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Nitric oxide enhances substance P-induced itch-associated responses in mice

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1 Substance P (SP) elicits itch and itch-associated responses in humans and mice, respectively. In mice, NK_1 tachykinin receptors are involved in SP-induced itch-associated responses, scratching, and mast cells do not play a critical role. The present study was conducted to elucidate the role of nitric oxide (NO) on SP-induced scratching in mice.

2 An intradermal injection of SP (100 nmol site^{-1}) elicited scratching in mice, and it was suppressed by an intravenous injection of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), but not by its inactive enantiomer D-NAME. Intradermal injections of L-NAME (100 nmol site^{-1}), another NOS inhibitor 7-nitroindazole (10 nmol site^{-1}) and the NO scavenger haemoglobin (0.01–10 nmol site^{-1}) also inhibited SP-induced scratching.

3 L-NAME (100 nmol site^{-1}) did not affect scratching induced by an intradermal injection of 5-hydroxytryptamine (100 nmol site^{-1}).

4 Intradermal injections of L-arginine (300 nmol site^{-1}) and the NO donor (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR3; 100 nmol site^{-1}) increased scratching induced by SP. Intradermal injections of L-arginine (1–1000 nmol site^{-1}) or NOR3 (1–100 nmol site^{-1}) alone were without effects on scratching.

5 Intradermal injections of SP (10–100 nmol site^{-1}) increased the intradermal concentration of NO in a dose-dependent manner in mice. An increase in NO levels induced by SP was inhibited by L-NAME and the NK_1 tachykinin receptor antagonist L-668,169, but not by the NK_2 tachykinin receptor antagonist L-659,877.

6 SP (1–10 μM) elicited NO production in cultured human keratinocytes and the SP-induced NO production was inhibited by L-NAME and L-668,169.

7 We conclude that intradermal SP increases NO in the skin, possibly through the action on NK_1 tachykinin receptors on the epidermal keratinocytes and that NO enhances SP-induced itch-associated responses.

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Keywords: Substance P; nitric oxide; nitric oxide synthase; scratching; tachykinin receptors; keratinocytes; pruritus

Abbreviations: L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; SP, substance P

Introduction

Itch, a skin sensation that provokes a desire to scratch, is a common complaint and strong itch is an important issue related to the quality of life. Itch generally follows cutaneous diseases and systemic disorders such as atopic dermatitis, contact dermatitis, urticaria, chronic renal failure, and cholestasis. Although many endogenous substances elicit an itch sensation following intradermal injection, precise mechanisms of itching in most pruritic diseases remain unknown. Substance P (SP) a member of the tachykinin family of peptides, acts as a neurotransmitter or modulator in the mammalian peripheral and central nervous system (Pernow, 1983). Clinically, SP has been claimed to be involved in itch in several pruritic diseases, including atopic dermatitis and chronic renal failure (Cho *et al*., 1997; Ostlere *et al*., 1995; Tobin *et al*., 1992). This peptide causes an itch sensation in human subjects when applied to the skin (Hägermark *et al*., 1978). The H_1 histamine receptor antagonist chlorcyclidine inhibits an itch sensation induced by an intradermal injection of low, but not high, doses of SP (Hägermark *et al*., 1978), suggesting the presence of histamine-dependent and independent mechanisms. In mice, SP elicits scratching (Kuraishi *et al*., 1995). Scratching is also induced by other pruritogens, but not by algogens (Kuraishi *et al*., 1995). SP-elicited scratching is inhibited by an opioid antagonist (Andoh *et al*., 1998). These findings suggest that SP-induced scratching is itch-associated response. Although direct action on NK_1 tachykinin receptors on the primary afferents may be at least partly involved in the SP-induced scratching (Andoh *et al*., 1996; 1998), the release of itch mediators from the skin is also responsible. For example, SP acts on the epidermal keratinocytes to release leukotriene B_4 (Andoh *et al*., 2001), which elicits itch-associated responses in mice (Andoh & Kuraishi, 1998). The inhibition of leukotriene B_4 action results in the inhibition of SP-induced itch-associated responses (Andoh & Kuraishi, 2000; 2002; Andoh *et al*., 2001).

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Nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) (Palmer *et al*., 1988). In the skin, NO has been shown to be involved in inflammation (Ialenti *et al*., 1992), hyperalgesia (Nakamura *et al*., 1996) and wound healing (Benrath *et al*., 1995). NO production may be increased in the skin of patients with pruritic skin diseases such as atopic dermatitis and psoriasis (Ormerod *et al*., 1998; Taniuchi *et al*., 2001). In the skin, NOS is present in several kinds of cells, including keratinocytes and mast cells (Baudouin & Tachon, 1996; Bidri *et al*., 1997). SP acts on similar kinds of cells, including keratinocytes (Staniek *et al*., 1998). In addition, SP acts on NOS-expressing cells to produce NO (Bull *et al*., 1996). Therefore, in the present study, we investigated whether an intradermal injection of SP would increase cutaneous concentration of NO and whether NO would be involved in SP-induced itch-associated responses.

Methods

Animals

Male ICR mice (Japan SLC., Shizuoka, Japan) of 5–6 weeks-of-age (30–35 g body weight) were used. They were housed under controlled temperature (23–25°C) and light (lights on from 08:00 h to 20:00 h) conditions. Food and water were freely available. Procedures in these animal experiments were approved by the Committee for Animal Experiments at Toyama Medical and Pharmaceutical University.

Materials

SP and (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR3) were purchased from Peptide Institute (Minoh, Japan) and Dojindo Laboratory (Kumamoto, Japan), respectively. 5-Hydroxytryptamine and 7-nitroindazole were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). N^G-nitro-L-arginine methyl ester (L-NAME), its D-enantiomer (D-NAME), cyclo(Gln-D-Trp(*N*Me)Phe(R)-Gly[ANC-2]Leu-Met)2 (L-668,169), and cyclo(Gln-Trp-Phe-Leu-Met) (L-659,877) were purchased from Research Biochemicals International (Natick, MA, U.S.A.). L-Arginine, D-arginine and haemoglobin were purchased from Wako Pure Chem. (Osaka, Japan). 7-Nitroindazole was dissolved in dimethyl sulfoxide (Wako Pure Chem.) and diluted with physiological saline. Other agents were dissolved in physiological saline.

Behavioural experiments

The hair was removed from the rostral part of the back using hair clippers and on the next day SP was injected intradermally in a volume of 50 μl. The other agents were injected intravenously 5 min before SP injection or intradermally together with SP. Before experiments, the animals were individually put into an acrylic cage (13 × 9 × 30 cm) for 1 h for acclimation. After SP injection, they were put back into the same cage and the behaviours were recorded using a video camera in unmanned conditions. Playing back of the video served for the counting of scratching of the injected site by the hind paws (Andoh *et al*., 1998). The mouse generally demonstrated scratching for about 1 s and a series of these movements was counted as one bout of scratching (Kuraishi *et al*., 1995).

Cell culture

Normal adult human epidermal cells (Kurabo Co., Osaka, Japan) were cultured at 10^6 cells per well in HuMedia-KG (Kurabo Co.) containing 10 μg ml^{-1} insulin, 0.1 ng ml^{-1} recombinant epidermal growth factor, 0.5 μg ml^{-1} hydrocortisone, 50 μg ml^{-1} gentamycin, 50 ng ml^{-1} amphotericin B and 4% bovine pituitary extract at 37°C in 5% CO_2 humidified atmosphere. SP was added in a volume of 50 μl to 450 μl of cell culture, and L-668,169, L-659,877, L-NAME and D-NAME were added together with SP.

Measurement of nitric oxide

The cutaneous concentration of NO was measured using the cutaneous microdialysis method (Andoh & Kuraishi, 1997). This series of experiments was conducted under ethyl carbamate (1.5 g kg^{-1}, i.p.) anaesthesia, without immobilization. The skin was perfused with a solution containing 0.1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide and perfusate was reacted on-line with Griess' reagent, 1.0% sulfanilamide and 0.1% naphtylethenediamine dihydrochloride in 2.5% phosphoric acid.

For *in vitro* experiments, human keratinocytes (10^6 cells) were cultured for 10 min in Humedia-KG2 medium containing 0.1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide and then SP was added. Five minutes later, an aliquot of medium was mixed with Griess's reagent, and the azo dye coloration formed was determined with a spectrophotometer at 540 nm, using sodium nitrite as standard. The concentration of NO was calculated from the concentration of nitrite; in *in situ* experiments, the cutaneous concentration of NO was calculated on the basis of 55% recovery.

Data processing

All data are presented as mean ± s.e.mean. The statistical significance of the time course of SP-induced scratching was analysed using the two-way repeated measures analysis of variance and the others using the one-way analysis of variance followed by Dunnett's or Student-Newman-Keuls multiple comparisons; $P<0.05$ was considered significant.

Results

SP-induced scratching

An intradermal injection of SP (100 nmol site^{-1}) elicited scratching of the injected site by the hind paws (Figure 1); the effect peaked in the first 10-min period and almost subsided by 30 min following injection. SP-induced scratching was inhibited by intravenous pretreatment with the NOS inhibitor L-NAME (0.1–10 mg kg^{-1}) in a dose-dependent manner (Figure 2). The inhibitory effect of L-NAME (10 mg kg^{-1}) was reversed by the co-injection of the NOS substrate L-

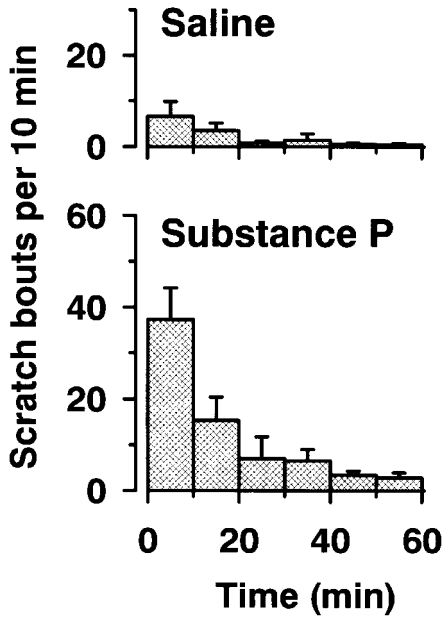

Figure 1 Scratching following an intradermal injection of substance P in mice. Mice were given an intradermal injection of saline or substance P (100 nmol site^{-1}), and the scratching of the injection site by the hind paws was counted at 10-min intervals. Values are the means and s.e.mean for eight animals. Two-way repeated measures analysis of variance, main effect of substance P, $F(1,14)=19.83$, $P<0.001$; group × time interaction, $F(5,70)=6.26$, $P<0.0001$.

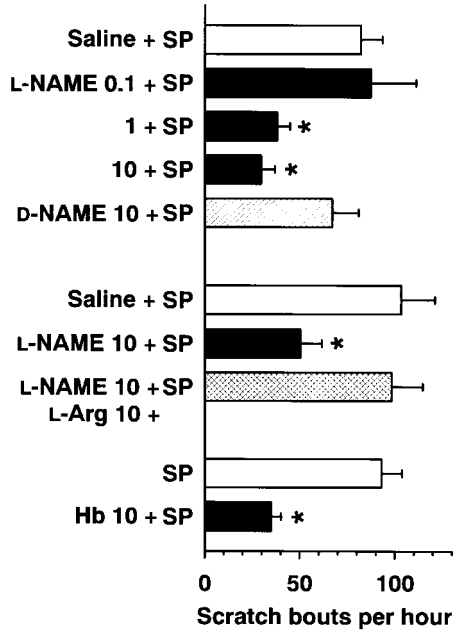

Figure 2 Inhibitory effects of nitric oxide synthase inhibitor and haemoglobin on substance P-induced scratching in mice. Mice were given an intradermal injection of substance P (SP; 100 nmol site^{-1}) and scratching was counted for 1 h. L-NAME (0.1–10 mg kg^{-1}) and the inactive enantiomer D-NAME (10 mg kg^{-1}) were injected intravenously 5 min before SP, and L-arginine (L-Arg; 10 mg kg^{-1}) was injected together with L-NAME. Haemoglobin (Hb; 10 nmol site^{-1}) was injected intradermally together with SP. Values are the means and s.e.mean for eight animals. *P <0.05 vs SP control (Dunnett's test).

arginine (10 mg kg^{-1}) (Figure 2). The inactive enantiomer D-NAME (10 mg kg^{-1}) was without effect on SP-induced scratching (Figure 2). Intravenous injection of L-NAME (10 mg kg^{-1}) and D-NAME (10 mg kg^{-1}) alone did not produce abnormal behaviours including scratching.

To determine the local action of NO, a NO scavenger and NOS inhibitors were injected intradermally together with SP (100 nmol site^{-1}). The NO scavenger haemoglobin (0.01–10 nmol site^{-1}) produced a dose-dependent inhibition of SP-induced scratching; the effect of a dose of 10 nmol site^{-1} is shown in Figure 2. L-NAME (100 nmol site^{-1}) and another NOS inhibitor 7-nitroindazole (10 nmol site^{-1}) significantly inhibited SP-induced scratching; the number of scratch bouts per hour following SP alone, SP plus L-NAME, and SP plus 7-nitroindazole were 107 ± 15, 45 ± 7, and 20 ± 5 ($n=8$ each), respectively. In contrast to SP, scratching elicited by an intradermal injection of 5-hydroxytryptamine (100 nmol site^{-1}) was not affected by L-NAME (100 nmol site^{-1}); the number of scratch bouts per hour was 242 ± 33 and 256 ± 48 ($n=8$ each) following 5-hydroxytryptamine alone and 5-hydroxytryptamine plus L-NAME, respectively.

Scratching induced by SP (100 nmol site^{-1}) was almost completely inhibited by a simultaneous injection of the NK_1 receptor antagonist L-668,169 (50 nmol site^{-1}), as compared with saline control (Figure 3). L-NAME (100 nmol site^{-1}) produced a partial inhibition of SP-induced scratching and did not enhance the effect of L-668,169; in other words, the number of scratch bouts following a combination of SP, L-668,169 and L-NAME was similar to that of saline control (Figure 3).

Intradermal injections of L-arginine (1–1000 nmol site^{-1}) and the NO donor NOR3 (1–100 nmol site^{-1}) did not significantly increase scratching as compared with saline (data not shown). Although neither L-arginine (300 nmol site^{-1}) nor SP (10 nmol site^{-1}) alone produced significant effects, their combination significantly increased scratching (Figure 4). SP (30 nmol site^{-1}) alone elicited scratching, and a combination with L-arginine (300 nmol site^{-1}) resulted in an increase in scratching (Figure 4). Similarly, NOR3 (100 nmol site^{-1}) also increased scratching induced by SP (10 nmol

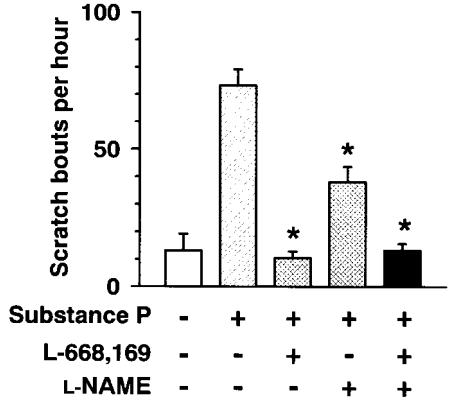

Figure 3 Inhibitory effects of nitric oxide synthase inhibitor and NK_1 receptor antagonist on substance P-induced scratching. Mice were given an intradermal injection of saline or substance P (100 nmol site^{-1}). L-NAME (100 nmol site^{-1}) and L-668,169 (50 nmol site^{-1}) were injected together with substance P. Values are the means and s.e.mean for eight animals. *$P<0.05$ vs substance P alone (Dunnett's test).

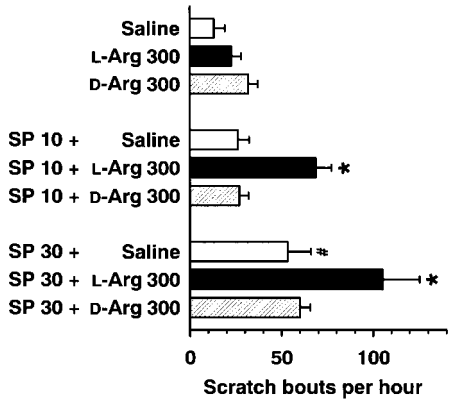

Figure 4 Enhancing effect of L-arginine (L-Arg) on substance P (SP)-induced scratching. L-Arg (300 nmol site^{-1}) and the isomer D-Arg (300 nmol site^{-1}) were injected alone or together with SP (10, 30 nmol site^{-1}). Values are the means and s.e.mean for eight animals. *$P<0.05$ vs SP plus saline control. #$P<0.05$ vs saline alone (Student-Newman-Keuls test).

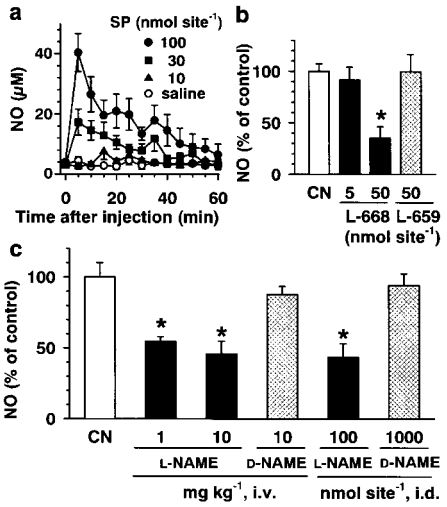

Figure 5 Substance P (SP)-induced production of nitric oxide in the mouse skin. Procedures for the determination of cutaneous nitric oxide are described in Methods. (a) Time-course and dose-dependence of nitric oxide production following intradermal SP. SP (10–100 nmol site^{-1}) or saline was injected intradermally into the rostral back. $n=5-6$. (b) Effects of tachykinin receptor antagonists on SP-induced nitric oxide production. L-668,169 (L-668) and L-659,877 (L-659) were injected intradermally together with SP (100 nmol site^{-1}). $n=8$. *$P<0.05$ vs SP alone (CN). (c) Effects of L-NAME on SP-induced nitric oxide production. L-NAME and D-NAME were injected intravenously (i.v.) 5 min before or intradermally (i.d.) together with SP (100 nmol site^{-1}). $n=6$. *$P<0.05$ vs SP alone (CN).

site^{-1}); the number of scratch bouts following NOR3, SP and NOR3 plus SP was 12 ± 3, 39 ± 5 and 78 ± 12 ($n=8$ each), respectively. D-Arginine (300 nmol site^{-1}) neither elicited scratching nor affected SP-induced scratching (Figure 4).

SP-induced NO production in the skin

Although saline and SP (10 nmol site^{-1}) were without effects, intradermal injections of SP (30 and 100 nmol site^{-1}) produced a dose-dependent increase in cutaneous NO concentration; the effects peaked after 5 min and subsided by 40–60 min (Figure 5a). An intradermal injection of the NK_1 tachykinin receptor antagonist L-668,169 (50, but not 5, nmol site^{-1}) significantly reduced (65% inhibition) the SP-induced NO production (Figure 5b). The NK_2 tachykinin receptor antagonist L-659,877 (50 nmol site^{-1}) was without effect (Figure 5b). Intravenous pretreatment with L-NAME (1 and 10 mg kg^{-1}) partially but significantly suppressed SP (100 nmol site^{-1})-induced NO production, with no suppression by D-NAME (10 mg kg^{-1}) (Figure 5c). Similar suppression was also obtained by an intradermal co-injection of L-NAME (100 nmol site^{-1}), but not D-NAME (1000 nmol site^{-1}) (Figure 5c).

SP-induced NO production in keratinocytes

Itch is a sensation of epidermis, which is mainly composed of keratinocytes. In this series of experiments, therefore, we examined whether SP would stimulate NK_1 tachykinin receptors on the keratinocytes to produce NO. Since NO production after intradermal SP peaked at 5 min, the NO production in cultured human keratinocytes was determined 5 min after SP administration. In control preparations, the concentration of NO was below the detection limit (0.2 μM nitrite), and SP itself affected neither the reaction with Griess' reagent nor the determination of nitrite (data not shown). SP (1–10 μM) elicited NO production in keratinocytes in a dose-dependent manner (Figure 6a). SP (10 μM)-induced NO production was dose dependently suppressed by L-668,169 at concentrations of 1–10 μM (Figure 6b). It was also dose dependently suppressed by L-668,169 at concentrations of 1–10 μM; L-NAME (10 μM) produced almost complete inhibition, whereas D-NAME (10 μM) was without effect (Figure 6c).

Discussion

NO and itch-associated responses

The principal result of the present study is that NOS inhibitors and NO scavengers produced a dose-dependent inhibition of SP-induced scratching. L-NAME inhibited the scratching, while the inactive enantiomer D-NAME was without effect and the inhibition by L-NAME was reversed by L-arginine, suggesting that NO produced by NOS is involved in the SP action. The results showing that SP-induced scratching was suppressed by a co-injection of L-NAME or 7-nitroindazole and increased by a co-injection of L-arginine suggest that NO produced in the skin is responsible for the SP-induced scratching. This idea is supported by the observation that an intradermal injection of SP increased the concentration of NO in the skin, the time-course of which was similar to that of SP-induced scratching. Intradermal injections of SP at doses of 30 and 100, but not 10 nmol site^{-1}, elicited scratching; the result is similar to the previous one (Andoh *et al*., 1998). An increase in cutaneous NO was also induced by SP at doses of 30 and 100, but not 10, nmol site^{-1}. In addition, intravenous injections of L-NAME at doses of 1 and 10, but not 0.1, mg kg^{-1}, suppressed SP-induced scratching, and the effective

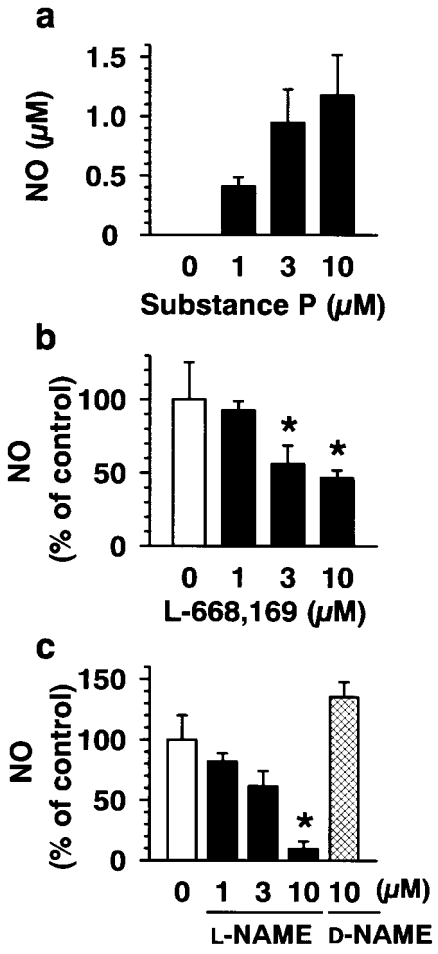

Figure 6 Substance P-induced production of nitric oxide in cultured human keratinocytes. (a) Dose-dependence of nitric oxide production induced by substance P (SP). (b) Suppressive effects of L-668,169 on nitric oxide production induced by SP (10 μM). (c) Suppressive effects of L-NAME on nitric oxide production induced by SP (10 μM). L-668,169, L-NAME and D-NAME were applied together with SP. The concentration of nitric oxide was determined 5 min after SP application. Values are the means and s.e.mean for six samples. $^*P<0.05$ when compared with SP alone.

doses also inhibited SP-induced NO production in the skin. Similarities in the time-course and dose-dependency between scratching and cutaneous NO production suggest a key role of NO in SP-induced scratching.

Intradermal injections of L-arginine (300 nmol site^{-1}) and NOR3 (100 nmol site^{-1}) increased SP-induced scratching. However, intradermal injections of L-arginine (1–1000 nmol site^{-1}) alone and NOR3 (1–100 nmol site^{-1}) alone did not increase scratching. Therefore, NO may be an enhancer of itching rather than an itch mediator or endogenous pruritogen. L-668,169 (50 nmol site^{-1}) almost completely inhibited SP-induced scratching; the result is similar to the previous one, in which L-668,169 and another NK_1 receptor antagonist spantide produced an almost complete inhibition (Andoh *et al*., 1998). On the other hand, L-668,169 at the same dose produced a partial inhibition of SP-induced NO production. Similarly, although a lower dose of 5 nmol site^{-1} significantly suppresses SP-induced scratching (Andoh *et al*., 1998), it was without effect on the NO production (the present experiment). These results taken together suggest that NK_1 receptors play a crucial role in SP-induced scratching and support the idea that NO is an enhancer of itching. Our results do not rule out the possibility of the presence of NK_1 receptor-independent mechanisms of SP-induced NO production because L-668,169 at doses tested produced only partial inhibitions of evoked NO production in *in situ* and *in vitro* experiments.

The serum level of nitrate, a product from NO, increases in patients with atopic dermatitis (Taniuchi *et al*., 2001). Repeated application of L-NAME decreases itch in 80% of atopic dermatitis patients (Morita *et al*., 1995). In addition, NO production is increased in the skin of patients with psoriasis, a pruritic skin disease (Kolb-Bachofen *et al*., 1994; Ormerod *et al*., 1998). Therefore, NO may be responsible for itch in some clinical pruritic diseases.

NO production in the skin

An intradermal injection of SP increased cutaneous NO concentration, which was inhibited by NK_1 tachykinin receptor antagonist. NOS1 and NOS3 require Ca^{2+} ions and calmodulin, while NOS2 does not require them (Busse & Mülsch, 1990; Schmidt *et al*., 1991; Yui *et al*., 1991). Stimulation of NK_1 receptors increases intracellular Ca^{2+} (Mochizuki-Oda *et al*., 1994). Therefore, NOS1 and NOS3, but not NOS2, may be involved in the SP-induced NO production.

An intradermal injection of 7-nitroindazole markedly suppressed the SP-induced scratching. As 7-nitroindazole is a selective inhibitor of NOS1 (Ayajiki *et al*., 2001), the results suggest an important role of NOS1 in the SP-induced NO production in the skin. However, since 7-nitroindazole also suppresses NOS3 at higher concentrations (Ayajiki *et al*., 2001), we do not rule out the involvement of NOS3. Several kinds of cells such as keratinocytes, primary sensory neurons, mast cells, fibroblasts, macrophages and endothelial cells can release NO in the skin (Moncada *et al*., 1991). In this study, SP acted on keratinocytes to produce NO *in vitro*, and this action was inhibited by the NK_1 tachykinin receptor antagonist. Keratinocytes constitutively express NOS1 and NOS3 (Baudouin & Tachon, 1996; Sasaki *et al*., 2000; Shimizu *et al*., 1997). Since itch is a nociceptive sensation of the epidermis, in which the keratinocyte is the predominant cell population, epidermal keratinocyte may be a leading candidate for itch-related NO production induced by SP. The keratinocytes induce NOS2 after stimulation, such as ultraviolet irradiation (Deliconstantinos *et al*., 1995), the ligation of CD23 by IgE/anti-IgE immune complexes (Bécherel *et al*., 1994), and contact hypersensitivity (Ross *et al*., 1998). Therefore, the present results do not exclude the possibility that NO produced by NOS2 is involved in itching, especially under dermatitis conditions.

NOS1 is also present in mast cells (Shimizu *et al*., 1997). SP acts on them to release several mediators including histamine, but this action is not mediated by NK_1 tachykinin receptors (Krumins & Broomfield, 1993; Mousli *et al*., 1990). In human subjects, mast cells and its mediator histamine play a role in SP-induced itching (Hägermark *et al*., 1978). However, SP elicits scratching in mast cell-deficient mice (Andoh *et al*., 1998), and SP-induced scratching is not suppressed by the H_1 histamine receptor antagonist chlor-

phenilamine in normal mice (Andoh & Kuraishi, 2002). The present study does not rule out the possibility that SP acts on mast cells to produce NO, but mast cells may not be primarily involved in SP-induced scratching.

Primary sensory neurons express NOS1 (Thippeswamy *et al*., 2001) and NK_1 tachykinin receptor (Andoh *et al*., 1996). Therefore, SP can be considered to act on NK_1 tachykinin receptors on afferent terminals to release NO. To ascertain this possibility, colocalization of NOS1 and NK_1 tachykinin receptors in sensory neurons should be elucidated.

Endothelial cells in the skin express NOS3 (Shimizu *et al*., 1997) and substance P stimulates the release of NO from dermal microvascular endothelial cells (Bull *et al*., 1996). Thus, it is also possible for intradermal SP to release NO from endothelial cells in the skin.

Site of action of NO in the skin

Although the present study does not provide convincing evidence for the site of action of NO induced by SP, we can give some suggestions. NO enhances the increase of intracellular calcium induced by bradykinin in primary sensory neuron (Yamada *et al*., 1997). NO may activate directly primary afferents (Meller *et al*., 1990). Thus, the primary afferent terminal is a likely candidate for the site of NO action. Another candidate is the vascular smooth muscle; SP dilates them through the release of NO from endothelial cells (Kuroiwa *et al*., 1995; Nguyen *et al*., 1995). Increase in cutaneous bloodstream rises skin temperature, which may elicit or enhance itching. SP-induced scratching was inhibited by NOS1-selective inhibitor; however, NOS1 is not present in the endothelial cells (Shimizu *et al*., 1997). 5-Hydroxytryptamine as well as SP releases NO from endothelial cells and dilates vascular smooth muscles (Ohnuki & Ogawa, 1997; Valentin *et al*., 1996). However, L-NAME suppressed SP-induced scratching, but not 5-hydroxytryptamine-induced scratching. Mosquito-induced immediate allergic response elicits scratching and plasma extravasation (Ohtsuka *et al*., 2001). In our preliminary experiments, L-NAME inhibited the plasma extravasation but not the scratching. Therefore, although the present results do not exclude the action of NO induced by SP on the vascular smooth muscles and endothelial cells, these cells may not play an important role in scratching.

Itch signals are mediated mainly by C-fibres (Schmelz *et al*., 1997), especially by capsaicin-sensitive primary afferents (Andoh *et al*., 1998; Tóth-Kása *et al*., 1986). The direct actions of SP (Andoh *et al*., 1996) and leukotriene B_4 released from keratinocytes (Andoh *et al*., 2001) on the primary afferents may generate itch signals and NO produced by SP may increase these actions to enhance itching.

Conclusion

Intradermal SP increases NO production, especially through the action on epidermal keratinocytes, and NO may play an enhancing role in SP-induced itch-associated responses. NO and NOS may be new targets for antipruritic agents.

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