

# Anti-Inflammatory Activity of Sertaconazole Nitrate Is Mediated *via* Activation of a p38–COX-2–PGE<sub>2</sub> Pathway

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Sertaconazole nitrate is an antifungal agent that exhibits anti-inflammatory activity; however, the mechanism for this action was unknown. We investigated the cellular mechanisms by which sertaconazole exerts its anti-inflammatory activity in keratinocytes and human peripheral blood mononuclear cells (PBMCs). Paradoxically, sertaconazole was found to activate the proinflammatory p38 mitogen-activated protein kinase. Treatment with sertaconazole also resulted in the induction of cyclooxygenase-2 (COX-2) and the subsequent release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Knocking down p38 in keratinocytes using small interfering RNA resulted in an inhibition of sertaconazole-induced PGE<sub>2</sub> release confirming that activation of p38 was required for PGE<sub>2</sub> production. Additionally, in stimulated keratinocytes and human PBMCs, sertaconazole was found to suppress the release of cytokines. Treatment with anti-PGE<sub>2</sub> antiserum or the COX-2 inhibitor NS398 reversed the inhibitory effects of sertaconazole on the release of proinflammatory cytokines, linking endogenous PGE<sub>2</sub> with the anti-inflammatory effects. Finally, in an *in vivo* mouse model of tetradecanoyl phorbol acetate (TPA)-induced dermatitis, the sertaconazole-mediated inhibition of TPA-induced ear edema was reversed by NS398. Biochemical analysis of tissue biopsies revealed increase in PGE<sub>2</sub> levels in sertaconazole-treated mice. Thus, activation of the p38–COX-2–PGE<sub>2</sub> pathway by agents such as sertaconazole provides anti-inflammatory therapeutic benefits.

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## INTRODUCTION

The p38 mitogen-activated protein (MAP) kinase plays a central role in numerous proinflammatory responses mainly via the post-transcriptional control of inflammatory gene expression (Saklatvala, 2004; Schieven, 2005). There are four isoforms of p38 kinase:  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$  (Saklatvala, 2004) and cells can express multiple isoforms. Keratinocytes, for example, are reported to express p38  $\alpha$ ,  $-\beta$ , and  $-\delta$  isoforms (Efimova *et al.*, 2003). Once activated by dual phosphorylation on Thr180 and Tyr182 by upstream MAP kinase kinases (Pearson *et al.*, 2001), p38 positively regulates expression of many genes involved in inflammation, one of which is cyclooxygenase-2 (COX-2). COX-2 mRNA has an AU-rich element in the proximal 3'-untranslated region that makes the

COX-2 message unstable, and this effect is counteracted by MAPKAPK-2, a downstream substrate of p38 MAP kinase (Lasa *et al.*, 2000; Sully *et al.*, 2004). COX-2 regulates the conversion of arachidonic acid into intermediate cyclic endoperoxides that on further enzymatic action generates prostaglandins (Robertson, 1998).

Prostaglandins are an important group of inflammatory mediators that are synthesized and released during tissue trauma and inflammation (Vane, 1971; Treede *et al.*, 1992). Although these eicosanoids are rapidly catabolized (Campbell and Halushka, 1996), during chronic injury, physiological levels of prostanoids in tissues can be maintained for extended periods of time (Vane *et al.*, 1994; Beiche *et al.*, 1996; Hay *et al.*, 1997). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is generated in substantial amounts in local sites of inflammation and plays distinct roles in inflammation that are tissue- and cell-type-specific (Serhan and Levy, 2003). Although PGE<sub>2</sub> induces effects that mediate several of the cardinal features of inflammation such as edema (Moncada *et al.*, 1973), it has been reported to resolve inflammation in a mouse model of mast cell-dependent allergic inflammation by suppressing mediator release (Raud *et al.*, 1988). Thus, PGE<sub>2</sub> may produce both pro- and anti-inflammatory effects.

We previously reported that of a panel of eight antifungal agents, sertaconazole nitrate was the most potent antifungal studied in reducing the release of cytokines from activated

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Abbreviations: COX-2, cyclooxygenase-2; IL, interleukin; PBMC, peripheral blood mononuclear cells; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PHA, phytohemagglutinin; P. acnes, Propionibacterium acnes; TPA, tetradecanoyl phorbol acetate; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

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peripheral blood mononuclear cells (PBMCs) and was effective against contact hypersensitivity and irritant contact dermatitis, whereas none of the other antifungal compounds evaluated significantly reduced inflammation in all dermal irritation models (Agut *et al.*, 1996; Liebel *et al.*, 2006). In this study, we sought to determine the mechanism by which the antifungal agent, sertaconazole nitrate, exerts its anti-inflammatory activity. In an unexpected finding, we demonstrate that sertaconazole activates the p38 MAP kinase pathway resulting in the induction of COX-2 and the subsequent release of PGE<sub>2</sub> in keratinocytes and PBMCs. Using pharmacological inhibitors and small interfering RNA (siRNA) to p38, the sertaconazole-induced release of PGE<sub>2</sub> is abolished. Furthermore, using an anti-PGE<sub>2</sub> antiserum or the COX-2 inhibitor, NS398, the release of PGE<sub>2</sub> was found to be the primary factor in the anti-inflammatory activity of sertaconazole-inhibiting cytokine release from stimulated keratinocytes and PBMCs. Finally, in a tetradecanoyl phorbol acetate (TPA)-induced ear edema model, we confirmed that topical application of sertaconazole enhanced PGE<sub>2</sub> release and inhibition of PGE<sub>2</sub> formation was found to reverse the anti-inflammatory effect of sertaconazole. These results elucidate a paradoxical anti-inflammatory pathway whereby sertaconazole exerts its anti-inflammatory effects via the p38-COX-2-PGE<sub>2</sub> pathway.

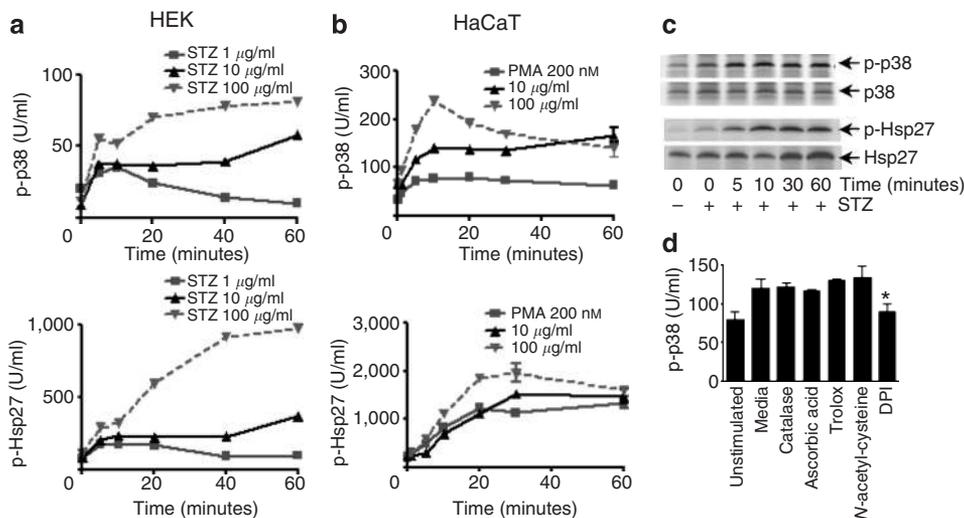
## RESULTS

### Activation of p38 MAP kinase by sertaconazole nitrate

We previously reported that sertaconazole exhibited anti-inflammatory activity *in vitro* by inhibiting the release of proinflammatory cytokines from human PBMCs and also exhibited efficacious anti-inflammatory activity *in vivo*

against a broad spectrum of dermal inflammation models (Liebel *et al.*, 2006). Because most of these inflammatory responses are mediated by different proinflammatory cytokines and the p38 MAP kinase plays a central role in the post-transcriptional control of numerous proinflammatory cytokine gene expression (Schieven, 2005), we sought to determine whether sertaconazole inhibited activation of p38 MAP kinase. Initial experiments indicated that sertaconazole did not inhibit p38 but paradoxically induced p38 MAP kinase activation. Co-treatment with sertaconazole and either tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or phorbol 12-myristate 13-acetate in primary human keratinocytes resulted in a greater activation of p38 MAP kinase than stimulation with TNF- $\alpha$  or phorbol 12-myristate 13-acetate alone (data not shown). Sertaconazole was also found to induce activation of p38 MAP kinase in the absence of co-stimulation. In both the primary keratinocytes (Figure 1a) and HaCaT keratinocytes (Figure 1b), sertaconazole treatment resulted in the activation of p38 MAP kinase and its downstream substrate, the small heat-shock protein 27 (Hsp27), in a dose- and time-dependent manner. HaCaT cells were treated with phorbol 12-myristate 13-acetate for different time intervals as a positive control. These results were verified by western blotting of whole-cell extracts. Consistent with the ELISA results, it was observed that in both normal keratinocytes (data not shown) and HaCaT cells, 1  $\mu$ g/ml sertaconazole activated p38 MAP kinase and Hsp27 in a time-dependent manner (Figure 1c). The phospho-blot was reblotted with p38 and Hsp27 total protein antibodies to account for equal protein loading.

One of the mechanisms by which p38 MAP kinase is activated is by stimulation of NAD(P)H oxidase and genera-



**Figure 1. Sertaconazole nitrate activates p38 MAP kinase and Hsp27 in keratinocytes.** (a) Normal human epidermal keratinocytes (HEK) were serum-starved for 24 hours followed by treatment with the indicated concentrations of sertaconazole nitrate for 5, 10, 20, 40, and 60 minutes and (b) HaCaT keratinocytes were serum-starved for 24 hours and treated with the indicated concentrations of sertaconazole nitrate or phorbol 12-myristate 13-acetate as control for 1, 5, 10, 20, 30, and 60 minutes. Phospho-p38 and phospho-Hsp27 levels were measured in the lysates by ELISA. Data are representative of one of three separate experiments. (c) HaCaT keratinocytes were serum-starved and treated with 1  $\mu$ g/ml sertaconazole (STZ) or vehicle for the indicated times. Whole-cell extracts (20  $\mu$ g of protein) were subjected to western blotting and probed with phospho-p38 and phospho-Hsp27 antibodies. The blots were reblotted with p38 and Hsp27 antibodies to verify equal protein loading. Data are representative of one of three separate experiments. (d) HaCaT keratinocytes were either left untreated or treated with 0.01% catalase, 50  $\mu$ M ascorbic acid, 50  $\mu$ M trolox, 10 mM N-acetyl-cysteine, or 5  $\mu$ M diphenylene iodonium for 30 minutes followed by 10  $\mu$ g/ml sertaconazole treatment for an additional 30 minutes. Unstimulated lane did not get any sertaconazole treatment. Phospho-p38 levels were measured in the lysates by ELISA. Data are representative of one of three separate experiments. \* $P < 0.05$  compared with keratinocytes treated with sertaconazole alone.

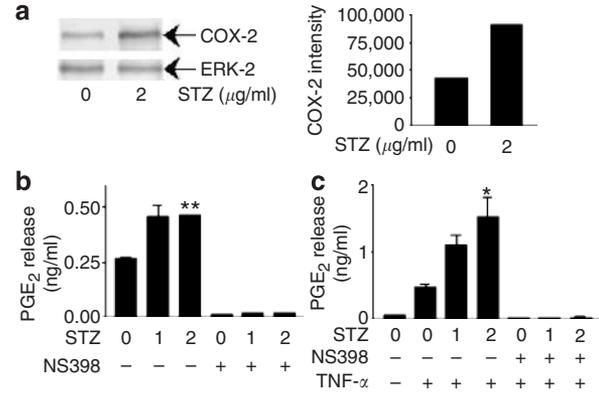
tion of reactive oxygen species such as superoxide anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Griendling and Ushio-Fukai, 2000; Blanc *et al.*, 2003; Mitra and Abraham, 2006). To assess how sertaconazole activated p38 MAP kinase, HaCaT keratinocytes were treated with pharmacological inhibitors for 30 minutes followed by treatment with sertaconazole. As observed in Figure 1d, only inhibitors of superoxide signaling affected the activation of p38. Diphenylene iodonium treatment that inhibits NAD(P)H oxidase prevented the sertaconazole-mediated p38 activation and a similar effect was also observed in the presence of superoxide dismutase (data not shown), which converts O<sub>2</sub><sup>•-</sup> into the more stable H<sub>2</sub>O<sub>2</sub> (Griendling and Ushio-Fukai, 2000). These results suggest that the mechanism of action for sertaconazole-stimulated p38 MAP kinase activation involves superoxide radical formation.

**Sertaconazole induces PGE<sub>2</sub> production via COX-2 in keratinocytes**

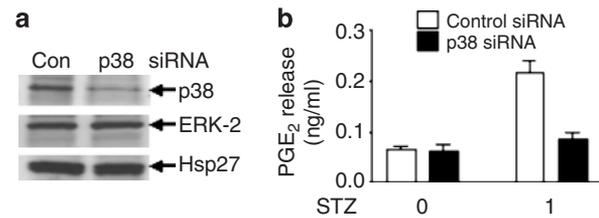
On activation, p38 MAP kinase induces the expression of its downstream substrate COX-2, which further catalyzes the production of prostanoids such as PGE<sub>2</sub>, which have been shown to play diverse roles in inflammation (Schieven, 2005). Because sertaconazole resulted in the activation of p38 MAP kinase, we asked whether sertaconazole induced COX-2 expression and affected PGE<sub>2</sub> production. To this end HaCaT keratinocytes were treated with the indicated concentrations of sertaconazole for 6 hours and western blot analysis of whole-cell extracts showed that sertaconazole treatment resulted in a 50% induction of expression of COX-2 protein at 2 μg/ml concentration (Figure 2a). The same blot was blotted with ERK-2 antibody to show equal protein loading. Next, we evaluated the effect of different concentrations of sertaconazole on PGE<sub>2</sub> production from HaCaT keratinocytes. Figure 2b shows that sertaconazole treatment results in a twofold increase in PGE<sub>2</sub> release. To assess the involvement of COX-2 in sertaconazole-mediated PGE<sub>2</sub> release, cells were preincubated with NS398, a specific inhibitor of COX-2 (Gierse *et al.*, 1995) before sertaconazole treatment. Sertaconazole-mediated PGE<sub>2</sub> production was inhibited in the presence of the COX-2 inhibitor (Figure 2b), suggesting the involvement of COX-2 in PGE<sub>2</sub> production. Next, we evaluated whether sertaconazole regulated the TNF-α-dependent PGE<sub>2</sub> production in these cells and interestingly it was observed that in a similar manner sertaconazole upregulated TNF-α-induced PGE<sub>2</sub> release (Figure 2c). This PGE<sub>2</sub> release was inhibited in the presence of the COX-2 inhibitor NS398 suggesting the involvement of COX-2 in TNF-α and sertaconazole-mediated PGE<sub>2</sub> production (Figure 2c).

**Sertaconazole-mediated induction of PGE<sub>2</sub> is dependent on p38 activation**

To confirm further the involvement of p38 MAP kinase in sertaconazole-mediated induction of PGE<sub>2</sub> production, we examined the sertaconazole-mediated PGE<sub>2</sub> release in the absence of p38 MAP kinase. To this end, we knocked down p38 MAP kinase in HaCaT keratinocytes by transfecting with



**Figure 2. Sertaconazole induces COX-2 protein expression and PGE<sub>2</sub> production in keratinocytes.** (a) HaCaT keratinocytes were treated with the indicated concentrations of sertaconazole (STZ) for 6 hours. Whole-cell extracts (20 μg of protein) were western-blotted and probed with COX-2 antibody and ERK-2 antibody to verify equal protein loading. The bar chart represents densitometric analysis of band intensities of COX-2 protein. Data represent one of three separate experiments. (b) HaCaT keratinocytes were treated with the indicated concentrations of sertaconazole in the presence or absence of 10 μM COX-2 inhibitor NS398 for 8 hours. PGE<sub>2</sub> levels in the supernatants were quantified using ELISA. Results represent mean ± SD from at least three different experiments. \*\*P < 0.01 compared with untreated control keratinocytes. (c) HaCaT keratinocytes were stimulated with 100 ng/ml TNF-α and treated with vehicle or the indicated concentrations of sertaconazole in the presence or absence of 10 μM COX-2 inhibitor NS398 for 24 hours. PGE<sub>2</sub> levels were quantified using ELISA. Results represent mean ± SD from at least three different experiments. \*P < 0.05 compared with keratinocytes treated with TNF-α plus vehicle.



**Figure 3. Sertaconazole-mediated PGE<sub>2</sub> production is dependent on p38 activation.** (a) HaCaT keratinocytes were transfected with control siRNA (100 nM) or p38 MAPK siRNA (20 nM) for 72 hours. Whole-cell extracts (20 μg protein) were subjected to western blotting and probed with p38 antibody to visualize p38 knockdown. The same blot was reprobed with ERK-2 and Hsp27 antibodies to confirm equal protein loading and siRNA specificity. (b) HaCaT keratinocytes were transfected with control siRNA or p38 MAPK siRNA for 48 hours after which the cells were treated with the indicated concentrations of sertaconazole for another 24 hours. Supernatants were assessed for PGE<sub>2</sub> levels by ELISA. Results represent mean ± SD from at least three different experiments.

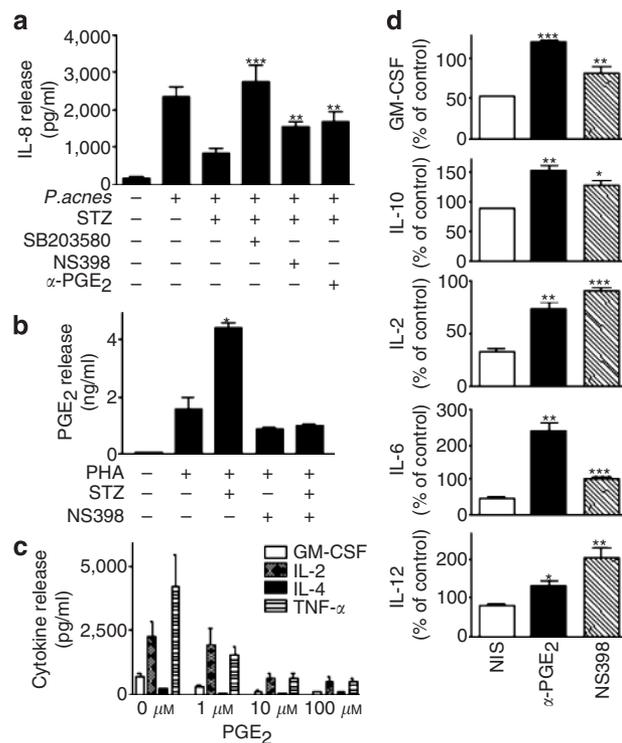
p38-specific siRNA for 72 hours. The levels of total p38 were determined by western blotting of whole-cell extracts. In p38 siRNA-transfected cells, p38 expression was downregulated by about 80% compared with control siRNA-transfected cells (Figure 3a). The same blot was reblotted with ERK-2 and Hsp27 antibodies to show equal protein loading. Next, we assessed sertaconazole-mediated PGE<sub>2</sub> release in control siRNA and p38 siRNA-transfected cells. In control siRNA-transfected cells, there was an induction of PGE<sub>2</sub> release at

1  $\mu\text{g/ml}$  sertaconazole as expected and in p38 siRNA-transfected cells, these levels were brought down to baseline levels indicating that p38 MAP kinase is involved in the regulation of PGE<sub>2</sub> production mediated by sertaconazole (Figure 3b). We also confirmed this using the p38 inhibitor SB203580 that showed sertaconazole-mediated PGE<sub>2</sub> release was inhibited in the presence of the inhibitor (data not shown).

#### Involvement of PGE<sub>2</sub> in the suppression of cytokine production by sertaconazole in keratinocytes and PBMCs

To assess whether sertaconazole-mediated PGE<sub>2</sub> release played an anti-inflammatory role, we looked at the effect of sertaconazole on *Propionibacterium acnes*-induced interleukin-8 (IL-8) production in keratinocytes. *P. acnes* is an important factor in the development of inflammatory acne lesions and bacteria-induced IL-8 production has been thought to play an important role in the pathophysiology of acne (Chen *et al.*, 2002). Typically, keratinocytes were left unstimulated or stimulated with *P. acnes* in the presence or absence of sertaconazole. The result showed that 1  $\mu\text{g/ml}$  sertaconazole treatment inhibited *P. acnes*-induced IL-8 production in keratinocytes (Figure 4a). Preincubation with the p38 inhibitor SB203580 or the COX-2 inhibitor NS398 before *P. acnes* or sertaconazole treatment reversed the inhibitory effects of sertaconazole on IL-8 production suggesting the involvement of p38, COX-2, and possibly PGE<sub>2</sub> in mediating this effect (Figure 4a). To confirm the involvement of PGE<sub>2</sub>, cells were treated with sertaconazole in the presence of anti-PGE<sub>2</sub> antiserum for 24 hours. Anti-PGE<sub>2</sub> antiserum reversed the inhibitory effects of sertaconazole on IL-8 production in keratinocytes suggesting the involvement of sertaconazole-induced endogenous PGE<sub>2</sub> in mediating this effect (Figure 4a).

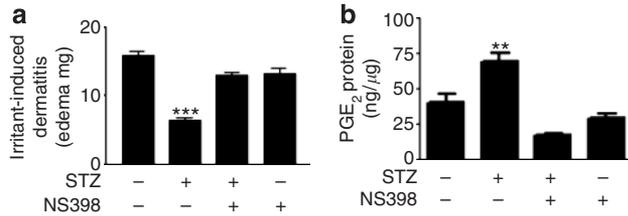
Our previous work showed that sertaconazole dose dependently inhibited cytokine release from phytohemagglutinin (PHA)-stimulated human PBMCs (Liebel *et al.*, 2006). We therefore assessed whether sertaconazole induced PGE<sub>2</sub> release in these immune cells and whether PGE<sub>2</sub> mediates the inhibition of cytokine release from these cells. Human PBMCs were stimulated with PHA in the presence or absence of sertaconazole. As seen from Figure 4b, PHA induced PGE<sub>2</sub> release from PBMCs and sertaconazole potentiated the PHA-induced PGE<sub>2</sub> release by about twofold. Preincubation with the COX-2 inhibitor NS398 before PHA or sertaconazole treatment inhibited PGE<sub>2</sub> release suggesting the involvement of COX-2 in sertaconazole-mediated PGE<sub>2</sub> release. Addition of exogenous PGE<sub>2</sub> dose dependently inhibited the release of proinflammatory cytokines from these cells (Figure 4c), supporting the hypothesis that sertaconazole results in the inhibition of cytokine release from PBMCs via induction of PGE<sub>2</sub>. To confirm this hypothesis, cells were treated with PHA and sertaconazole in the presence of non-immune serum or anti-PGE<sub>2</sub> antiserum or COX-2 inhibitor NS398. Anti-PGE<sub>2</sub> antiserum and NS398 reversed the suppressive effects of sertaconazole on cytokine production confirming the involvement of endogenous PGE<sub>2</sub> in this effect (Figure 4d).



**Figure 4. PGE<sub>2</sub> is involved in sertaconazole-mediated suppression of cytokine production in keratinocytes and PBMCs.** (a) Normal human epidermal keratinocytes were stimulated with *P. acnes* in the absence or presence of 1  $\mu\text{g/ml}$  sertaconazole nitrate alone or combined with 10  $\mu\text{M}$  SB203580 or 10  $\mu\text{M}$  NS398 or anti-PGE<sub>2</sub> antiserum. After 24 hours, release of IL-8 was analyzed. Results represent mean  $\pm$  SD from at least three different experiments. \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared with keratinocytes treated with 1  $\mu\text{g/ml}$  sertaconazole nitrate. (b) Lymphocyte-enriched human PBMCs were stimulated with 10  $\mu\text{g/ml}$  purified PHA in the presence or absence of 1  $\mu\text{g/ml}$  sertaconazole nitrate or 10  $\mu\text{M}$  NS398. After 24 hours, release of PGE<sub>2</sub> from activated PBMCs was assayed using ELISA. The results shown are mean  $\pm$  SD of PGE<sub>2</sub> release from PBMCs derived from three independent donors. \* $P < 0.05$  compared with PBMCs treated with PHA plus vehicle. (c) PBMCs were stimulated with PHA in the absence or presence of exogenously added 1, 10, and 100  $\mu\text{M}$  PGE<sub>2</sub>. After 24 hours, supernatants were collected and cytokine levels were analyzed. (d) PBMCs were preincubated with 1,000-fold diluted non-immune serum or anti-PGE<sub>2</sub> antiserum or 10  $\mu\text{M}$  NS398 for 30 minutes, and then stimulated with PHA in the presence of 1  $\mu\text{g/ml}$  sertaconazole. After 24 hours, supernatants were collected and cytokine levels were analyzed. Results represent mean  $\pm$  SEM from at least three different experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , or \*\*\* $P < 0.001$  compared with PBMCs treated with non-immune serum.

#### Sertaconazole-mediated PGE<sub>2</sub> release is involved in the suppression of TPA-induced ear edema in mice

TPA is the main active compound found in croton oil, producing vasodilation, erythema, and edema within 5 hours after contact with the skin (Rao *et al.*, 1993). In a mouse model of TPA-induced ear edema, sertaconazole nitrate significantly reduced the TPA-induced edema response. The mean ear weight of TPA-challenged animals treated with sertaconazole nitrate (1%) was  $6.29 \pm 0.43$  mg compared with  $15.88 \pm 0.64$  for controls, indicating a statistically significant reduction (60.4%) in irritant dermatitis ( $P < 0.0001$ ; Figure 5a). Treating the ears with both sertaco-



**Figure 5. Reduction of TPA-induced murine dermatitis by topical application of sertaconazole nitrate.**

(a) CD-1 mice were treated with TPA applied to the left ear; the right remained untreated. Immediately after application of TPA (1 μg/ear), sertaconazole (1%), and/or NS398 (0.1%) were applied to the TPA-treated ear ( $n=7$  per group). The results shown are mean  $\pm$  SD; \*\*\* $P<0.0001$  indicates a significant reduction in inflammation compared with the TPA plus vehicle-treated group determined using two-tailed Student's  $t$ -test. (b) Biopsies of mouse ears that had been challenged with TPA only or treated with sertaconazole or NS398 after TPA challenge were homogenized and supernatants were assessed for PGE<sub>2</sub> levels using ELISA. Total protein content of the biopsy samples was determined and PGE<sub>2</sub> levels represented as ng/μg protein. The results shown are mean  $\pm$  SD; \*\* $P<0.01$  indicates a significant increase in PGE<sub>2</sub> levels compared with the TPA plus vehicle-treated group determined using two-tailed Student's  $t$ -test.

nazole and the COX-2 inhibitor NS398 abrogated the anti-inflammatory effects of sertaconazole (Figure 5a). Quantitation of PGE<sub>2</sub> levels in mouse ear biopsies showed that in sertaconazole-treated ears, there was an induction of PGE<sub>2</sub> production ( $68.8 \pm 15$  ng/μg protein) as compared with TPA controls ( $40.7 \pm 12$  ng/μg protein) and this induction of PGE<sub>2</sub> was suppressed by the COX-2 inhibitor NS398 (Figure 5b). Taken together, these results suggest that sertaconazole reduced inflammation via inducing PGE<sub>2</sub> production and the COX-2 inhibitor blocks sertaconazole from exerting its anti-inflammatory effects.

## DISCUSSION

Our results indicate that the antifungal agent sertaconazole nitrate activates the p38 MAP kinase pathway as a mechanism of action for the anti-inflammatory activity. This is a paradoxical finding because the main biological response of p38 activation involves the production of proinflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2, and other proteins that promote the inflammatory process (Saklatvala, 2004; Kaminska, 2005). The anti-inflammatory activity of sertaconazole is also dependent on the secondary production of PGE<sub>2</sub>, which is also a paradoxical finding because prostanoids primarily induce proinflammatory responses such as vasodilation and plasma extravasation (Moncada et al., 1973). Thus, it appears that sertaconazole elicits an anti-inflammatory response through a non-classical anti-inflammatory pathway.

The p38 MAP kinase is activated in response to various extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines, and growth factors (Zarubin and Han, 2005). Many of these stimuli signal to p38 MAP kinase by the stimulation of NAD(P)H oxidase and generation of reactive oxygen species such as superoxide anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Griendling and Ushio-Fukai, 2000; Blanc et al., 2003; Mitra and Abraham, 2006). In our

studies, we found that the sertaconazole-induced activation of p38 MAP kinase was inhibited in the presence of diphenylene iodonium an inhibitor of NAD(P)H oxidase (Griendling et al., 1994) and by superoxide dismutase (Figure 1d), which converts O<sub>2</sub><sup>•-</sup> into the more stable H<sub>2</sub>O<sub>2</sub> (Griendling and Ushio-Fukai, 2000). Incorporation of antioxidants (trolox, ascorbic acid, and *N*-acetyl cysteine (NAC)) had no effect on the phosphorylation of p38 by sertaconazole. Sertaconazole did not increase H<sub>2</sub>O<sub>2</sub> formation in human keratinocytes (data not shown) and treatment with catalase was ineffective in reducing the phosphorylation of p38 by sertaconazole, suggesting that the peroxide formation was not a factor in p38 activation. In contrast to sertaconazole, another antifungal agent, miconazole nitrate, was shown to induce H<sub>2</sub>O<sub>2</sub> formation (Kobayashi et al., 2002); however, miconazole does not inhibit cytokine release or elicit anti-inflammatory activity comparable with sertaconazole (Agut et al., 1996; Liebel et al., 2006) and therefore does not probably activate the same signal transduction cascade. Thus, from these results, we believe that sertaconazole stimulates superoxide radical formation to induce p38 MAP kinase.

The results from this study indicate that sertaconazole activates p38 MAP kinase to induce anti-inflammatory activity. There are four isoforms of p38 kinase:  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$  (Saklatvala, 2004). Keratinocytes express only the p38 $\alpha$ , - $\beta$ , and - $\delta$  isoforms. The siRNA used in these studies to inhibit p38 MAP kinase signaling are non-selective for the P38 isoforms and will result in downregulation of all isoforms, therefore the current results do not address which isoform(s) is activated by sertaconazole. Multiple p38 MAP kinase isoforms provides the potential for each of these isoforms to differ in their substrate specificity and respond in a cell-type-specific manner (Enslin et al., 1998; Eckert et al., 2003). Whereas all four P38 isoforms can be activated by stress stimuli, such as proinflammatory cytokines TNF- $\alpha$  and IL-1 (Goedert et al., 1997; Kumar et al., 1997; Hu et al., 1999), the p38 $\delta$  isoform is solely activated in response to stimuli such as epigallocatechin-3-gallate (Efimova et al., 2002) and plays a key role in the regulation of keratinocyte differentiation and apoptosis (Eckert et al., 2003). Additionally, p38 $\alpha$  and p38 $\gamma$  were found to have opposing effects on the activation of activator protein-1 (AP-1)-driven reporter genes in mammalian cells (Askari et al., 2007). Recent studies have demonstrated that resveratrol, a stilbene isolated from the skin and seeds of grapes, both induces the activation of p38 MAP kinase- $\beta$  and inhibits p38 MAP kinase- $\alpha$  in cardiomyocytes to induce a cardioprotective effect during ischemia (Das et al., 2006). Thus, simultaneous activation or inhibition of multiple p38 isoforms may also add to the signal transduction complexity in the p38 MAP kinase pathway. Future studies will investigate the isoforms of p38 MAP kinase, which mediate the anti-inflammatory activity of sertaconazole.

Several lines of evidence suggest that production of PGE<sub>2</sub> is the downstream mediator for the anti-inflammatory activity induced by sertaconazole. First treating human keratinocytes or PBMCs in culture with sertaconazole resulted in a dose-

dependent stimulation of PGE<sub>2</sub> production (Figures 2b and 4a). In addition, topical treatment with sertaconazole resulted in an increase in murine tissue content of PGE<sub>2</sub> (Figure 5b). Treatment with a pharmacological inhibitor of COX-2, NS398 abolished the anti-inflammatory activity induced by sertaconazole in keratinocytes (Figure 4a) and PBMCs (Figure 4d), suggesting that a cyclooxygenase product is involved in the anti-inflammatory signaling. Topical application of the phorbol ester TPA to the skin of mice produces a long-lasting edema that is associated with a marked influx of neutrophils and mononuclear cells as well as the predominant formation of leukotriene B<sub>4</sub> (Rao *et al.*, 1993; Lloret and Moreno, 1995). Sertaconazole inhibited TPA-induced ear edema by 60%, and NS398 (Figure 5a) and ibuprofen, a non-selective COX inhibitor, also abrogated this effect (data not shown). Co-treatment with sertaconazole and a neutralizing antibody to PGE<sub>2</sub> established that the cyclooxygenase product responsible for the anti-inflammatory activity was PGE<sub>2</sub> (Figure 4a and 4d). Furthermore, exogenous treatment with PGE<sub>2</sub> resulted in a reduction in cytokine release from PBMCs comparable to the treatments with sertaconazole (Figure 4c). Taken together, these results establish that the sertaconazole-induced PGE<sub>2</sub> production mediates the anti-inflammatory activity of the antifungal agent.

Although PGE<sub>2</sub> is primarily associated with proinflammatory activity, the prostanoid has been also shown to elicit anti-inflammatory effects. PGE<sub>2</sub> has been shown to be beneficial for treatment of immunological-based diseases such as psoriasis where topical application of a gel containing PGE<sub>2</sub> was found to improve psoriatic lesions (Remy *et al.*, 1986) and inhibition of PGE<sub>2</sub> formation with non-steroidal anti-inflammatory drugs were found to exacerbate the skin condition (Katayama and Kawada, 1981). PGE<sub>2</sub> has also been reported to resolve mast cell-dependent allergic inflammation in mice by suppressing mediator release (Raud *et al.*, 1988). In human peripheral blood polymorphonuclear neutrophils, PGE<sub>2</sub> was found to cause a switch from proinflammatory to anti-inflammatory strategy by switching eicosanoid biosynthesis from predominantly 5-lipoxygenase-initiated leukotriene B<sub>4</sub> production to 15-lipoxygenase-initiated lipoxin A<sub>4</sub> production that carry "stop signals" for inflammation (Levy *et al.*, 2001). PGE<sub>2</sub> activates at least four separate EP receptor subtypes (EP1-EP4), which are coupled to different intracellular signal transduction pathways (Chung, 2005). Human PBMCs have been reported to express only the EP2 and EP4 receptors (Strong *et al.*, 2001). It is also well established that in PBMCs and other cells, PGE<sub>2</sub> signals via the EP2 and EP4 receptors to induce adenylyl cyclase activity by signaling through a G<sub>s</sub> protein, resulting in enhanced intracellular cAMP formation which negatively regulates cytokine gene expression (Nigg *et al.*, 1985; Betz and Fox, 1991; Marcinkiewicz and Chain, 1993; Vassiliou *et al.*, 2003). cAMP induces activation of protein kinase-A and subsequent phosphorylation of the cAMP-responsive element-binding protein, resulting in inhibition of cytokine genes containing cAMP-responsive element in their promoters (Gonzalez and Montminy, 1989; Masquillier and Sassone-Corsi, 1992). In keratinocytes, PGE<sub>2</sub> activation of

the EP2 and a subtype of the EP3 receptor have been shown to reduce the stimulated release of the chemokine CCL27 (Kanda *et al.*, 2004). PGE<sub>2</sub> has also been shown to downregulate UVB-induced IL-8 release in keratinocytes (Grandjean-laquerriere *et al.*, 2005). Presumably, the anti-inflammatory activity induced by sertaconazole is derived from the PGE<sub>2</sub>-stimulated production of cAMP and subsequent inhibition of cytokine production from keratinocytes and PBMCs.

In this study, we demonstrate that the antifungal agent sertaconazole exhibits anti-inflammatory properties via the p38-COX-2-PGE<sub>2</sub> pathway. We previously evaluated the anti-inflammatory activity of eight antifungal agents and found that only sertaconazole nitrate reduced the release of cytokines from activated lymphocytes and mitigated inflammation in animal models of irritant contact dermatitis and neurogenic inflammation (Agut *et al.*, 1996; Liebel *et al.*, 2006). Treatment of keratinocytes under basal conditions with the antifungal agents butoconazole, fluconazole, sertaconazole nitrate, terconazole, tioconazole, or ketoconazole indicated that only sertaconazole resulted in a direct stimulation of P38 MAP kinase (data not shown). Thus, it seems unlikely that activation of the p38-COX-2-PGE<sub>2</sub> pathway contributes to the antifungal activity of the compound, although a similar activation of the p38-COX-2-PGE<sub>2</sub> pathway may mediate or contribute additional activity to other agents. For example, the immunomodulatory compound cyclosporine A has been indirectly shown to stimulate p38 in a human erythroleukemic cell line (Sawafuji *et al.*, 2003) and cyclosporine A also inhibits mitogenic stimulation of PBMCs and increases the release of PGE<sub>2</sub> from PBMCs (McMillen *et al.*, 1991). This could suggest that other immunomodulatory agents could partially work through a similar pathway as sertaconazole. Indeed, because the notion that stimulation of p38 activation and PGE<sub>2</sub> release can produce an anti-inflammatory effect is paradoxical, it may be overlooked as a pathway to explain anti-inflammatory mechanisms of action. Our studies suggest that activation of the p38-COX-2-PGE<sub>2</sub> pathway by agents such as sertaconazole nitrate provides anti-inflammatory therapeutic benefits.

## MATERIALS AND METHODS

### Materials

Sertaconazole nitrate was obtained from Ferrer Pharmaceuticals (Barcelona, Spain). Phytohemagglutinin (PHA) was obtained from Remel (Lenexa, KS). TPA, non-immune serum, anti-PGE<sub>2</sub> antiserum and all routine reagents were obtained from Sigma (St Louis, MO). Phospho-p38, p38, phospho-Hsp27, Hsp27, and ERK-2 antibodies were obtained from Cell Signaling Technology (Danvers, MA). COX-2 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Lipofectamine 2000 transfection reagent was obtained from Invitrogen Corporation (Carlsbad, CA). PGE<sub>2</sub> and NS398 were obtained from Cayman Chemical (Ann Arbor, MI). *P. acnes* was obtained from ATCC (Strain 11828; Manassas, VA).

### Cells and cell culture

Human HaCaT keratinocytes (a gift from Dr NE Fusenig, Heidelberg, Germany) were maintained in DMEM (Invitrogen Corporation)

containing 10% fetal bovine serum, 4.5 mg/ml glucose, 2 mM L-glutamine, 1% penicillin, and streptomycin. Cells were maintained at <80% confluency at 37°C in 5% CO<sub>2</sub> (v/v). Normal human epidermal neonatal keratinocytes were obtained from Cascade Biologics (Portland, OR) and maintained in serum-free Epilife medium (Cascade Biologics) supplemented with human keratinocyte growth supplement containing 0.2% (v/v) bovine pituitary extract, 5 µg/ml bovine insulin, 0.18 µg/ml hydrocortisone, 5 µg/ml bovine transferrin, and 0.2 ng/ml human epidermal growth factor.

### Phospho-p38/Hsp27 ELISA

Keratinocytes were plated at a density of 10,000 cells per well in 96-well plates in Epilife media with growth supplement HKGS (Cascade Biologics). The media were replaced with serum-free media for 24 hours. The cells were then treated with sertaconazole nitrate or 200 nM TPA (positive control) for different time points. The cells were then washed with cold phosphate-buffered saline and lysed on ice for 30 minutes in 50 µl of cell extraction buffer (Biosource International; Camarillo, CA). Phospho-p38 and phospho-Hsp27 levels were measured in the lysates by using p38 MAPK [pTpY180/182] and Hsp27 [pS82] ELISA kits (Biosource International) according to the manufacturer's instructions. Briefly, lysates were incubated in a 96-well plate precoated with a monoclonal antibody specific to p38 MAPK or Hsp27, then incubated with a phospho-p38 or phospho-Hsp27 detection antibody. After extensive washing, phospho-p38/Hsp27 levels were detected by incubation with horseradish peroxidase-labeled antibodies, followed by colorimetric enzyme assays.

### Measurement of PGE<sub>2</sub> release

Keratinocytes were incubated with or without 100 ng/ml TNF-α in the presence of vehicle or indicated concentrations of sertaconazole for 8 or 24 hours, respectively. The supernatant PGE<sub>2</sub> amounts were measured by ELISA according to manufacturer's instructions (Assay Designs Inc., Ann Arbor, MI).

### Western blotting

Keratinocytes were grown in six-well plates to 80% confluency. Cells were treated with sertaconazole for indicated times periods. Cells were then washed with phosphate-buffered saline and lysed with RIPA lysis buffer containing 65 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8), 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail (Sigma). Lysates were centrifuged and total protein was estimated in the supernatants. Protein (20 µg) was loaded on SDS-PAGE followed by immunoblotting with the specific antibodies and detection using the ECL chemiluminescence detection system (Amersham Life Sciences; Arlington Heights, IL).

### Knockdown of p38 using siRNA

HaCaT keratinocytes were plated in 24-well plates at a density of 4 × 10<sup>4</sup> cells per well and incubated overnight at 37°C, 5% CO<sub>2</sub>. Cells were transfected with SignalSilence negative control siRNA or SignalSilence pool p38 MAPK siRNA (Cell Signaling Technology; Danvers, MA) at 20 nM concentration using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's

instructions. This p38 MAP kinase siRNA will non-specifically target all p38 MAPK isoforms. Cells were then grown for 72 hours at 37°C, 5% CO<sub>2</sub>.

### *P. acnes* stimulation of keratinocytes

Normal human epidermal keratinocytes were plated on 96-well plates at a density of 10,000 cells/well in media (200 µl/well) and incubated at 37°C, 5% CO<sub>2</sub>. Stationary phase *P. acnes* at a density of 1 × 10<sup>8</sup> CFU/ml was obtained and 3 ml of this was spun and the bacterial pellet resuspended in 10 ml Epilife media without antibiotics or growth supplement HKGS. Sertaconazole, SB203580, and NS398 were diluted in Epilife media without antibiotics or HKGS to 2 × concentration. Media were removed from wells and replaced with 100 µl of treatment compounds and 100 µl of *P. acnes*. Samples were preincubated with SB203580, NS398, and anti-PGE<sub>2</sub> antiserum for 30 minutes before stimulation with *P. acnes*. Plates were incubated for 24 hours at 37°C, 5% CO<sub>2</sub>. Supernatants were removed and analyzed for IL-8 (Upstate; Charlottesville, VA) content using the Luminex100 system.

### PBMC activation

Cytokine release from PBMCs was performed as described previously (Agut *et al.*, 1996; Liebel *et al.*, 2006). PBMCs were prepared from three different healthy adult male donors by differential centrifugation on Ficoll-Hypaque (Biological Specialty Corporation, Colmar, PA). For antibody treatments, PBMCs were preincubated with NS398 or 1,000-fold diluted non-immune serum or anti-PGE<sub>2</sub> antiserum for 30 minutes before PHA stimulation. PBMCs were then incubated at 37°C at 5% CO<sub>2</sub> for 24 hours after which supernatants were collected and cytokine release assayed.

### TPA-induced ear edema (irritant dermatitis) and quantitation of PGE<sub>2</sub> levels in mouse ear biopsies

TPA-induced ear edema was performed as described previously (Agut *et al.*, 1996; Liebel *et al.*, 2006). The Institutional Animal Care and Use Committee at Johnson & Johnson approved all procedures used in these experiments. Biopsies of 7 mm diameter were taken from mouse ears that had been challenged with TPA only or treated with sertaconazole or NS398 after TPA challenge. Biopsies were homogenized on ice in 800 µl cold phosphate-buffered saline containing protease inhibitor cocktail (Sigma Aldrich; St Louis, MO) with a Polytron homogenizer. Samples were centrifuged at 1,000 r.p.m. for 10 minutes at 4°C and supernatants were removed. PGE<sub>2</sub> content of the supernatant was determined using PGE<sub>2</sub> Detection Kit (Assay Designs Inc.; Ann Arbor, MI). Total protein content of the biopsy samples was determined using BCA protein assay kit (Pierce Biotechnology; Rockford, IL) and PGE<sub>2</sub> levels represented as ng/µg protein.

### Statistical analysis

Data are presented as mean ± SD. Cytokine release experiments were individually performed from three separate donors of PBMCs. Student's *t*-test was used for comparisons between two groups. A value of *P* < 0.05 was considered significant.

### CONFLICT OF INTEREST

All authors are employees of Johnson & Johnson.

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