Short Communication

Repeated Treatment With the Traditional Medicine Unsei-in Inhibits Substance P-Induced Itch-Associated Responses Through Downregulation of the Expression of Nitric Oxide Synthase 1 in Mice

Tsugunobu Andoh¹, Ali Al-Akeel¹, Kenichiro Tsujii¹, Hiroshi Nojima¹, and Yasushi Kuraishi¹,²,*

¹Department of Applied Pharmacology, Faculty of Pharmaceutical Sciences, and ²21st Century COE Program, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

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Abstract. Unsei-in inhibits substance P (SP)-induced scratching of mice after repeated administration. The involvement of cutaneous nitric oxide (NO) in the SP-induced scratching led us to investigate the effects of Unsei-in on the cutaneous NO system in mice. Seven-day oral administration of Unsei-in (300, but not 100, mg/kg daily) significantly inhibited scratching and the increase of cutaneous NO after intradermal SP injection. The NO synthase 1 (NOS1) inhibitor 7-nitroindazole (1 nmol/site) decreased SP-induced scratching and NO production. Repeated administration of Unsei-in (300 mg/kg) reduced the cutaneous NOS1 level. The results suggest that the inhibition of cutaneous NOS1 expression and NO production participates in the antipruritic action of Unsei-in.

Keywords: Unsei-in, nitric oxide synthase 1, itch-associated response

Unsei-in is a traditional medicine that is composed of Rehamanniae Radix, Paeoniae Radix, Cnidii Rhizoma, Angelicae Radix, Scutellariae Radix, Phellodendric Cortex, Coptidis Rhizoma, and Gardeniae Fructus. It is used to treat pruritic cutaneous diseases such as eczema and skin eruptions. With regard to the experimental evidence for the antipruritic effects of Unsei-in, we have recently found that although single oral administration of Unsei-in (300 and 1,000 mg/kg) does not affect substance P (SP)-induced scratching (itch-associated response), repeated administration of the dose of 300 mg/kg significantly inhibits the scratching in mice (1). In addition, the same dosage of Unsei-in significantly downregulated the expression of NK1 tachykinin receptors in the mouse skin (1).

SP acts on mast cells to release histamine, which may not be mediated by the activation of tachykinin receptors, but instead by the direct action on G protein (2). On the other hand, an intradermal injection of SP elicits scratching in mice (3), which is inhibited by the NK1 tachykinin receptor antagonist (4), but not by the H1 histamine receptor antagonist (5). SP-induced scratching is suppressed by the nitric oxide (NO) synthase inhibitor (6). SP increases NO production through the action on the skin, especially keratinocytes, which is inhibited by the NK1 tachykinin receptor antagonist (6). Considering these findings, the inhibitory effect of Unsei-in on the expression of NK1 tachykinin receptors (1) raises the possibility that the suppression of the production of NO is involved in the inhibitory effect of Unsei-in on the SP-induced scratching. To test this possibility, in the present experiments, we determined whether repeated administration of Unsei-in would affect SP-induced production of NO in the skin.

SP-induced scratching is inhibited by 7-nitroindazole, an NO synthase 1 (NOS1) inhibitor (7), in mice, suggesting the involvement of NOS1 in this SP action (6). The NOS1 gene has the consensus sequence for the cyclic AMP response element (CRE) in the promoter region (8). The promoter region of the NK1 tachykinin receptor also contains a sequence for CRE (9). Therefore, we also examined the effect of repeated administration of Unsei-in on the expression level of NOS in murine skin.

Male ICR mice (Japan SLC, Shizuoka), 4 weeks of age at the start of experiment, were used. They were housed under controlled temperature (23 – 25°C) and light conditions (lights on from 08:00 to 20:00). Food
and water were freely available. Procedures for the animal experiments were approved by the Committee for Animal Experiments of Toyama Medical and Pharmaceutical University.

SP (Peptide Institute, Minoh) was dissolved in physiological saline and the 100 nmol/site dose was injected intradermally to the rostral back. The extract of Unsei-in, a gift from Kanebo (Tokyo), was suspended in 5% Arabic gum and administered orally once a day for 7 days; SP was injected 30 min after the last injection of Unsei-in. 7-Nitroindazole (Sigma Chem., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide and diluted with physiological saline. This agent was injected intradermally together with SP.

The hair was clipped over the rostral part of the back the day before the pruritogen injection. Before behavioral recording, the mice were individually put into the cells (13 × 9 × 30 cm) of an acrylic cage for at least 1 h for acclimation. Immediately after the intradermal injection, the animals were put back in the same cells and their behaviors were videotaped for 1 h with any persons kept out of the observation room. Playing back of the video served for counting scratching behavior. Mice generally scratch several times for about 1 s and a series of these movements was counted as one bout of scratching (3).

The skin was excised from the rostral back. Proteins were extracted from the skin (a diameter of 1.7 cm) with a lysis buffer [20 mM Tris HCl (pH 7.5), 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin]. The proteins (20 μg) were separated by electrophoresis using a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk solution for 1 h, the membrane was reacted with anti-NOS1 or anti-β-actin antibody overnight at 4°C. Subsequently, it was incubated with horseradish peroxidase-conjugated IgG for 1 h at room temperature and then treated with chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ, USA). Chemiluminescent signals were detected using x-ray film and analyzed using the NIH Image program. The data was normalized with β-actin.

The pruritogen-injected skin with a diameter of 1.7 cm was isolated 5 min after the SP injection. The skin was homogenized in 0.1 M phosphate buffer (0.072 M NaH₂PO₄, 0.028 M NaH₂PO₄·2H₂O, pH 7.4) and the homogenate was centrifuged. The supernatant was used for the measurements of nitrite, one of the metabolites of NO, and protein concentration. For the detection of nitrite, the supernatant (100 μl) was reacted with Griess’ reagent (1% sulfanilamide, 0.1% N-1-naphthylethylene-diamine dihydrochloride, 2.5% phosphoric acid). The azo dye formed was determined with a spectrophotometer at 540 nm, using sodium nitrite as a standard. The protein concentration was determined by using a Bio-Rad protein assay kit.

All data are presented as the mean and S.E.M. Statistical significance was analyzed by Student’s t-test or Dunnett’s multiple comparisons. P<0.05 was considered significant.

An intradermal injection of SP (100 nmol/site), but not saline, elicited scratching of the injected skin by the hind paws; the effect peaked in the initial 10 min and almost subsided by 60 min (Fig. 1: A and B). Repeated oral administration of Unsei-in (300 mg/kg, but not 100 mg/kg) inhibited the SP (100 nmol/site)-induced scratching (Fig. 1C). In our preliminary experiments, we found that NOS1 is present in the epidermis of normal

Fig. 1. Effects of Unsei-in and 7-nitroindazole on substance P (SP)-induced scratching in mice. Mice were given an intradermal injection of SP (100 nmol/site) and their scratching of the injected site was counted for 1 h. A: Scratching after an intradermal injection of saline (SLN). B: Scratching after an intradermal injection of SP (100 nmol/site). C: Effects of Unsei-in and 7-nitroindazole on SP-induced scratching. Unsei-in (100 and 300 mg/kg) or vehicle was administered orally once a day for 7 days. SP was injected 30 min after the last Unsei-in administration. 7-Nitroindazole (0.1 and 1 nmol/site) was injected together with SP. The number of scratch bouts (mean ± S.E.M.) of the control (CNT) groups was 88 ± 13 and 127 ± 15 in the Unsei-in and 7-nitroindazole experiments, respectively. Values are the means and S.E.M. for eight animals. *P<0.05 vs CNT, by Dunnett’s multiple comparisons.
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Therefore, as the control experiments, we examined the effects of a selective NOS1 inhibitor on the SP-induced scratching. SP (100 nmol/site)-induced scratching was dose dependently inhibited by intradermal co-injections of 7-nitroindazole (0.1 and 1 nmol/site) (Fig. 1C).

An intradermal injection of SP (100 nmol/site) produced a significant increase in the NO concentration (determined by nitrite concentration) in the skin, which was completely abolished by a co-injection of 7-nitroindazole (1 nmol/site) (Fig. 2A). Repeated treatment with Unsei-in (100 and 300 mg/kg) produced a dose-dependent inhibition of SP (100 nmol/site)-induced production of NO in the skin (Fig. 2B). The basal concentration of NO was not affected by repeated treatment with Unsei-in (100 and 300 mg/kg), although it tended to decrease (Fig. 2B).

Western blotting using anti-NOS1 antibody gave a clear single band corresponding to NOS1 in the murine skin (Fig. 3A). Repeated treatment with Unsei-in (300 mg/kg) produced a significant 68% decrease in the NOS1 level (Fig. 3B). NOS2 was not detected in the normal mouse skin (data not shown).

We have previously shown that although single oral administration of Unsei-in (300 and 1000 mg/kg) is without effect, repeated administration of Unsei-in (300 mg/kg) inhibits SP-induced scratching in mice (1). In the present experiments, we reconfirmed the effectiveness of repeated administration of Unsei-in at a dose of 300 mg/kg and found that the lower dose of 100 mg/kg was without effect. The effective dose is similar to those in other experiments using mice, such as carrageenan-induced edema (10) and acetic acid-induced abnormal constriction (11). The clinical dose of Unsei-in is 2,400 mg per day. Given that the body weight is 60 kg, the dose per body weight is 40 mg/kg. Thus, the effective dose in mice is 7.5-fold higher than the daily dose in humans. Differences in effective dose between humans and mice might be at least due to differences in the absorption and/or metabolism of this medicine.

![Fig. 2.](image2.png) Effects of 7-nitroindazole (7-NI) and Unsei-in on substance P (SP)-induced NO production in the mouse skin. Mice were given an intradermal injection of SP (100 nmol/site) or saline (SLN); and 5 min later, a 1.7-cm diameter, round section of the skin including the injected site was isolated and served for the determination of NO concentration using the Griess’ method (see Materials and Methods). A: 7-NI (1 nmol/site) was injected together with SP. B: Unsei-in (100 and 300 mg/kg) or vehicle (VH) was administered orally once a day for 7 days, and SP or SLN was injected intradermally 30 min after the last administration. The dotted lines indicate the concentration of nitrite in the untreated mouse skin. Values are the means and S.E.M. for six animals. *P<0.05 vs SLN; ^P<0.05 vs SP alone (A) and VH (B), by Dunnett’s multiple comparisons.

![Fig. 3.](image3.png) Inhibition by Unsei-in on the expression level of nitric oxide synthase 1 (NOS1). Unsei-in (300 mg/kg) and vehicle were administered orally once a day for 7 days. The skin of the rostral back was isolated 30 min after the last administration and served for the Western blot determination of NOS1. A: A typical example of the levels of NOS1 and β-actin. B: The expression level of NOS1 normalized with β-actin. Values are the means and S.E.M. for four animals. *P<0.05, by Student’s t-test.
Repeated administration of Unsei-in at a dose of 300, but not 100, mg/kg, inhibited NO production in the skin and scratching induced by an intradermal injection of SP, suggesting that the inhibition of NO production is involved in the inhibitory effect of Unsei-in on the SP-induced scratching. This idea is supported by the results that an intradermal injection of the NOS1 inhibitor 7-nitroindazole (1 nmol/site) together with SP markedly suppressed the NO production as well as scratching induced by SP.

Single administration of the NOS1 inhibitor 7-nitroindazole suppressed the SP-induced scratching and NO production. On the other hand, single administration of Unsei-in (300 and 1000 mg/kg) does not affect SP-induced scratching (1), suggesting that the inhibitory effect of Unsei-in on scratching and NO production induced by SP is not due to the inhibition of NOS activity. Repeated treatment with Unsei-in markedly decreased the expression level of NOS1 in the skin. This reduction may play a key role in the inhibitory effect of Unsei-in on the SP-induced scratching and NO production.

Unsei-in suppresses the expression of NK1 tachykinin receptors in the skin (1). This action may be also involved in the inhibitory action of Unsei-in on the SP-induced NO production. In addition, the activation of NK1 tachykinin receptors in the skin and keratinocytes results in the release of leukotriene B4 in mice (12). This lipoxygenase product elicits scratching in mice at low intradermal doses (13). Therefore, although the 26% inhibition in NK1 tachykinin receptor expression (1) was smaller than the 68% inhibition in NOS1 (present experiments), the former may also play a role in the effect of Unsei-in on the SP-induced scratching.

The inhibitory mechanisms of Unsei-in on the expression of NOS1 (the present data) and NK1 tachykinin receptor (1) are unclear. The promoter regions of NOS1 gene (8) and NK1 tachykinin receptor gene (9) have binding sites for CRE-binding protein (CREB). In our preliminary experiments, the repeated treatment with Unsei-in did not inhibit the expression of the transcription factor CREB. The critical feature of CREB protein activation is the phosphorylation of CREB, which is required for CREB-mediated stimulation of transcription. CREB is phosphorylated by several kinases such as calmodulin-dependent protein kinase II (14), protein kinase A (14), and and p38 MAP kinase (15). Thus, Unsei-in might inhibit the activity or the expression of protein kinase(s) after repeated administration.

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