Prostaglandin D₂ improves IL-31-induced alloknesis: itch-stimulation becomes pain-stimulation in mouse skin

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Introduction

Recent studies on itch have been based on the human pruriceptive sense, and have found no discernable differences between the nociceptive stimuli examined. Since itching elicits a strong desire to scratch, the measurement of scratching is useful for evaluating itching [1]. In a previous investigation that measured spontaneous scratching in NC/Nga mice, an animal model of atopic dermatitis [2], two kinds of scratching behavior were observed: long-lasting
scratching (LLS, over 1.5 s) and short-lasting scratching (SLS, 0.3 – 1.5 s). In that study, LLS was frequently seen in spontaneous skin-lesioned NC/Nga mice, but not in other strains of mice. In contrast, SLS was frequently seen in both skin-lesioned NC/Nga mice and other strains of mice. These results suggest that SLS is a form of social and/or hygiene behavior, while LLS is the true itching response in these mice. Therefore, we investigated LLS as an indicator of itching [3, 4].

Previously, we reported that topical application of prostaglandins (PGs), especially PGD₂, significantly suppressed LLS counts in skin-lesioned NC/Nga mice, via a specific prostanoid DP1 receptor [5]. It is known that the itch sensation can be reduced by the painful sensations caused by scratching. The inhibition of itching by painful stimuli has been experimentally demonstrated by the use of various painful stimuli. We demonstrated that the scratching of mouse skin with a stainless steel wire brush (mechanical scratching) was associated with a significant elevation of cutaneous PG levels [6], and these PGs suppressed the itch-associated scratching behavior (LLS) observed in mite-infected mice [7]. On the other hand, it is well known that PGs are associated with inflammation, and their administration was found to enhance pain [8].

Touch- or brush-evoked pruritus around an itching site has been termed “aloknesis” [9, 10], whereas pin prick-evoked itching sensations around an itching site have been termed “hyperknesis”. Recent studies in patients with chronic itching have demonstrated that repetitive application of painful stimuli, such as electrical or noxious heat stimuli, or scratching stimuli distal to an itchy stimuli, may be perceived as an itch [11, 12]. This may also explain why scratching aggravates itching and induces a vicious cycle of scratching-induced itching [13]. Clarification of the precise mechanisms of itchy skin could have major therapeutic implications, but a suitable animal model for studying this phenomenon does not yet exist. Pruritus is an important symptom of atopic dermatitis; however, the major pruritogen(s) have not yet been identified. Interleukin-31 (IL-31) is a possible mediator of itching, and induces both severe pruritus and dermatitis in mice [14]. Recently, we reported that intradermal injection of pruritogens and algogens in BALB/c mice that were cohoused with skin-lesioned NC/Nga mice or pretreated with IL-31 increased itch-associated scratching (LLS) counts, and suggested that this phenomenon might be similar to “aloknesis” or “hyperknesis” [15]. However, the sites of action of IL-31 have not been clarified. In this study, we investigated the relationship between IL-31, an alloknesis-inducer, and PGD₂, an alldynia-inducer, to elucidate the regulatory mechanism of itch and pain.

Materials and Methods

Animals

Male 8-week-old BALB/c mice were purchased from SLC Japan (Shizuoka, Japan). The animals were housed under conditions of controlled temperature (23±3 °C), humidity (50±20 %) and lighting (lights on from 7:00 am to 7:00 pm). All animals were given free access to food and tap water. All procedures for animal experiments were approved by the Committee for Animal Experimentation at the International University of Health and Welfare and were in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Reagents

Mouse IL-31 cDNA spanning amino acids 24-163 of IL-31 was cloned in frame with pET30A (Novagen, Darmstadt, Germany), and the construct was transformed in BL-21 cells (Novagen). After induction with isopropyl-β-D-thiogalactopyranoside, IL-31 protein was purified under denaturing conditions by nickel-chelating sepharose (Qiagen, Benelux B.V. Netherlands), and dialyzed in phosphate buffer solution [16]. IL-31 was injected intradermally (i.d.; 0.2 mL/site) to the rostral part of the back or intravenously (i.v.; 0.2 mL/body) in mice. Prostaglandin D₂ (PGD₂, Cayman), prostaglandin E₂ (PGE₂, Cayman) and prostaglandin I₂ (PGI₂, Cayman) were dissolved separately in ethanol (EtOH) and topically applied to the rostral part of the back (0.2 mL/site) or the footpad (0.04 mL/site) of mice. Ketotifen (Sigma-Aldrich) and dexamethasone (Wako Jyunyaku) were suspended in 1% Tween 80 and administered orally (p.o.; 10 mL/kg). Histamine (Wako Jyunyaku, Osaka, Japan), serotonin (5-HT; Sigma-Aldrich, St Louis, MO, USA), compound 48/80 (Sigma-Aldrich), acetylcholine (Wako Jyunyaku), bradykinin (Wako Jyunyaku) and capsaicin (Wako Jyunyaku) were dissolved separately in saline and injected intradermally (i.d.; 0.02 mL/site) into the back and neck of mice.

Measurement of scratching counts

Scratching was measured as described previously [17]. The number of scratches was detected automatically and evaluated objectively using MicroAct (Neuroscience, Tokyo, Japan) [18]. The analysis parameters for detecting waves were Threshold: 0.1 V, Event gap: 0.2 s, Minimum duration: 1.5 s, Maximum frequency; 20 Hz, and Minimum frequency; 2 Hz.

Effect of PGD₂ on IL-31 (acute administration)-induced itch-associated scratching behavior in mice
A single intradermal injection of IL-31 (1μg/site, i.d.) was carried out at 10:00 AM. This dosage is based on our previous report [15]. PGD₂ (0.1 %) was dissolved in EtOH and applied topically at 1:00 PM to the rostral part of the back of mice at 3 h after IL-31 injection. The scratch count was measured as described above and compared between groups.

Effects of drugs on IL-31 (repeated administration)-induced scratching behavior and DRG IL-31RA mRNA expression in mice

Injection of IL-31 (15 μg/kg, s.c.) was carried out every 12 h (7:00 and 19:00) for 3 days. PGD₂ (0.1 %) was dissolved in EtOH and applied topically to the rostral part of the back of mice. Ketotifen (10 mg/kg, p.o.) and dexamethasone (1 mg/kg, p.o.) were administered immediately after the IL-31 injection.

Measurement of irritant-induced scratch counts

To elicit scratching behavior, histamine (2 μmol/site, i.d.), 5-HT (300 nmol/site, i.d.), compound 48/80 (160 nmol/site, i.d.), acetylcholine (1.5 μmol/site, i.d.), bradykinin (200 nmol/site, i.d.) or capsaicin (60 nmol/site, i.d.) was injected 1 h after the administration of IL-31 (1 μg/body, i.v.). Immediately after the injection, the mice were placed in an observation chamber and scratching behavior was monitored every 10 min for 1 h.

Quantitative real-time PCR

Total RNA was extracted from the dorsal skin of each mouse by Trizol (Invitrogen-Carlsbad, CA, USA) and digested using amplification-grade DNase I (Invitrogen), according to the manufacturer’s instructions. cDNA was synthesized by the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with SYBR Green Master Mix, using an Applied Biosystems 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR primers for IL-31 were designed using PRIMER 3 software, and primers for β-actin were purchased from TAKARA BIO (Otsu, Shiga, Japan). Primer sequences were as follows: IL-31RA (‘5’-CCA GAA GCT GCC ATG TCG AA-3’ and 5’-TCT CCA ACT CGG TGT CCC AAC-3’), β-actin (5’-TGA CAG GAT GCA GAA GGA GA-3’ and 5’-GCT GGA AGG TGG ACA GTG AG-3’). Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer’s
technical bulletin. A standard curve was generated using fluorescent data from four-fold serial dilutions of total RNA of the sample with the highest expression. This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding \( \beta \)-actin RNA transcript in the skin samples.

**Measurement of the nociceptive effect of prostaglandins**

The hot-plate test was adapted from Eddy and Leimbach (1953). Mice were placed on a hot-plate maintained at 51 ± 0.5 °C, and the latency to either paw-lick or an attempt to escape by jumping was recorded. PGD\(_2\), PGE\(_2\) and PGI\(_2\) dissolved in EtOH at 0.1% (w/v) were topically applied to the footpad of mice. Animals were tested before and 30, 60, 90, 120, 150, 180 min after the application of PGs. To prevent tissue damage, mice that showed no response within 60 sec were removed from the hot-plate and assigned a score of 60 sec. The percentage of nociception (nociceptive index) was calculated according to the formula: \( [(T_1-T_0)/(T_2-T_0)] \times 100 \), where \( T_0 \) and \( T_1 \) were the latencies observed before and after the application of PGs, respectively, and \( T_2 \) was the cut-off time (60 sec).

**Data analysis**

Parameters are reported as the mean ± S.E. Nociceptive effects are represented by the area under the curve that was calculated for each mouse by the trapezoidal method and expressed as a mean percentage for the vehicle-treated group. The parametric Student’s t-test with the Bonferroni correction was used to analyze the data. \( P \) values of less than 0.05 were considered statistically significant.

**Results**

**Effect of PGD\(_2\) on IL-31 (acute administration)-induced itch-associated scratching behavior in mice**

In vehicle (PBS)-injected and ethanol (EtOH)-applied mice, LLS counts were slightly increased at night (Fig. 1A, green line). There was no change in LLS counts in vehicle-injected and 0.1% prostaglandin D\(_2\) (PGD\(_2\))-applied mice during the experimental period (Fig. 1A, purple line), but the total LLS count was significantly decreased compared to that in vehicle-injected and EtOH-applied mice (Fig. 1B). On the other hand, IL-31 (acute administration 1 \( \mu \)g/site, i.d.)-injected and EtOH-applied mice showed a gradual increase in LLS counts over 24 h, especially at night.
(Fig. 1A, blue line). The LLS caused by IL-31 was suppressed by topical application of 0.1% PGD₂ (Fig. 1, orange line). Total LLS counts with vehicle + EtOH, vehicle + PGD₂, IL-31 + EtOH and IL-31 + PGD₂ were 57.5±9.9, 27.4±7.2, 186.0±21.5 and 86.9±20.1 counts/24 h, respectively (Fig. 1B).

Effects of PGD₂, dexamethasone and ketotifen on IL-31(repeated administration)-induced LLS and DRG IL-31 mRNA expression in mice

The effects of prostaglandin D₂ (PGD₂), dexamethasone (Dex) and ketotifen (Ket) on IL-31 (repeated administration)-induced scratching behavior in mice were examined. Repeated administration of IL-31 (15 μg/kg, s.c.)
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every 12 h for 3 days induced an apparent increase in LLS. This increase in LLS was significantly suppressed by pretreatment with 0.1 % PGD$_2$ or Dex (1 mg/mL, p.o.), but not Ket (10 mg/mL, p.o.). Total LLS counts for last 24 h of treatment with PBS, vehicle + IL-31, PGD$_2$ + IL-31, Dex + IL-31 and Ket + IL-31 were 92.5±19.9, 211.3±44.4, 120.3±13.6 and 120.3±13.6, respectively (Fig. 2A). Repeated administration of IL-31 every 12 h for 3 days significantly increased IL-31RA mRNA expression compared with that in vehicle-injected mice. Pretreatment with PGD$_2$, Dex or Ket did not affect the changes in DRG IL-31RA mRNA expression (Fig. 2B).

Effects of the topical application of PGD$_2$ on irritant-induced LLS and SLS in mice with itchy skin caused by pretreatment with IL-31

Figure 3A shows traces of saline-, histamine (2 μmol/site, i.d.), 5-HT (300 nmol/site, i.d.), compound 48/80 (160 nmol/site, i.d.), acetylcholine (1.5 μmol/site, i.d.), bradykinin (200 nmol/site, i.d.) and capsaicin (60 nmol/site, i.d.)-induced scratching behavior in mice with itchy skin caused by IL-31 (50 μg/kg, i.v.). These pruritogens and algogens elicited many SLS and a few LLS in EtOH (topical)-treated mice (Fig. 3A, left side). In contrast, 0.1 % PGD$_2$ (topical)-treated mice, the expression of LLS but not SLS seemed to decrease (Fig. 3A, right side). Indeed, a detailed data analysis revealed that the topical application of PGD$_2$ in mice with itchy skin caused by IL-31 significantly decreased pruritogen- or algogen-induced LLS counts (Fig. 3B). However, no significant differences in SLS were observed between the EtOH- and PGD$_2$-treated groups, except that acetylcholine-induced SLS was significantly decreased in the PGD$_2$-treated group (Fig. 3C).

Effects of topical application of PGD$_2$ on irritant-induced LLS and SLS in normal-skin mice

In mice that were treated topically with EtOH, saline, histamine (2 μmol/site, i.d.), 5-HT (300 nmol/site, i.d.), compound 48/80 (160 nmol/site, i.d.), acetylcholine (1.5 μmol/site, i.d.), bradykinin (200 nmol/site, i.d.) and capsaicin (60 nmol/site, i.d.) elicited many SLS and a few LLS (Figs. 4A and 4B). On the other hand, in mice that were treated topically with 0.1 % PGD$_2$, these pruritogen- and algogen-elicited SLS and LLS were slightly decreased, although most of these changes were not significant. The topical application of PGD$_2$ significantly decreased only 5-HT- and bradykinin–induced LLS (Fig. 4A), and 5-HT-induced SLS (Fig. 4B).

Effects of PGs on the pain threshold for thermal stimuli

![Figure 4. Effects of PGD$_2$ on pruritogen- or algogen-induced scratching behavior in normal-skin mice. LLS; long-lasting scratching (counts/1h) (A), and SLS short-lasting scratching (counts/1h) (B). Saline; scratch counts after saline was injected intradermally into the rostral part of the back of mice, Hist; histamine (10 μg/mouse), 5-HT; serotonin (5 μg/mouse), Co48/80; Compound 48/80 (10 μg/mouse), ACh (acetylcholine, 10 μg/site, i.d.), BK (bradykinin, 10 μg/site, i.d.) or Cap (capsaicin, 1 μg/site). The green column indicates vehicle + EtOH-treated mice, and the yellow column indicates vehicle + prostaglandin D$_2$ (PGD$_2$)-treated mice. Each value represents the Mean±S.E. *P<0.05 compared with the respective values in EtOH-treated mice.](http://www.smartscitech.com/index.php/itp)
estimated by the hot-plate test in mice.

In vehicle (EtOH)-applied mice, latency was not changed during the experimental period (Fig. 5A, blue line). In contrast, PGD$_2$ (orange line), PGE$_2$ (green line) and PGI$_2$ (purple line) decreased the latency to escape behavior from 30 to 120 min after application (Fig. 5A). The nociceptive index (AUC$_{30-120}$) for the vehicle, PGD$_2$, PGE$_2$ and PGI$_2$ was 2.64±5.70, -37.42±3.95, -21.94±11.30, and -33.79±10.72 respectively (Fig. 5B). The nociceptive activities of these PGs were in the order PGD$_2$ > PGI$_2$ > PGE$_2$.

Discussion

Itch-associated scratching behavior is an important symptom for the development of dermatitis in NC/Nga mice, but the major pruritogens have not yet been identified. We previously reported that LLS counts correlated with the dermatitis score in NC/Nga mice, and LLS was inhibited by dexamethasone or PGD$_2$, but not by ketotifen [3, 4]. In this study, PGD$_2$ and dexamethasone significantly suppressed IL-31-induced LLS counts, while ketotifen had no effect. These effects are similar to those on spontaneous scratching behavior in NC/Nga mice, suggesting that IL-31 participates in the sensation of itching and promotes LLS in skin-lesioned NC/Nga mice. However, neither PGD$_2$ nor dexamethasone suppressed the expression of DRG IL-31RA mRNA in mice. This result indicates that the suppressive effect of PGD$_2$ on IL-31-induced LLS was not due to a decrease in the expression of DRG IL-31 receptor A. It has been reported that mechanical scratching of the skin significantly increases the cutaneous PGD$_2$ level [6] and the cutaneous application of PGD$_2$ significantly suppresses LLS in skin-lesioned NC/Nga mice [7]. Considering these reports, the present findings suggest that PGD$_2$ may keep an itch in check through functional antagonism for alloknesis or hyperknesis caused by IL-31.

Tissue injury results in the release of many chemical mediators, such as ATP, bradykinin, amines, protons, prostanoids, cytokines and peptide [19]. These inflammatory mediators also act to modify the response properties of primary afferent neurons to subsequent stimuli (peripheral sensitization). Alternatively, the responses to noxious stimuli may be enhanced (hyperalgesia) or normally innocuous stimuli may produce pain (allodynia). PGE$_2$ and PGI$_2$ have been shown to influence inflammation, and their administration was found to reproduce the major signs of inflammation including augmented pain [8, 20]. They are synthesized by the constitutive enzyme cyclooxygenase-1 (COX-1) and its isof orm enzyme COX-2, which is induced in peripheral tissue by inflammatory stimuli [21]. Interestingly, it has been reported that COX-1-deficient mice show reduced nociceptive activity [22]. On the other hand, the topical
application of PGD₂, PGE₂ and PGI₂ significantly suppressed LLS in skin-lesioned NC/Nga mice, and their inhibitory activities were in the order PGD₂ >> PGI₂ > PGE₂. In this study, PGD₂ significantly suppressed IL-31-induced LLS. In addition, PGD₂, PGE₂ and PGI₂ increased nociceptive effects and their nociceptive activities were in the order PGD₂ > PGI₂ > PGE₂, which is the same as the order of their anti-pruritic activities [5]. Our previous data showed that topical application of the selective COX-1 inhibitor CS-560, but not the selective COX-2 inhibitor NS-398, clearly increased LLS [23]. Furthermore, the cutaneous level of PGD₂ in COX-1-deficient mice was significantly lower than that in wild type, while the cutaneous levels of PGE₂, PGF₂α and PGI₂ in COX-1-deficient mice were almost the same as those in wild type [24]. These previous and present findings suggest that cutaneous PGD₂ could be mainly produced by COX-1, and may play a critical role in the regulation of the sensation of both itch and pain.

Recently, we investigated LLS induced by several pruritogens and algogens i.e., histamine, serotonin, compound 48/80, acetylcholine, bradykinin and capsaicin, in mice with itchy skin. In NC/Nga mice with itchy skin caused by mite-infestation, LLS induced by pruritogens (histamine, serotonin and compound 48/80) or algogens (acetylcholine, bradykinin and capsaicin) was significantly increased compared to that in non-mite-infested mice [15]. NC/Nga mice with itchy skin caused by mite-infestation showed increased cutaneous IL-31 mRNA expression [25, 26]. Based on these previous findings, in the present study we examined the effects of pruritogens and algogens on LLS in mice that had been systemically pretreated with IL-31. As a result, in mice with itchy skin caused by intravenous pretreatment with IL-31, not only pruritogens but also algogens significantly increased LLS compared to that in vehicle-pretreated mice. These previous and present findings suggest that IL-31 may cause alloknesis or hyperknesis, i.e., it changes non-selective irritant stimulation into itch-stimulation in mouse skin. Therefore, it is possible that pain and itch are transmitted on the same nerve fibers, and a sensation is perceived as pain or itch depending on the operation of IL-31. Furthermore, the present study also demonstrated that PGD₂ decreased LLS by the cutaneous injection of pruritogens or algogens in itchy skin caused by IL-31. These results indicate that PGD₂ improves IL-31-induced alloknesis or hyperknesis, i.e., it changes non-selective irritant stimulation into

Figure 6. Schematic diagram of the putative roles of IL-31 and PGD₂ in the regulation of the sensation of cutaneous itch and pain in mice. IL-31 can change non-selective-stimulation into itch-stimulation. In contrast, PGD₂ can change non-selective-stimulation into pain-stimulation transmitted by the primary nerves of C-fibers and by second-order nerves and spinothalamic tract neurons in the spinal cord. This suggests that IL-31 and PGD₂ regulate the perception of sense (pain or itch) through their mutual functional antagonism.
pain-stimulation in mouse skin. If we consider all of these data together, the sensation of pain and itch may be regulated by PGD$_2$ (allodynia-inducer) and/or IL-31 (alloknesis-inducer), through their functional antagonism (Fig. 6). For example, when a mite irritates the skin, IL-31 is expressed in that region of the skin, and an itch sensation is produced in response to various kinds of cutaneous stimulation. On the other hand, PGD$_2$ is produced in response to inflammation such as that in response to a burn, and this may be involved in the onset of pain in response to various kinds of cutaneous stimulation.

Conflicting interests

The authors have declared that no conflict of interests exist.

Abbreviations


Author contributions

Iwao Arai designed the study, conducted the study, collected the data and prepared the manuscript, Minoru Tsuji collected the data and helped in the preparation of the manuscript, Kazuya Miyagawa collected and analysed the data, Hiroshi Takeda conducted the study and helped in the preparation of the manuscript, Nobutake Akiyama contributed essential reagents, Saburo Saito conducted the study and helped in the preparation of the manuscript. All authors read and approved the final manuscript.

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