observed after exposure to the highest concentration of NVA (3.2 mM) and, in fact, substantial shrinkage in the diameter was observed 90 min after the exposure to NVA at this concentration. This shrinkage was almost coincident with oedema formation, which was detectable with concentrations of NVA above 3.2 mM (Table 1).

Figure 3 shows the concentration (0.032–10.0 mM)-response curves for NVA obtained 30 (upper curve) and 60 (lower curve) min after the drug application. These concentration curves were bell-shaped and the threshold concentration of NVA for the vasodilator response 30 or 60 min after topical application seemed to be around 0.1 mM (Fig. 3), indicating that rabbit ear skin is, to a considerable extent, sensitive to the vasodilator effect of capsaicin.

NVA-induced oedema formation: At the concentration of 1.0 mM, no change around the artery compared to the matched control ointment base was observed after the exposure to the ointment. At concentrations of 3.2 and 10 mM, dose-dependent formations of oedema induced by NVA were observed 30 or 60 min after the application of NVA (Table 1).

Effect of L-NAME on the resting artery and NVA-induced oedema formation: L-NAME (3 mg/kg, i.v.) had no effect on the resting vessels. Furthermore, compared with the formation measured in the rabbit treated with control physiological salt solution, L-NAME did not alter the course of the NVA-induced oedema formations in the rabbit ear skin (Table 1), indicating that nitric oxide is not involved in oedema formation under these conditions.

Effects of atropine on NVA-induced vasodilatation: In the second series of experiments, the prolonged vasodilator response to 0.32 mM NVA has been characterized with various pharmacological agents. Figure 4 shows the effects of pretreatment of muscarinic acetylcholine (ACh) receptor antagonist, atropine (1 mg/kg i.v.) on the resting and NVA

Fig. 3. Dose-related vasodilative effects of NVA on the diameter of the ear artery in conscious rabbits. NVA ointment was applied topically to the ear skin in concentrations of 0.032–10.0 mM. The responses to NVA were plotted as percentage of baseline diameter vs log concentration of NVA 30 (a) or 60 (b) min after application. Each point is the mean $\pm$ SEM (vertical bars) of five animals. * $P < 0.05$ and ** $P < 0.01$, as compared to control ointment base (COB) by Student's t test.

(0.32 mM)-induced vasodilative ear artery. In this experiment, rabbits were given atropine 3 min before the NVA application and the vessel diameters were measured before and 30 min after the NVA application. Although the resting and NVA-induced vasodilative vessels were slightly inhibited by atropine, these inhibitory effects were not

Fig. 4. The effects of pretreatment of atropine (1 mg/kg, i.v.) and propranolol (80 μ g/kg, i.v.) on vasodilator response to topical NVA (0.32 mM) in the ear artery of conscious rabbits. Rabbits were given atropine or propranolol 3 min before the application of NVA. Vessel diameters were measured with precise dial caliper before and 30 min after NVA application. Each column represents percentage of baseline diameter with SEM shown by the vertical bars ($n = 5$). Data were analysed by Tukey's multiple test. NS=not significant; * $P < 0.05$, as compared to control ointment base (COB).

specific for NVA-induced vasodilatation, and were not statistically significant.

Effects of propranolol on NVA-induced vasodilatation: Figure 4 also shows the effect of pretreatment of adrenergic β receptor antagonist, propranolol (80 μ g/kg, i.v.) on the resting and NVA (0.32 mM)-induced vasodilative artery. In this experiment, rabbits were given propranolol 3 min before the NVA application and the vessel diameters were measured before and 30 min after the NVA application. Although the resting and NVA-induced vasodilative vessels were slightly inhibited by propranolol, these inhibitory effects were not specific for NVA-induced vasodilatation, and were not statistically significant.

Effects of L-NAME and D-NAME on NVA-induced vasodilatation: Figure 5 shows effects of a low dosage of L-NAME, which suppresses the formation from L-arginine to L-citrulline, on the resting and NVA (0.32 mM)-induced vasodilative ear artery. The vasodilator response to topical NVA was completely suppressed by L-NAME (3 mg/kg, i.v.), while the resting diameter of the artery was not affected by L-NAME. Furthermore, the inactive stereoisomer D-NAME (3 mg/kg i.v.) did not affect the NVA-induced vasodilator response, indicating that the NVA-induced vasodilatation is dependent on the formation of NO.

DISCUSSION

The present study has shown that NVA induces vasodilator effects on the artery of rabbit ear skin and that the vasodilator response to low concentrations of NVA was completely suppressed by the NO synthase inhibitor [21, 22, 46, 47], L-NAME (Fig. 5), but not the inactive stereoisomer, D-NAME (Fig. 5). These results strongly suggest a possible role of nitric oxide formation in the

Fig. 5. Inhibitory effect of pretreatment of N^G-nitro-L-arginine methyl ester (L-NAME) on vasodilator response to topical NVA in the artery of conscious rabbits. Rabbits were given L-NAME (3 mg/kg, i.v.) or N^G-nitro-D-arginine methyl ester (D-NAME, 3 mg/kg, i.v.) 3 min before the application of NVA. Vessel diameters were measured with precise dial caliper before and 30 min after NVA application. Each column represents percentage of baseline diameter with SEM shown by the vertical bars ($n = 5$). Data were analysed by Tukey's multiple test. NS=not significant; * $P < 0.05$, as compared to control ointment base (COB). # $P < 0.05$, as compared to NVA + L-NAME group.

vasodilatation induced by topical NVA. At high concentrations of NVA, however, it was observed that NVA produced substantial shrinkage in the diameter (Fig. 2). This shrinkage was almost coincident with oedema formation, which was detectable with concentrations of NVA above 3.2 mM (Table 1). Substance P and CGRP, which all increase in the vascular permeability leading to oedema formation [56], are considered to be the principal mediators of neurogenic inflammation in the skin. In the present experiments, unlike the vasodilator component, the exudative component of the neurogenic inflammatory response to NVA was not affected by L-NAME. Although it is known that protein leakage induced by substance P involves endothelium-derived NO, which may add to the leakage of plasma protein and fluid [34], Lippe *et al.* [40] reported that the exudative component of the neurogenic inflammatory response to mustard oil, which stimulates capsaicin sensitive afferent neurons, does not seem to involve NO directly. Thus, it is possible that nitric oxide was not involved in the NVA-induced oedema formation in the present study, although the possibility remains that the L-NAME used was not sufficient to suppress the oedema formation since a low dose of L-NAME was used in the present experiments. Also, it is feasible that the leakage of fluid from blood vessels to interstitial space induced by oedema formation [40] might suppress the NVA-induced vasodilator response and then produce substantial shrinkage in the diameter, since, except for the swollen parts of vessels, only the net diameter of vessels was measured in the present experiment.

There are considerable species differences in the sensitivity of afferent neurons to capsaicin [28, 29]. It has been said that the rabbit is less sensitive to capsaicin analogues, although no systematic investigation has been done [28, 29]. Our results show that threshold concentration of NVA to the rabbit ear skin is 0.032–0.1 mM from changes in the blood vessel diameters (Fig. 3). Roberts *et al.* [48] reported that the threshold concentration of capsaicin to the human skin is 0.033–0.16 mM by laser Doppler blood flux [48]. Since it has been reported that the rabbit ear artery is innervated by capsaicin-sensitive peptidergic afferent neurons [44], it thus seems likely that sensory neurons in the skin of humans and rabbits are similarly sensitive to the vasodilator effect of topical capsaicin analogue, although it is difficult to compare the extent of vasodilator responses measured by these different methods.

In the rabbit, intradermal injection of capsaicin induces a vasodilator response [6–8] which is inhibited by the CGRP antagonist CGRP_(8–37) [31, 32]. This suggests that CGRP is the important vasodilator neuropeptide which is released from capsaicin-sensitive nerves in skin. It is well known that substance P is often co-localized with CGRP in capsaicin-sensitive nerves. However, substance P, despite established potent vasoactive effects in rat skin [38], is extremely weak when injected intradermally into the rabbit [4, 5] and a selective neurokinin-1 antagonist [3] had no effect on capsaicin-induced blood flow in rabbit skin [33]. Thus, it is quite possible that NVA-induced vasodilatation of rabbit ear skin is attributable to CGRP, but not substance P, released from sensory neurons, since recent studies suggest that CGRP is an important mediator of arteriolar dilatation whilst substance P is an essential mediator of increased vascular permeability [23, 31, 38, 39, 41, 58].

Our results that the resting and NVA-induced vasodilative vessels were slightly inhibited by atropine suggest that there may be some role of ACh in regulation of artery tone *in vivo*. However, these inhibitory effects were not significant (Fig. 4). The present result is consistent with earlier findings that atropine fails to block, and eserine fails to potentiate, antidromic vasodilatation in the rabbit ear [27]. It is thus unlikely that ACh is a major mediator of capsaicin sensitive nerve-mediated vasodilatation induced by the suprathreshold concentration of NVA. In the present study, a tendency to inhibition of the resting and NVA-induced vasodilative vessels by adrenoceptor $\beta_{1,2}$ -receptor antagonist, propranolol, was observed. Similar results were obtained from extensive experiment in which the effect of pretreatment of the specific adrenoceptor β_2 -receptor antagonist, ICI 118551 on the NVA-induced vasodilator response was investigated [51, 52]. This suggests that the tendency to inhibit the resting and NVA-induced vasodilative artery may be attributable to action of the β_2 -receptor antagonist, and that noradrenaline may have some role in regulation of artery tone *in vivo*. However, the inhibitory effect of propranolol or ICI 118551 was not specific for NVA-induced vasodilatation and it may thus be necessary to subtract the effect of propranolol or ICI 118551

on the resting diameter from that on NVA-induced vasodilative artery. The mechanism of NVA-induced vasodilatation, therefore, remains unexplained. However, the NVA-induced vasodilator response was completely suppressed by L-NAME (Fig. 5), a nitric oxide synthase inhibitor [21, 22, 46, 47], but not by an inactive stereoisomer, D-NAME (Fig. 5). The finding that the action of L-NAME on the vasodilative artery was enantiomer-specific indicates this drug acts by inhibition of the L-arginine: NO pathway [45, 47]. Furthermore, this concentration of L-NAME we used had no influence on the resting diameter (Fig. 5). Therefore, it is concluded that the L-arginine: NO pathway is specifically involved in capsaicin induced vasodilatation. It is thus conceivable that NVA-induced vasodilatation may be produced by NO formed either in the capsaicin sensitive sensory neuron or vascular endothelial cell, although the current investigation cannot discriminate between these possibilities.

Capsaicin has highly specific actions on polymodal nociceptive neurons [6–8, 13, 50]. This involves membrane depolarization and the opening of a unique, cation-selective, ion channel which can be selectively and competitively antagonized by capsazepine [13, 18, 43, 50]. Furthermore, it has been demonstrated that the functional capsaicin receptor antagonist, ruthenium red [8, 14], completely suppressed the vasodilator response to the topical capsaicin analogue [51, 52]. These data suggest that the NVA-induced vasodilator response may be mediated by a capsaicin receptor on polymodal nociceptive neurons in the rabbit ear skin. On the other hand, Hughes and Brain [31] reported that capsaicin-induced vasodilator response was totally inhibited by the CGRP antagonist, CGRP_(8–37). Moreover, they reported that L-NAME has an inhibitory effect on the increased blood flow induced by CGRP, especially at lower doses of CGRP [32]. It is known that CGRP can act via a selective rise in levels of cyclic AMP to induce vascular relaxation [24, 37]; or, in the rat aorta where L-NAME inhibits CGRP-induced relaxation [25], CGRP can act via an endothelial, nitric oxide-dependent mechanism to cause vascular relaxation [5, 24, 25]. This dual mechanism of vasodilator action of CGRP is in keeping with the finding that the hypotensive response to intravenous CGRP in the rat can be partially attenuated by nitric oxide synthase inhibitors [1, 22]. Hence, based on our results and analysis of the literature, it seems likely that in the rabbit ear skin there is a cascade of events in which vanilloid receptor activation induced by NVA results in the release of the sensory neuropeptides, such as CGRP, which in turn stimulate endothelial NO which is responsible for the observed arterial vasodilatation, although the effect of NVA on the membrane receptor was not investigated in the present experiments. Alternatively, NO might be involved in sensory-motor neuromodulation of sympathetic vasoconstriction by CGRP in the rabbit central ear artery, since such a hypothesis has been proposed for the artery [44].

The present study provides additional important evidence

that with low suprathreshold doses of NVA, but not with higher doses, desensitization to NVA did not take place. These data suggest that the extent to which desensitization to capsaicin analogues develops is related to the dose of the drug, as well as the time of exposure to the drug, and the time interval between consecutive dosings [28]. It is known that, with low suprathreshold doses of capsaicin analogues given at appropriate intervals, desensitization does not necessarily occur so that excitation of thin sensory neurons [35, 36], pain reactions [15, 16, 26, 54, 55], and peptide release from peripheral sensory nerve endings [2, 17] can be reproduced. Also, it may be possible that NVA-induced release of NO from endothelial cells could be reproduced with each application of low dose of this agent. The complexity of capsaicin-induced responses of sensitization and desensitization appears to limit the usefulness of capsaicin as a constituent of the classical counterirritant, or as a new analgesic for an attenuation of neurogenic inflammation [18] in clinical use [30], however, the application technique with low suprathreshold doses of capsaicin analogues given at appropriate intervals, may release capsaicin from the limitation of usefulness in clinical use [53].

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