Induction of Prostaglandin D_2 through the p38 MAPK Pathway Is Responsible for the Antipruritic Activity of Sertaconazole Nitrate

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Prostaglandin D_2 (PGD_2) is known to have antipruritic activity by suppressing histamine release. However, agents that can topically induce PGD_2 for itch relief are not well established. The antimycotic sertaconazole nitrate (STZ) has been shown to exhibit anti-itch properties; however, the mechanism for this activity has not been elucidated. STZ mitigated degranulation of RBL-2H3 (rat basophilic leukemia) mast cells induced by compound 48/80, a pruritogenic agent known to promote the release of histamine, and augmented PGD_2 production in mast cells and macrophages. Addition of exogenous PGD_2 abrogated compound 48/80-induced degranulation by acting through the prostanoid D receptor 1 (DP1). STZ induced p38 mitogen-activated protein kinase (MAPK) phosphorylation in mast cells and a pharmacological inhibitor of p38 MAPK, SB203580, resulted in the attenuation of PGD_2 levels. Finally, in a murine model of pruritus, the scratching behavior induced by compound 48/80 was mitigated by topical application of STZ. This effect was reversed by the addition of the cyclooxygenase inhibitor, ibuprofen, or a DP1 receptor antagonist (MK0524). Collectively, these results suggest that STZ mediates its anti-itch effects by boosting the antipruritic agent, PGD_2, by the activation of the p38-MAPK pathway. This is the first report to demonstrate a promising approach to topically induce PGD_2 for improving pruritus.

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INTRODUCTION

Pruritus (itching) has been characterized as an unpleasant sensation that elicits the desire to scratch (Rothman, 1941) and can range in severity from acute to intractable. Pruritus can lead to a vicious itch–scratch cycle resulting in disrupted skin integrity and subsequent decreased barrier resistance to infections (Yosipovitch, 2004; Yosipovitch and Papoiu, 2008). Itching has also been characterized as a major symptom in several forms of dermatitis, including atopic dermatitis (AD) (Williams *et al.*, 1994). The act of scratching leads to the release of inflammatory mediators that potentially induce or aggravate the skin condition resulting in the itch–scratch cycle (Ständer *et al.*, 2006, 2008). Dermal mast cells act very closely with nerve fibers and contain substances, such as histamine, which act as both direct and indirect mediators of pruritus (Ständer *et al.*, 2008).

Several lines of evidence suggest that Prostaglandin D_2 (PGD_2), which is produced primarily by skin mast cells (Lewis *et al.*, 1982; Peters *et al.*, 1984) and macrophages (Cao *et al.*, 2008) functions as a natural antipruritic agent (Arai *et al.*, 2004; Honma *et al.*, 2005) by suppressing histamine release from mast cells (Chan *et al.*, 2000; Hashimoto *et al.*, 2005). PGD_2 is a metabolite of arachidonic acid released from membrane phospholipids membrane by phospholipase A_2, and produced by the action of cyclooxygenase (COX) and prostaglandin D synthase (PGDS) enzymes (Larsen and Henson, 1983). In the biosynthesis pathway, there are two PGD_2 synthases that isomerize PGH_2 to PGD_2: lipocalin-type PGDS and hematopoietic PGDS (Kanaoka and Urade, 2003). Although the lipocalin-type PGDS is found in the central nervous system and male genital organs, the hematopoietic PGDS is expressed and localized in immune and inflammatory cells, such as antigen-presenting cells, mast cells, and megakaryocytes (Urade and Hayaishi, 2000; Kanaoka and Urade, 2003). Two receptors for PGD_2 have been characterized: prostanoid D receptor (DP)1 and DP2. Both these Prostanoid D receptors belong to the G protein-coupled receptor family. DP1 is the most studied PGD_2 receptor and its activation leads to G_s-mediated elevation in cAMP. The DP2 or chemoattractant receptor-homologous molecule expressed on T helper2 cells receptor is homologous to the

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Abbreviations: AD, atopic dermatitis; COX, cyclooxygenase; DP1, prostanoid D receptor 1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PGD_2, Prostaglandin D_2; PGE_2, Prostaglandin E_2; RBL-2H3, rat basophilic leukemia; STZ, sertaconazole nitrate

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chemoattractant receptor that is expressed on T-helper type 2 cells. The two receptors vary in their cellular expression: the DP1 receptor is the subtype found in mast cells, whereas the DP2 receptor is expressed in T-helper type 2 cells, basophils, etc. (Park and Christman, 2006).

PGD_2 is involved in the regulation of several physiological and pathophysiological processes, such as sleep induction, vasodilatation, recruitment of inflammatory cells, allergic asthma, platelet aggregation, etc. (Kanaoka and Urade, 2003). There is also emerging evidence pointing toward the potential role of this prostanoid in skin and wound repair (Kämpfer *et al.*, 2003, 2005). Recent studies have provided new insights into the role of PGD_2 in the suppression of scratching behavior in mice (Arai *et al.*, 2004; Honma *et al.*, 2005; Futaki *et al.*, 2007). Using NC/Nga mice as a model for AD, it was observed that topical application of PGD_2 resulted in a remarkable inhibition of itching in mice, even at low doses (Arai *et al.*, 2004). In the same strain of mice, cutaneous barrier disruption caused by mechanical scratching led to elevated levels of prostaglandins, with marked increases observed for PGD_2 and PGE_2 levels, suggesting a physiological role of these prostanoids in the repair of the skin damaged by scratching (Futaki *et al.*, 2007). Scratching caused by ovalbumin injected into ovalbumin-sensitized mice was also suppressed by PGD_2, suggesting that this prostanoid decreased IgE-mediated scratching by inhibiting the corresponding histamine release from activated mast cells (Hashimoto *et al.*, 2005).

The broad-spectrum antifungal agent sertaconazole nitrate (STZ) (Pfaller and Sutton, 2006) has been shown to exhibit anti-inflammatory and anti-itch activities (Agut *et al.*, 1996; Liebel *et al.*, 2006). Using a murine model of Substance P-induced itch, we previously demonstrated that STZ inhibits the scratching response in mice The anti-inflammatory activity of STZ was recently shown to be mediated through the p38–COX-2–PGE_2 mechanism, in which STZ induced PGE_2 production through the p38-mitogen-activated protein kinase (MAPK) pathway (Sur *et al.*, 2008a). However, signaling mechanism(s) that govern the antipruritic activity of STZ are not fully understood. In this study, we explore the pathway by which STZ mediates its anti-itch activity. This is the first study to show that a topical antimycotic agent, STZ mediates its antipruritic activity by enhancing PGD_2 levels in mast cells and macrophages by induction of the p38-MAPK pathway. Elevated PGD_2 levels attenuate mast cell degranulation, leading to anti-itch response. The antipruritic activity of STZ may contribute significantly to its application in fungal infections in which itch is a predominant symptom. The use of this particular antimycotic agent could provide additional benefits to patients with fungal infections toward relieving multiple conditions, including inflammation and itch.

RESULTS

STZ reverses compound 48/80-induced degranulation of mast cells and stimulates PGD_2 levels through the p38-MAPK pathway

Degranulation of mast cells leading to the release of histamine, the enzymes tryptase and β-hexosaminidase, and other mediators, has been confirmed as one of the most important factors inducing pruritus and an important target for curing chronic itch (Yamashita *et al.*, 2007). Thus, compounds that suppress degranulation of mast cells can potentially be of high value in anti-itch therapy. Therefore, we first looked at compound 48/80-induced degranulation in rat basophilic leukemia (RBL-2H3) mast cells to assess antipruritic activity. The secretagogue, compound 48/80 was added alone or in conjunction with the indicated concentrations of STZ, and a time course study was performed to look at time points ranging from 15 minutes to 6 hours (data for 1–6 hours time points not shown). Treatment with compound 48/80 for 30 minutes led to the release of ~21% of the granule marker β-hexosaminidase from mast cells. STZ was found to significantly reverse the degranulatory effect of compound 48/80 in RBL-2H3 cells as early as 15 (Figure 1a) and 30 minutes (Figure 1b) after treatment with the secretagogue by approximately 30–40% overall. The antimycotic did not exert a significant effect on mast cell degranulation when evaluated in the absence of compound 48/80 induction (data not shown). To further dissect the mechanism, PGD_2 levels were analyzed from the same supernatants that were initially tested for β-hexosaminidase release by using competitive PGD_2 ELISA. STZ enhanced the levels of PGD_2 (1,400–1,800 $pg\,ml^{-1}$) produced by compound 48/80 starting at the 30-minute time point (Figure 1c), and this increase was more pronounced at higher time points. In the absence of compound 48/80 induction, STZ alone led to ~40% increase in PGD_2 levels (data not shown). A pharmacological inhibitor of p38 MAPK, SB203580, was also tested to evaluate the pathway by which STZ mediates its anti-itch effects. Pretreatment with SB203580 suppressed the above-mentioned effects of STZ on mast cell degranulation and PGD_2 production (Figures 1a–c). The inhibitor alone led to 11.1 ± 2.3% increase in mast cell degranulation and no change in PGD_2 release when compared with the corresponding vehicle control (in the absence of any inducing agent) after 30 minutes of treatment (data not shown). As mast cell degranulation is a good measure for pruritic activity, suppression of β-hexosaminidase release in combination with the induction of PGD_2 by STZ provides strong evidence that this antifungal agent mediates its antipruritic activity by augmenting PGD_2 levels.

STZ enhances PGD_2 levels in LPS-stimulated macrophages and compound 48/80-stimulated mast cells after 24 hours of treatment through the p38-MAPK pathway

PGD_2 is primarily expressed in skin mast cells and macrophages (Lewis *et al.*, 1982; Peters *et al.*, 1984; Cao *et al.*, 2008); therefore, we further evaluated the levels of PGD_2 in both these cell types after stimulation with compound 48/80 or lipopolysaccharide (LPS) in conjunction with STZ. To verify whether the previously observed increase in PGD_2 production was a transient or long-term effect, murine macrophages RAW-264.7 and RBL-2H3 mast cells were treated with LPS (Figure 2a) or compound 48/80 (Figure 2b) alone or in combination with the indicated concentrations of STZ for 24 hours. GD_2 levels were measured 24 hours after

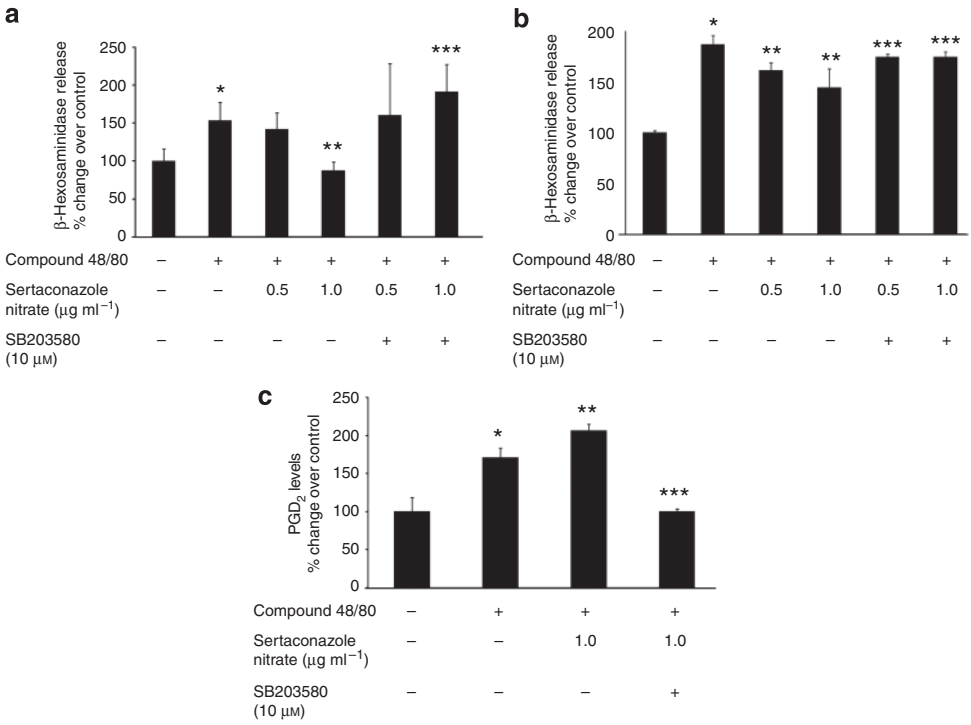

Figure 1. Sertaconazole nitrate reverses compound 48/80-induced degranulation of mast cells and stimulates PGD_2 levels through the p38-MAPK pathway. RBL-2H3 mast cells were pretreated with the p38 MAPK inhibitor, SB203580 (10 μM), for (**a**, **c**) 30 or (**b**) 45 minutes, followed by another pretreatment with the indicated concentrations of STZ for 30 minutes. After the pretreatments, compound 48/80 (30 μg ml^{-1}) was added to the cells for (**a**) 15 or (**b**) 30 minutes. The supernatants and pellets were assayed for the release of the granule marker β-hexosaminidase as an indicator of mast cell degranulation. (**c**) Supernatants collected after 30 minutes of compound 48/80 treatment were also used to assess PGD_2 levels by ELISA. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance, $*P<0.05$ vs. uninduced control; $**P<0.05$ vs. 48/80; $***P<0.05$ vs. 48/80 + corresponding dose of STZ. MAPK, mitogen-activated protein kinase; PGD_2, Prostaglandin D_2; RBL-2H3, rat basophilic leukemia; STZ, sertaconazole nitrate.

treatment by ELISA. Data confirmed that STZ enhanced LPS- or compound 48/80-induced PGD_2 levels in macrophages and mast cells, respectively, at 24 hours. This effect was reversed by the addition of the p38 inhibitor, SB203580, emphasizing that STZ exerts its effects through the p38-MAPK pathway (Figures 2a and b). The inhibitor alone led to a 4.4 ± 3.9% decline in mast cell degranulation when compared with the corresponding vehicle control (in the absence of any inducing agent) after 24 hours of treatment (data not shown). These observations are significant in confirming the role of STZ in enhancing PGD_2 levels in both the primary cell types that express PGD_2 synthase.

Exogenous PGD_2 reverses the degranulatory effects of compound 48/80 in mast cells. A selective DP1 receptor antagonist, MK0524 blocks the effects of PGD_2 and STZ on mast cell degranulation

The degranulation and PGD_2 ELISA results discussed previously suggest that STZ suppresses degranulation of mast cells by enhancing PGD_2 levels. To confirm the role of PGD_2 as an antipruritic agent, we investigated whether addition of exogenous PGD_2 would have a direct effect on mitigating compound 48/80-induced degranulation of RBL-2H3 mast cells. Cultured cells were pretreated with mutiple doses of exogenous PGD_2 for 30 minutes, followed by treatment with compound 48/80 for 30 minutes. At that point, supernatants (culture media) and pellets were separated and analyzed for the degranulation marker β-hexosaminidase. Exogenously added PGD_2 directly suppressed degranulation in mast cells caused by compound 48/80 in a dose-dependent manner by >50%, thus underlining the role of this prostanoid as a natural antipruritic agent (Figure 3a). Mast cells have been shown to express the DP1 receptor for PGD_2 (Park and Christman, 2006). To demonstrate that PGD_2 acts through this receptor in curtailing mast cell degranulation, we used a selective DP1 receptor antagonist, MK0524, to block those effects. Cultured RBL-2H3 cells were pretreated with two doses (20 and 50 μM) of MK0524 for 1 hour, followed by treatment with exogenous PGD_2 and/or compound 48/80 for 30 minutes each. Samples were analyzed for the degranulation marker β-hexosaminidase. Although the lower dose of the receptor antagonist (20 μM) did not have a significant effect on mast cell degranulation caused by compound 48/80, the higher dose (50 μM) significantly enhanced degranulation. Pretreatment of cells with MK0524 (50 μM), followed by addition of exogenous PGD_2 resulted in abrogation of the effects of PGD_2 on decreasing degranulation (Figure 3b). The DP1 receptor antagonist was also successful in reversing the activity of STZ on decreasing compound 48/80-induced mast cell degranulation (Figure 3c), thus confirming that STZ exerts its antipruritic effects through PGD_2. In control experiments, treatment of mast cells with MK0524 in the

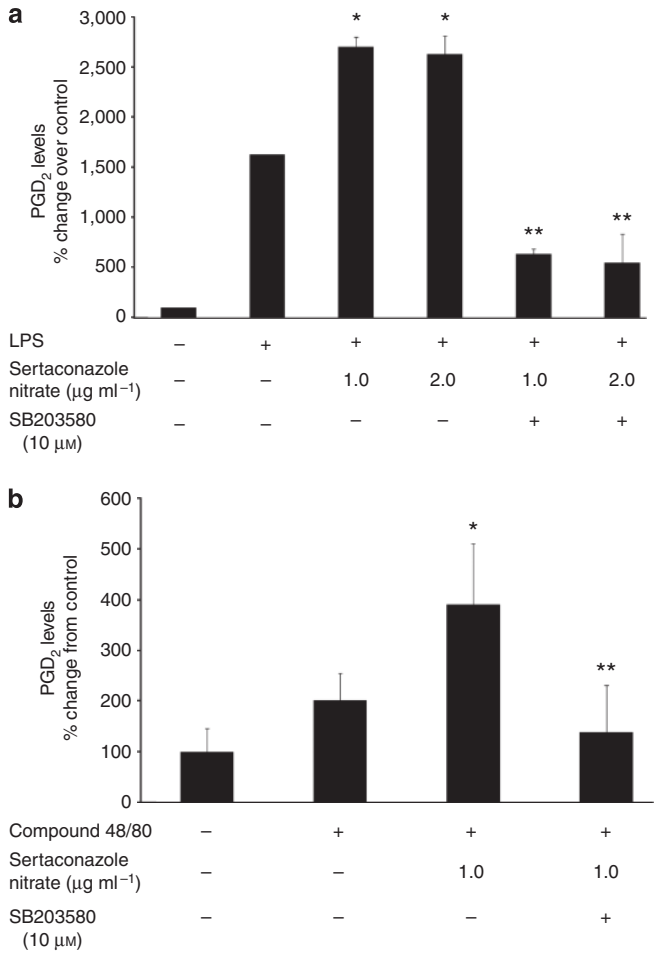

Figure 2. Sertaconazole nitrate enhances PGD_2 levels after 24-hour treatments in LPS-stimulated macrophages and compound 48/80-stimulated mast cells through the p38-MAPK pathway. (**a**) Murine macrophages RAW-264.7 and (**b**) RBL-2H3 mast cells were treated with (panel **a**) LPS (100 ng ml^{-1}) or (panel **b**) compound 48/80 (30 μg ml^{-1}) alone or in combination with the indicated concentrations of sertaconazole nitrate (STZ) after 30-minute pretreatment with the p38 MAPK inhibitor, SB203580 (10 μM), and PGD_2 levels were measured 24 hours later by ELISA. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. *$P<0.01$ vs. (panel **a**) LPS or (panel **b**) compound 48/80; **$P<0.01$ vs. (panel **a**) LPS + corresponding dose of STZ or (panel **b**) compound 48/80 + STZ. LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PGD_2, Prostaglandin D_2; RBL-2H3, rat basophilic leukemia.

absence of any inducing agent, for 1 hour, led to 7.3 ± 5.3% decline in β-hexosaminidase release when compared with the corresponding vehicle. Similar results were obtained when the cells were treated with exogenous PGD_2 without compound 48/80 induction; a decline of 7.9 ± 5.1% in mast cells degranulation was observed by using 100 μM dose of PGD_2 (data not shown). Overall, these results provide further evidence that PGD_2 acts through the DP1 receptor in its scope as a physiological antipruritic agent, and are consistent with previously published reports on the anti-itch activity of PGD_2 in murine models of AD and mast cells (Chan *et al.*, 2000; Arai *et al.*, 2004; Hashimoto *et al.*, 2005; Honma *et al.*, 2005; Futaki *et al.*, 2007).

STZ leads to the activation of p38 MAPK in mast cells

We have previously shown that STZ induces the p38-MAPK pathway in primary human keratinocytes, leading to an anti-inflammatory response by the production of PGE_2 (Sur *et al.*, 2008a). Results discussed so far confirm the role of p38 MAPK in lowering degranulation and augmenting PGD_2 levels by STZ (Figures 1 and 2). To corroborate these results, we sought to determine whether this antifungal agent leads to the direct activation of the p38-MAPK pathway in mast cells. RBL-2H3 cells were treated with STZ (1 μg ml^{-1}) for a time-course study ranging from 15 to 60 minutes. Western blotting with the phospho-p38 antibody confirmed that STZ led to the activation of p38 MAPK in mast cells, with more than two-fold increase in the phosphorylation of the p38 protein at the 15-minute time point (Figure 4). The same blot was then stripped and reprobed with total p38 MAPK and α/β-tubulin antibodies to show equal protein loading.

Topical treatment with STZ inhibits scratching in a murine model of pruritus through the PGD_2 pathway

To validate our *in vitro* results, we used ICR mice, which are a good model for evaluating itch mediators and mechanisms (Inagaki *et al.*, 2001). Control, shaved mice scratched an average of 8.0 ± 7.9 times, whereas those injected with the vehicle (saline) scratched 9.0 ± 9.6 times in a 30-minute time period. These mice were pretreated with a topical application of STZ for 30 minutes. Compound 48/80, prepared in sterile physiological saline, was injected into the interscapular part of the back of the mice, and the number of scratches was counted. STZ significantly inhibited the number of scratches elicited by compound 48/80 treatment in mice by 42.5 ± 7.2% as shown in Figure 5a. Pretreatment with EtoH vehicle alone for 30 minutes before compound 48/80 treatment had no significant effect on decreasing scratching, the average scratches from 80.7 ± 5.4 to 76.9 ± 4.3 times in a 30-minute time period. To further assess the mechanism of action, a nonselective COX inhibitor, ibuprofen, or a selective DP1 receptor antagonist, MK0524, was also topically applied for 30 minutes pretreatment, followed by sertaconazole or compound 48/80 treatments. The inhibitory effect of STZ on scratching was significantly reversed by the application of ibuprofen or the PGD_2 receptor antagonist, thus confirming that the anti-itch activity of STZ is mediated through PGD_2 (Figure 5b). It is also noteworthy that blocking the DP1 receptor by using MK0524 (in the absence of STZ treatment) augmented the scratching behavior in mice elicited by compound 48/80 (Figure 5b), thus confirming that PGD_2 acts as a physiological antipruritic agent.

DISCUSSION

We have previously shown that STZ, an imidazole-type antifungal agent elicits anti-itch response in a Substance P-induced murine model of pruritus (Liebel *et al.*, 2006). The anti-inflammatory properties of STZ were recently attributed to an increase in PGE_2 production through the p38-MAPK pathway (Sur *et al.*, 2008a). However, signaling mechanism(s) governing the anti-itch effects of this antimycotic are still poorly understood. In this study, we sought to elucidate

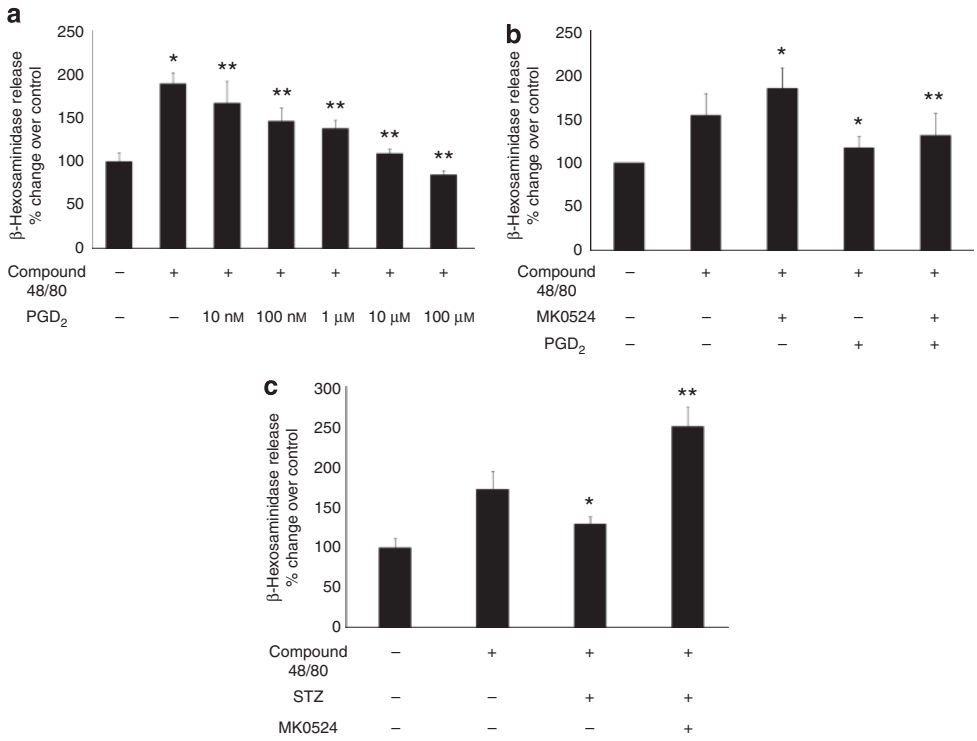

Figure 3. Exogenous PGD_2 reverses the degranulatory effects of compound 48/80 in mast cells in a dose-dependent manner. Selective DP1 receptor antagonist, MK0524, augments degranulation by blocking the effects of exogenous PGD_2 and sertaconazole nitrate. (**a**) Cultured RBL-2H3 mast cells were pretreated with the indicated doses of exogenous PGD_2 (10 nM–100 μM) for 1 hour, followed by treatment with compound 48/80 (30 μg ml^{-1}) for 30 minutes. Samples were analyzed for the degranulation marker β-hexosaminidase. Results show a dose-dependent effect of the prostanoid on abrogating mast cell degranulation. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. *$P<0.01$ vs. uninduced control; **$P<0.01$ vs. 48/80. (**b**) Mast cells were pretreated with two doses of the DP1 receptor antagonist MK0524 (50 μM) alone for 1 hour, and/or followed by cotreatment with exogenous PGD_2 (10 μM) for 30 minutes before the addition of compound 48/80 for 30 minutes as indicated. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. *$P<0.05$ vs. 48/80; **$P<0.05$ vs. 48/80 + MK0524. (**c**) RBL-2H3 cells were pretreated with MK0524 (50 μM) for 1 hour, followed by cotreatment with STZ (0.5 μg ml^{-1}) for 30 minutes before the addition of compound 48/80 for 30 minutes as indicated. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. *$P<0.01$ vs. 48/80; **$P<0.01$ vs. 48/80 + STZ. DP1, prostanoid D receptor 1; PGD_2, Prostaglandin D_2; RBL-2H3, rat basophilic leukemia; STZ, sertaconazole nitrate.

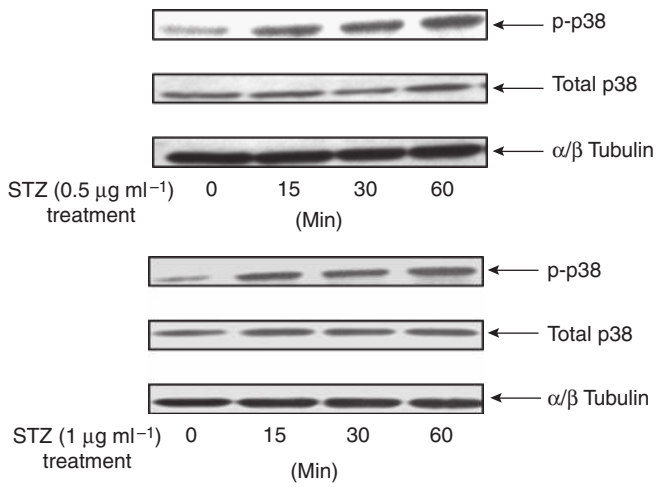

Figure 4. Sertaconazole nitrate leads to the activation of p38 MAPK in mast cells. RBL-2H3 mast cells were treated with STZ (0.5 or 1 μg ml^{-1}) for the indicated time periods, followed by protein analysis. Whole-cell extracts were western blotted and probed with respective antibodies as indicated. Results show that treatment with STZ resulted in the activation of p38 MAPK, without affecting the levels of total p38. MAPK, mitogen-activated protein kinase; RBL-2H3, rat basophilic leukemia; STZ, sertaconazole nitrate.

the mechanism of action by which STZ inhibits the itch response induced by the pruritogen, compound 48/80.

The 2H3 sublines of RBL-2H3 cells are analogs of mast cells that degranulate and have been used extensively to study the biochemical pathways for secretion in mast cells (Barsumian *et al.*, 1981). Upon activation, these cells release histamine and β-hexosaminidase, which are stored in the secretory granules and are used as markers for mast cell degranulation (Schwartz *et al.*, 1981; Yamada *et al.*, 2007). To mimic a pruritogenic response, we used compound 48/80, a mixture of polymers derived from *N*-methyl-*p*-methoxy-phenylethylamine, which belongs to a family of polybasic mast cell secretagogues (Chahdi *et al.*, 2000), and has been shown to induce histamine release from mast cells by multiple mechanisms (Rothschild, 1970; Koibuchi *et al.*, 1985).

Degranulation of mast cells is one of the most important factors in the induction of pruritus and an important target for chronic itch (Yamashita *et al.*, 2007). Therefore, we first elicited a degranulatory response in mast cells by the action of compound 48/80, and conducted a time-course study in which STZ was added to the cells in conjunction with this polybasic secretagogue. We observed that treatment with

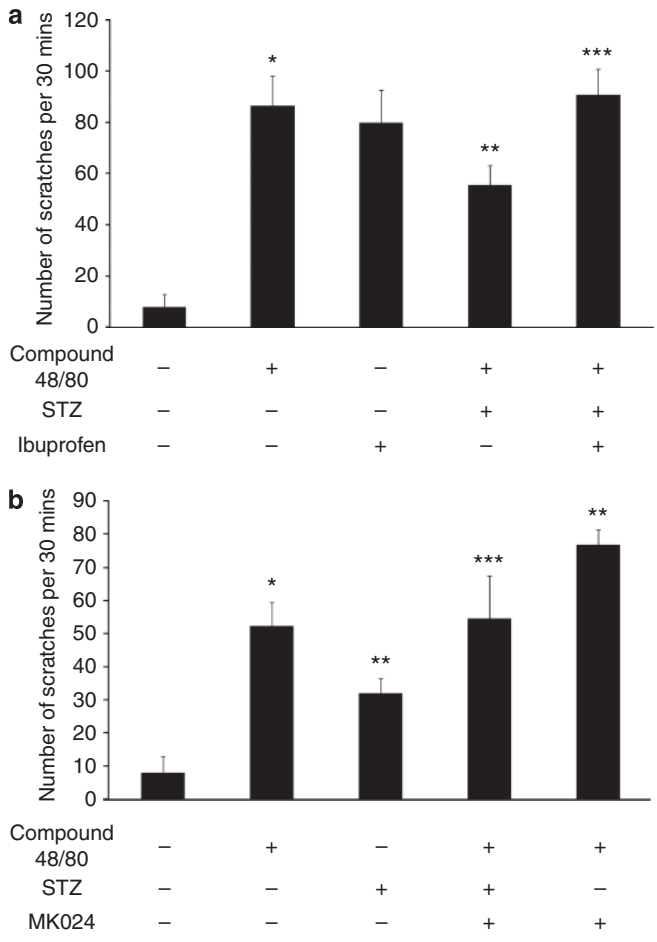

Figure 5. Topical treatment with sertaconazole nitrate inhibits scratching behavior in a murine model of pruritus through the PGD_2 pathway. ICR mice were pretreated for 30 minutes with topical application of 1% STZ. Compound 48/80 (50 μg/50 μl w/v) was injected into an area on the back of the mice, and the number of scratches was counted in a 30-minute period. (**a**) Compound 48/80 drastically enhanced scratching in mice ($n = 14$ mice), which was significantly inhibited by treatment with 1% STZ (($n = 10$ mice) (panel **a**). This reduction in itch was reversed by cotreatment of STZ with topical ibuprofen ((1% in ethanol) ($n = 8$ mice)). A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. $^*P < 0.05$ vs. no 48/80 treatment, $^{**}P < 0.05$ vs. 48/80, $^{***}P < 0.05$ vs. 48/80 + STZ. (**b**) To confirm the mechanism of action, 50 μl volume of a selective DP1 receptor antagonist, MK0524 (0.1% in ethanol), was topically applied for 30 minutes pretreatment ($n = 8$–11 mice), followed by STZ and/or compound 48/80 treatments (panel **b**). A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. $^*P < 0.05$ vs. no 48/80 treatment, $^{**}P < 0.05$ vs. 48/80, $^{***}P < 0.05$ vs. 48/80 + STZ. PGD_2, Prostaglandin D_2; STZ, sertaconazole nitrate.

compound 48/80 led to the degranulation of mast cells under the conditions we tested, whereas preincubation with STZ reversed those effects at 15 and 30 minutes exposure to the secretagogue (Figures 1a and b). Similar results were also recorded for longer time points evaluated (data not shown). Mitigation of mast cell degranulation by STZ is a good indicator of its antipruritic efficacy, falling along the lines of earlier studies reported by us (Liebel *et al.*, 2006).

There have been some previous reports in literature that RBL-2H3 mast cells do not respond to nonimmunological stimuli such as compound 48/80 without being cocultured with fibroblasts (Swieter *et al.*, 1993) or sensitized with the kinase inhibitor, quercetin (Senyshyn *et al.*, 1998; Chahdi *et al.*, 2000). We tested the response of RBL-2H3 cells to compound 48/80 in the absence and presence of prolonged exposure to quercetin and analyzed degranulation levels. In contrast, we recorded no significant difference in the levels of β-hexosaminidase release in RBL-2H3 cells pretreated with quercetin when compared with untreated cells under the conditions we tested (data not shown). It is possible that media conditions and high cell-passage number might have a role in the response of these cells to compound 48/80. We observed a trend toward lower degranulation in these cells at high passages. In addition, the end point being measured in this case (β-hexosaminidase release) is different from those looked at in some of the previous reports. As a result, studies mentioned in this paper were conducted in RBL-2H3 cells in the absence of any pretreatment with the kinase inhibitor, quercetin.

Several reports in literature suggest that PGD_2 might serve to attenuate pruritogenic (itch) responses in animal models of AD and mast cells (Chan *et al.*, 2000; Arai *et al.*, 2004; Hashimoto *et al.*, 2005; Honma *et al.*, 2005; Futaki *et al.*, 2007). As this prostanoid is primarily expressed in mast cells and macrophages (Lewis *et al.*, 1982; Peters *et al.*, 1984; Cao *et al.*, 2008), we decided to investigate whether STZ alters PGD_2 levels in RBL-2H3 mast cells and RAW-264.7 macrophages. Corresponding to the time points evaluated in the mast cell degranulation assay, PGD_2 levels were also measured using competitive ELISA. STZ boosted PGD_2 production in mast cells starting at 30 minutes treatment (Figure 1c). In addition to these short-term effects, we also observed that STZ boosted PGD_2 produced by mast cells in response to compound 48/80 after treatment for 24 hours (Figure 2a). Similar results were obtained in the macrophages, in which induction of PGD_2 production by LPS was further stimulated by STZ (Figure 2b) at the 24-hour time point. In addition, we also observed analogous results with STZ on IgE-mediated induction of PGD_2 in RBL-2H3 mast cells, confirming that these effects are not restricted only to 48/80-induced PGD_2 production (data not shown).

To further elucidate the endogenous antipruritic effects of PGD_2 and to confirm whether this postanoid has a direct influence on mast cell degranulation, we exogenously added PGD_2 to RBL-2H3 cells and monitored compound 48/80-induced degranulation. Pretreatment of mast cells with PGD_2 before compound 48/80 directly mitigated degranulation in a dose-dependent manner (Figure 3a). These effects were reversed in the presence of a selective DP1 receptor antagonist, MK0524 (Figure 3b). It is also notable that blocking the PGD_2 receptor by using MK0524 (at 50 μM dose) significantly increased mast cell degranulation caused by compound 48/80 (Figure 3b), and inverted the effects of STZ on decreasing degranulation (Figure 3c). Our results corroborate previously published reports (Chan *et al.*, 2000; Arai *et al.*, 2004; Hashimoto *et al.*, 2005; Honma *et al.*, 2005; Futaki *et al.*, 2007) on the anti-itch effects of PGD_2. It has also been previously reported that agents that induce and sustain

intracellular cAMP levels attenuate the release of mediators from mast cells (Peachell *et al.*, 1988; Weston and Peachell, 1998). PGD_2 and BW245C (DP receptor-specific agonist) have also been shown to enhance cAMP levels in CHO cells transfected with the DP1 receptor (Hirata *et al.*, 1994), and in rat peritoneal mast cells (Lau and Chan, 2001). Taken together, our results showing the inhibition of mast cell degranulation by PGD_2 through the DP1 receptor may potentially be attributed to the activation of adenyl cyclase by the G_s protein, leading to the increased production of cAMP.

The p38 MAPK is known to have a role in proinflammatory cytokine production in response to various external stimuli (Saklatvala, 2004; Borders *et al.*, 2008). We have also shown that activation of this kinase by STZ in keratinocytes can lead to nonclassical anti-inflammatory effects by the production of PGE_2 (Sur *et al.*, 2008a). To further dissect the mechanism for the antipruritic effects of STZ, we used a pharmacological inhibitor of p38 MAPK, SB203580. Pretreatment of RBL-2H3 mast cells with SB203580 reversed the effects of STZ on attenuation of mast cell degranulation (Figures 1a and b) and PGD_2 production in mast cells and macrophages, both (Figures 1a–c and 2a and b) highlighting the role of this signaling pathway in mediating the anti-itch effects of STZ. We also confirmed that STZ induces phosphorylation of the p38 MAPK protein in mast cells by western blotting (Figure 4). A time-course study was performed in which RBL-2H3 cells were treated with the antimycotic, and cell lysates were analyzed for phospho-p38 MAPK, followed by total p38 MAPK and α/β-tubulin to verify equal protein loading. STZ led to the activation of p38 MAPK within 15 minutes, and this induction remained unchanged until at least 60 minutes. These results corroborate our initial observations signifying that the p38-MAPK pathway is involved in the antipruritic effects of STZ by the production of PGD_2. Other antifungal agents, such as butoconazole, fluconazole, terconazole, tioconazole, or ketoconazole, failed to stimulate the p38-MAPK pathway (data not shown), suggesting that the above-described activity is independent of the azole structure, and is specific to STZ.

Owing to the fact that STZ resulted in abrogation of mast cell degranulation in the 15-minute time period, and escalated PGD_2 levels in 30 minutes, we hypothesized that under these conditions, the synthesis of PGD_2 might involve COX-1-mediated liberation of arachidonic acid. Using specific COX-1 and COX-2 inhibitors, it has been previously shown that abrogation of COX-1 is associated with increased scratching behavior in mice, which is improved by the topical application of PGD_2 (Sugimoto *et al.*, 2006a). In a separate study, mechanical scratching caused by a stainless-steel wire brush led to elevated PGD_2 levels in a 10-minute time period, signifying the involvement of the COX-1 isozyme, especially as COX-2 was shown to be induced after 3 hours (Sugimoto *et al.*, 2006b). In the same report, it was also shown that levels of PGD_2 and some other prostanoids were markedly lower in the scratched skin of COX-1-deficient mice. However, only PGD_2 levels were suppressed in the normal skin of COX-1-deficient mice, collectively suggesting that cutaneous PGD_2 could be primarily produced by the action of the COX-1 enzyme (Sugimoto *et al.*, 2006b).

Finally, using ICR mice as a model for evaluating itch mediators and mechanisms (Inagaki *et al.*, 2001), topical application of STZ in conjunction with compound 48/80, resulted in the abrogation of scratching behavior (Figure 5a). As COX enzymes have a crucial role in the biosynthesis of prostanoids, we used ibuprofen, a nonselective COX inhibitor, or the selective DP1 receptor antagonist, MK0524, to further delineate the signaling pathway. Topical application of ibuprofen and MK0524 for 30 minutes, followed by STZ or compound 48/80 treatments led to a reversal of the inhibition caused by STZ. It is also worth discussing that blocking the DP1 receptor by using MK0524 along with compound 48/80 augmented the scratching behavior in mice (Figure 5b) and this observation corroborates our *in vitro* mast cell degranulation results with the receptor antagonist. Activation of mast cells by compound 48/80 leads to the release of low levels of PGD_2 in addition to several itch mediators. PGD_2 acts as a natural anti-itch agent by suppressing mast cell degranulation, which is confirmed by our *in vitro* and *in vivo* data. The exact potential of compound 48/80 to elicit an itch response is evident by our *in vivo* results with the DP1 receptor antagonist. STZ enhances PGD_2 production and mitigates the pruritic effects of compound 48/80 through this mode of action. The *in vivo* results also suggest that topically applied STZ may affect mast cells, and possibly macrophages. Overall, our data reveal one of the plausible mechanisms through which this antimycotic agent might exert its anti-itch effects. There can also be other modes of action by which STZ affects pruritus. For instance, it has also been shown that Substance P evokes a scratching response in mast cell-deficient mice as well (Andoh *et al.*, 1998). Such an effect might be mediated by pathways different from those discussed in this study.

Fungal infections represent the second most frequently reported skin disease in the United States after acne (Weinstein and Berman, 2002), and the broad-spectrum antimycotic efficacy of STZ (Ertaczo, 2%) is well established (Weinstein and Berman, 2002; Pfaller and Sutton, 2006; Ribotsky, 2009). One of the most common dermatophyte infections tinea pedis (specific to feet) is characterized by irritation, burning, and itching related to severity of the disease (Weinstein and Berman, 2002). On the basis of our observations, topical induction of PGD_2 can potentially mitigate the itch response associated with several dermatological conditions. Collectively, our findings provide strong evidence that PGD_2 has a physiological role in the suppression of pruritus by acting through the DP1 receptor, and that the anti-itch effects of STZ are mediated by enhancing the release of this prostanoid through activation of the p38-MAPK pathway in mast cells and macrophages in the skin.

MATERIALS AND METHODS

Materials

STZ was obtained from Ferrer Pharmaceuticals (Barcelona, Spain). Compound 48/80, LPS, SB203580, and NAG (β-Nitrophenyl *N*-acetyl β-D-glucosaminide) were procured from Sigma (St Louis,

MO). Phospho-p38 and p38 MAPK and α/β-tubulin antibodies were purchased from Cell Signaling Technology (Danvers, MA). Lipofectamine 2000 transfection reagent was obtained from Invitrogen Corporation (Carlsbad, CA). PGD_2 and MK0524 (DP1 receptor antagonist) were procured from Cayman Chemical Company (Ann Arbor, MI).

Cell culture

RBL-2H3 mast cells and RAW-264.7 macrophages were purchased from ATCC (Manassas, VA) and cultured under the conditions specified by the company. In brief, RBL-2H3 cells were maintained in Eagle's MEM (ATCC) containing 15% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin at 37 °C in 5% CO_2. For all degranulation studies, mast cells were grown in the above-described media and treated in serum-free RPMI media (Invitrogen Corporation) without phenol-red and antibiotics. RAW-264.7 cells were maintained in DMEM (Invitrogen Corporation) containing 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C in 5% CO_2. Treatments were carried out in DMEM with 2% fetal bovine serum and 1% penicillin and streptomycin.

Mast cell degranulation assay

RBL-2H3 mast cells grown in 48-well plates were treated with compound 48/80 (final concentration 30 μg ml^{-1}) alone or after pretreatment with the indicated doses of STZ in the absence or presence of the p38 MAPK inhibitor, SB203580 (10 μM) for 15 or 30 minutes in phenol red-free and serum-free RPMI media. After incubation, the tissue culture (media) supernatants were collected and cellular pellets were lysed using 0.1% Triton X-100 in PIPES buffer. A small volume of the supernatants and pellets were collected and mixed with an equal volume of NAG dissolved in 0.1 M citrate buffer for 60–90 minutes. The reaction was stopped by the addition of 0.1 M sodium carbonate-bicarbonate buffer, and the plate was read at 405 nm. Degranulation was assessed by measuring levels of the granule marker: % β-hexosaminidase release was calculated as the optical density of supernatant at 405 nm × 100/(optical density of supernatant + optical density of pellet).

Measurement of PGD_2 release

Detection of PGD_2 in the supernatants of macrophages and mast cells was performed using a PGD_2 competitive ELISA kit (Cayman Chemical Company), according to manufacturer's protocol.

Western blotting

RBL-2H3 cells grown in 6-well plates were treated with STZ at a final concentration of 1 μg ml^{-1} for the indicated time periods. Cells were then washed with cold phosphate-buffered saline and lysed with RIPA lysis buffer. Lysates were centrifuged and total protein was estimated in the supernatants using a bicinchoninic acid protein assay kit (Pierce–Thermo Fisher Scientific, Rockford, IL). Protein (25 μg) was loaded on SDS-PAGE, followed by immunoblotting with specific antibodies (incubation with primary antibodies diluted 1:1,000 overnight at 4 °C) and detection using the ECL chemiluminescence detection system (Amersham Life Sciences–GE Healthcare, Piscataway, NJ).

In vivo scratching model

All procedures used in these experiments were approved by the Institutional Animal Care and Use Committee at Johnson and Johnson.

An itch-associated response was induced by intradermal injection of compound 48/80 in ICR mice (Sur *et al.*, 2008b) Mice were individually housed in a plastic cage for at least 1 hour before the experiment for acclimation, then pretreated for 30 minutes with topical application of vehicle control, or 10 mg ml^{-1} (1%) STZ prepared in 100% ethanol (50 μl final volume), to an area of the back that had been shaved 1 day before the experiment. Each group consisted of 8–14 mice. Compound 48/80 was prepared in sterile physiological saline, then 50 μl of a 1 μg μl^{-1} solution was injected into the interscapular part of the back, the number of scratches elicited during the 30-minute period after injection was determined as described previously (Liebel *et al.*, 2006), and the number of scratches counted by visual observation conducted independently by two individuals. Ibuprofen (1% solution in ethanol) or the PGD_2 receptor antagonist, MK0524 (0.1% in ethanol) were also used for 30 minutes pretreatment (50 μl volume topically applied), followed by STZ and/or compound 48/80 treatments.

Statistical analysis

Data are presented as mean ± SD. A one-way analysis of variance with Newman–Keuls *post hoc* test was used to determine significance. A value of $P<0.05$ was considered significant.

CONFLICT OF INTEREST

All authors are employees of Johnson & Johnson. Parts of this work have been presented in a poster format at the Society for Investigative Dermatology Annual Meeting, Montreal, 2009.

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