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Prostaglandin E_2 is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors

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Keratinocytes are the major target of sunlight, and they produce prostaglandin (PG) E_2 upon ultraviolet (UV) exposure. Although indomethacin, one of cyclooxygenase inhibitors, is known to suppress UV-induced acute skin inflammation, it remains uncertain whether endogenous PGE_2 is responsible for UV-induced skin inflammation, and which subtype of PGE_2 receptors mediates this process. UV-induced skin inflammation was investigated by using genetically and pharmacologically PGE_2 receptor-deficient mice. We applied UV-induced skin inflammation model to genetical and pharmacological PGE_2 receptor-deficient mice. We exposed UVB on these mice at 5 kJ/m², and examined the ear swelling and the histological findings. We also measured the blood flow using a laser doppler device to assess the intensity of UVB-induced inflammatory change. The UV-induced ear swelling at 48 h after exposure was significantly reduced in $EP2^{-/-}$, $EP4^{-/-}$ or wild-type mice treated with the EP4 antagonist compared to control mice. Consistently, inflammatory cell infiltration into the local skin, and local blood flow after UV exposure were significantly reduced by EP2 or EP4 signaling blockade. These data suggest that PGE_2-EP2/EP4 signaling is mandatory in UV-induced acute skin inflammation, presumably by enhancing blood flow in the microenvironment.
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Ultraviolet light (UV) radiation has a wide variety of actions, such as sunburn formation, immunosuppression, skin aging and cancer development. Acute skin inflammation is another effect of UV radiation and is characterized by erythema and edema. Vasodilatation at the early phase after UV exposure is a critical event, leading to skin accumulation of inflammatory cells, such as neutrophils and T cells.[1] In addition, UV exposure to the skin triggers the release of lipid mediators such as prostaglandins (PGs), which are produced via sequential pathways involving cyclooxygenase (COX) and each PG synthetase. Among PGs, PGE_2 is known to be produced abundantly by keratinocytes in the skin on UV exposure.[2,3]

PGE_2 exerts its actions by binding to four different types of G-protein-coupled receptors, known as EP1, EP2, EP3 and EP4.[4] EP2 and EP4 receptors bind to G_s and increase cAMP. EP1 receptors are coupled to G_q and EP3 receptors mostly to G_i.[4] We have previously generated mice individually deficient in each of the four subtypes of PGE_2 receptors and studied the *in vivo* significance of each PGE_2 receptor with novel findings in various aspects.[5–11]

It is well known in humans that indomethacin, one of COX inhibitors, inhibits UV-induced acute skin inflammation,[12] and an exogenous intradermal injection of PGE_2 increases skin microenvironmental blood flow.[13] Although these observations have suggested that PGE_2 serves as a mediator in the UV-induced skin alterations, it remains uncertain whether endogenous PGE_2 is responsible for UV-induced skin inflammation, and which subtype of PGE_2 receptors mediates this process. Using both

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genetical and pharmacological approaches in mice, we examined *in vivo* the potential role of PGE_2 in UV-induced skin inflammation.

Materials and methods

Animals

Mice lacking the EP1, EP2, EP3 and EP4 receptors individually ($EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$ and $EP4^{-/-}$ mice, respectively) were generated as described.[5–8] With the exception of $EP4^{-/-}$ mice, each mutant was backcrossed 10 times to C57BL/6CrSlc (Japan SLC, Shizuoka, Japan), and females of the F_2 progenies of N10 mice were used with C57BL/6 female mice as their controls. Mice were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

Reagents

EP4 antagonist, ONO-AE3-208, 4-(4-Cyano-2-(2-(4-fluoronaphthalen-1-yl) propionylamino) phenyl) butyric acid (AE3-208) was kindly provided by Ono Pharmaceutical Co., Osaka, Japan. AE3-208 was administered (10 mg/kg/day) orally in the drinking water 2 days before UV exposure and through the experiment. A volume of 20 μl of 1% wt/vol indomethacin in acetone was topically applied on the ears of mice immediately after UV irradiation.

UVB Irradiation

Sunlamps emitting 280–360 nm with a peak emission at 312.5 nm (Toshiba FL 20SE; Toshiba Electric Co.) were used as a source of UVB. The irradiance was 5.5 J/m^2/s at a distance of 40 cm, as measured by an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan). For UV-induced skin inflammation, mice were exposed to 5 kJ/m^2 of UVB. The ear swelling was measured at the indicated time points after irradiation using ear thickness gauge, and the ear thickness change was shown.[14]

Determination of PGE_2 Content in the Mouse Skin

The amounts of PGE_2 in the mouse abdominal skin at 0 and 48 h after irradiation with 5 kJ/m^2 of UVB were determined by enzyme immunoassay basically as manufacturer's protocol (Cayman Chemical). In brief, abdominal skin was excised and immediately dropped into liquid nitrogen. Frozen skin were weighted and homogenized with a polytron homogenizer in 10 ml ethanol containing 0.1 ml of 5 N HCl, which was precooled at -20°C. After centrifugation, PGE_2 in the ethanol extract were applied on SEP-PAK C18 cartridges (Waters Associates, Milford, MA, USA). The amounts of PGE_2 were measured by enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

Histology

The skin of the ear was excised and fixed in 10% formaldehyde. Sections of 5 μm thickness were prepared and subjected to staining with hematoxylin and eosin. The numbers of neutrophils and lymphocytes per field ($\times 20$) were counted at five randomized spots using microscopy. The diameter of randomly selected 50 blood vessels in the ear was measured.

Measurement of Skin Blood Flow

Abdominal area of mouse skin was shaved and irradiated with UVB. Blood flow was assessed using an Advance Laser (model ALF21R) Doppler flowmeter. The probe was held at six spots of abdominal area, and each reading was the mean of the six measurements.

Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test or one-way ANOVA followed by Dunnett multiple comparisons. *P*-value of <0.05 was considered to be significant.

Results

It is known that a single exposure of UVB to mice causes marked skin inflammation, most remarkably on the ears. The extent of inflammation was evaluated by measuring the ear swelling responses. When control C57BL/6 mice were exposed to at 5 kJ/m^2 to UVB, the ear swelling became detectable within 24 h after irradiation, and the response progressed over the next 24 h as reported previously.[15] Mice receiving topical application of 1% indomethacin in acetone immediately after UV exposure showed significantly reduced ear swelling responses at each time point tested, as compared to the control mice that received acetone alone (Figure 1). As indomethacin inhibits COX, an enzyme to produce PGs, it is assumed that PGs play a pivotal role in the induction of UV-induced skin inflammation. As PGE_2 is the major PG produced by keratinocytes, we tested the magnitude of swelling responses in mice lacking each receptor subtype. The responses were significantly reduced in $EP2^{-/-}$ mice, $EP4^{-/-}$ mice and EP4 antagonist-treated C57BL/6 mice compared to the control C57BL/6 or $EP4^{+/+}$ mice at 48 h, although the response was similar in EP1 and EP3 mice (Figure 1). The baseline

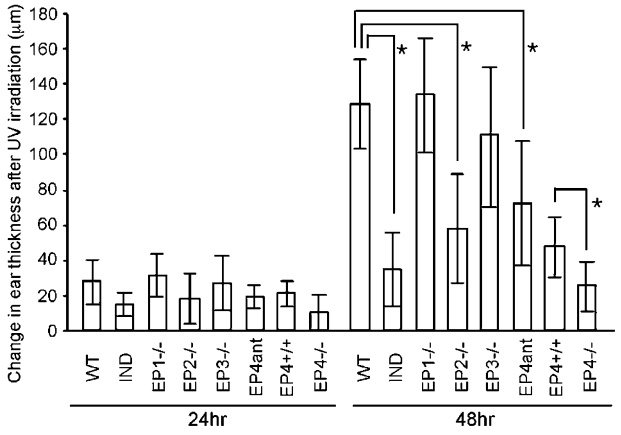

Figure 1 Impaired UV-induced ear skin swelling response by EP2 and EP4 receptor signaling blockade. C57BL/6 mice were treated without (WT, $n=15$) or with indomethacin (IND, $n=10$), or pretreated with EP4 antagonist (EP4 ant, $n=16$). Along with these mice, $EP1^{-/-}$ ($n=11$), $EP2^{-/-}$ ($n=8$), $EP3^{-/-}$ ($n=9$), $EP4^{+/+}$ ($n=6$) and $EP4^{-/-}$ ($n=6$) mice were exposed to 5 kJ/m^2 of UV radiation. They were examined in ear swelling responses 24 and 48 h later, and the increment in ear thickness was calculated (ear thickness after irradiation—ear thickness before irradiation). Data are a representative of three independent experiments and presented as the means $\pm$s.d. A Student's *t*-test was performed between the indicated groups and an asterisk indicates $P<0.05$.

ear thickness of C57BL/6 with or without the EP4 antagonist, and EP-deficient mice, all without UV radiation, was comparable between these groups (data not shown). These results suggested that endogenous PGE_2 mediates UV-induced skin inflammation through EP2 and EP4 receptors. As $EP4^{-/-}$ mice were F_2 progeny and individual mice possessed mixed genetic backgrounds, we examined the response of mice administered with the selective EP4 antagonist for further evaluation of EP4 serving as critical receptors.

We then examined the histology, which showed that the ear of C57BL/6 mice treated with or without the EP4 antagonist, and $EP2^{-/-}$ mice, all without UV exposure, were similar (Figure 1). And the histology of the ears of wild-type mice 48 h after UVB irradiation showed significant dermal edema, inflammatory cells infiltration in the upper dermis, and the marginal thickening of the epidermis. This edematous change in the dermis was milder in $EP2^{-/-}$ mice and C57BL/6 mice treated with the EP4 antagonist (Figure 2). Moreover, the infiltration of inflammatory cells was less intensive in those mice (Figure 2). Whereas neutrophils and lymphocytes in the ears of wild-type mice were increased after UVB irradiation, those numbers in $EP2^{-/-}$ mice or C57BL/6 mice treated the EP4 antagonist were lower than those in C57BL/6 mice after irradiation (Figure 3).

The edema response following UV irradiation is dependent on vascular blood flow and vascular permeability. Especially, vascular blood flow can alter fluid movement between the vascular lumen and the interstitial space by increasing the luminal hydrostatic pressure. It is known that increasing local-microenvironmental blood overflow at the early phase after UV exposure is essential to establish UV-induced skin inflammation.[1,16] We therefore measured the diameter of blood vessels in the skin 48 h after UVB irradiation in histological specimens. While blood vessels in wild-type mice were enlarged after UVB irradiation, those in $EP2^{-/-}$ mice or wild-type mice treated with the EP4 antagonist were significantly smaller (Figure 4). We then assessed the blood flow of the abdominal skin at 0, 12, 24, 36 and 48 h after UVB (5 kJ/m^2) irradiation using a laser doppler device, and found that the blood flow peaked around 24 h after UV exposure, and was impaired by the EP2 or EP4 signaling blockade (Figure 5). These data suggested that PGE_2-EP2/EP4 signaling is critical in acute skin inflammation by enhancing blood flow in the microenvironment.

The above results suggested that both EP2 and EP4 are essential for the development of acute skin inflammation. However, the suppressed level of inflammation by each signaling blockade tended to be lower than the indomethacin treatment. We therefore explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering $EP2^{-/-}$ mice with the EP4 antagonist. The administration of the EP4 antagonist further decreased the reduced ear swelling level of $EP2^{-/-}$ mice (Figure 6). To rule out the possibility that UV-induced PGE_2 expression is affected by EP signaling blockade, which eventually affect the skin inflammation, we measured the PGE_2 expression in the skin. PGE_2 level in the abdominal skin 48 h after irradiation from wild-type and $EP2^{-/-}$ mice with or without the EP4 antagonist were as follows (wild-type C57BL/6 mice; 90.8 ± 10.5, $EP2^{-/-}$ mice; 80.4 ± 12.8, $EP2^{-/-}$ mice with the EP4 antagonist; 76.3 ± 9.0 ng/g tissue: average $\pm$s.d., $n=4$ each). As a comparison, the baseline PGE_2 expression in the skin without UVB irradiation was 0.9 ± 0.3 ng/g tissue: average $\pm$s.d., $n=4$ each. These results suggest that a significant amount of PGE_2 was induced by UV exposure, and the concentration of PGE_2 in the skin was not affected by the EP signaling blockade.

Discussion

In this study, we demonstrated that UVB-induced ear swelling response, skin infiltration of neutrophils and lymphocytes, and local blood flow were reduced in $EP2^{-/-}$, $EP4^{-/-}$ or wild-type mice treated with the EP4 antagonist. These results suggest that endogenous PGE_2 mediates UVB-induced acute skin inflammation through EP2 and EP4 receptors.

The UVB-evoked acute swelling response seems to be derived mainly from vasodilatation and resultant exudates. PGE_2 exerts its physiological functions by binding to its specific receptors, EP1-EP4, which are well known to be expressed on blood vessels. Vasodilatation is performed through EP2

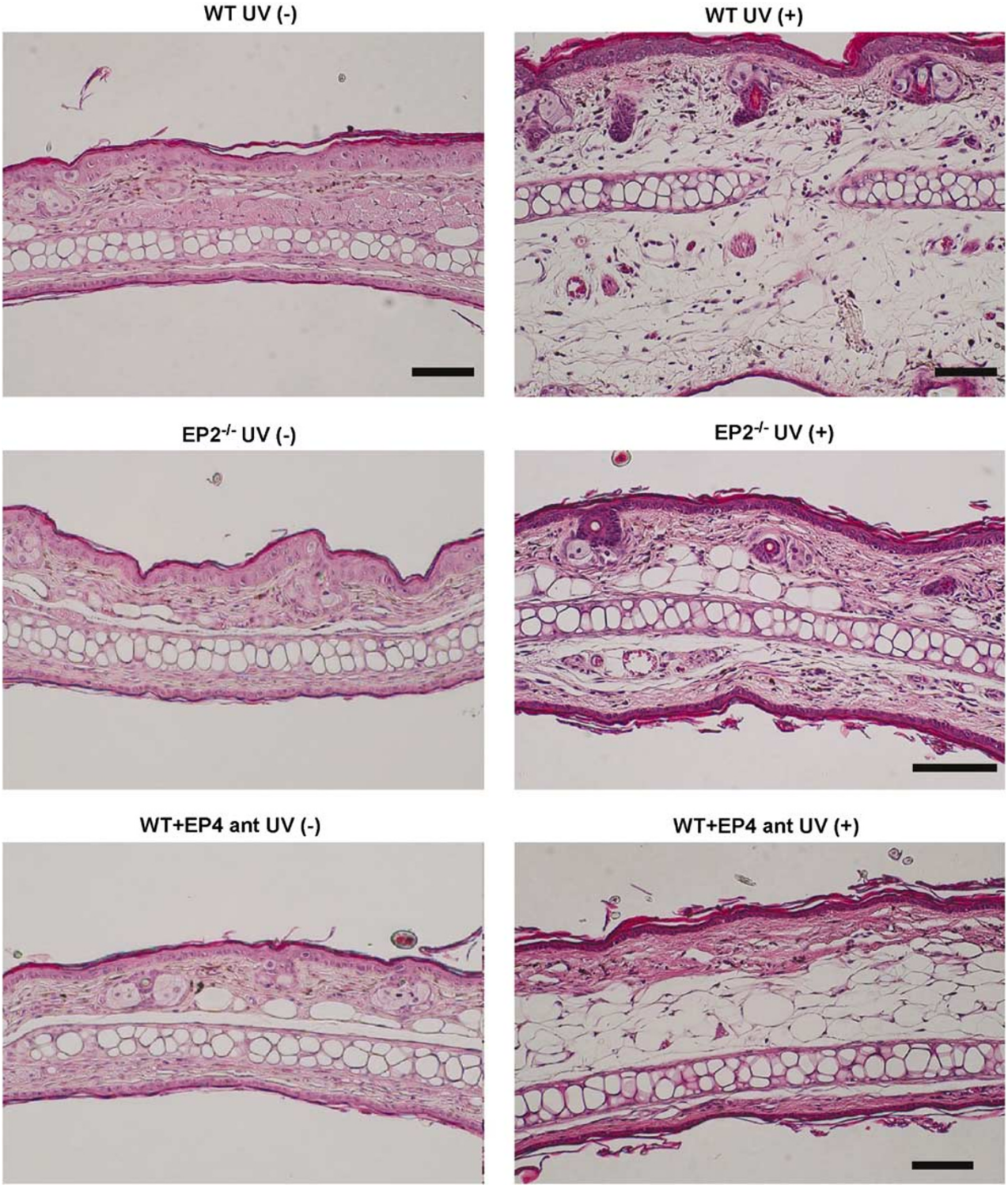

Figure 2 Histology of the skin after UV exposure. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised before (UV (−)) and 48 h after UV irradiation (UV (+)). The edema and inflammatory cell infiltration was notified in WT mice after irradiation, which was less significant in WT mice treated with EP4 antagonist or $EP2^{-/-}$ mice. Sections of 5 μm thickness were prepared and subjected to staining with hematoxylin and eosin. Scale bars, 50 μm.

and EP4 receptors in association with increased cyclic AMP levels by coupling G_s.[17–19] Moreover, PGI_2 receptor, IP and PGD_2 receptor, DP, are known to increase cyclic AMP levels via G_s.[4] In mice, however, PGD_2 was barely detected in the skin after UVB irradiation, and PGI_2 was not examined in that study.[20] We showed that the ear swelling response in EP2 mice (58 + 12.6 μm) or C57BL/6 mice treated

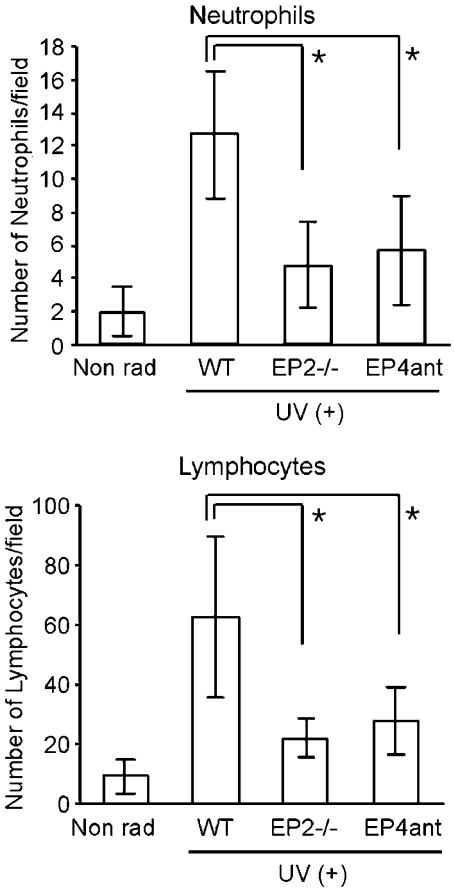

Figure 3 Number of inflammatory cells in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The numbers of neutrophils and lymphocytes infiltrating into the skin per field ($\times 20$) were counted at five randomized areas, and are presented as the means $\pm$s.d. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, unpaired two-tailed *t*-test).

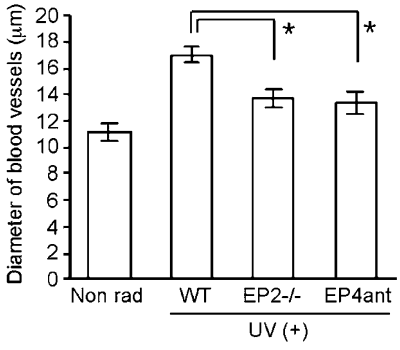

Figure 4 Diameter of blood vessels in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The diameter of 50 randomized blood vessels in the dermis were measured, and are presented as the means $\pm$s.e.m. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, unpaired two-tailed *t*-test).

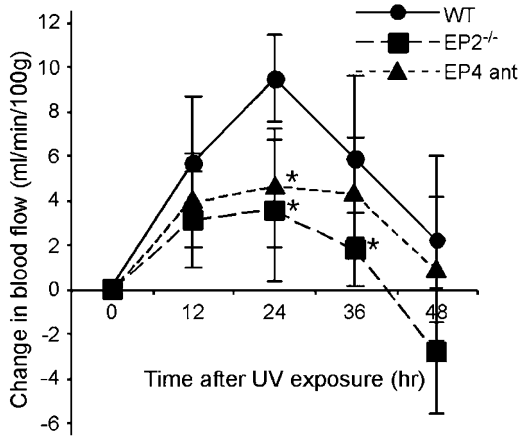

Figure 5 Blood flow of the skin after UV exposure. The blood flow of the abdomen of C57BL/6 mice treated with (EP4 ant, $n=5$) or without (WT, $n=5$) EP4 antagonist, and that of $EP2^{-/-}$ ($n=5$) mice were measured using laser Doppler flowcytometer at indicated time points. These data are a representative of three independent experiments and are presented as the means $\pm$s.d. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, Dunnett multiple comparisons).

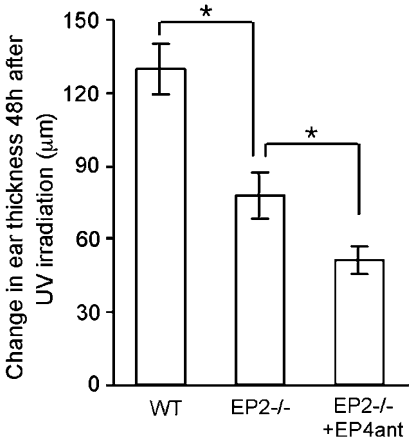

Figure 6 Enhanced impairment of UV-induced ear skin swelling response by combinational signaling blockade of EP2 and EP4 receptors. C57BL/6 (WT) mice and $EP2^{-/-}$ mice treated with or without EP4 antagonist were exposed to 5 kJ/m^2 of UV radiation ($n=5$, each). They were examined in the ear swelling response 48 h later. These data are a representative of two independent experiments and are presented as the means $\pm$s.d. Student's *t*-test was performed between the indicated groups and an asterisk indicates $P<0.05$.

with the EP4 antagonist ($72+14.3\,\mu$m) was not as low as mice treated with indomethacin ($35+8.6\,\mu$m). Therefore, we explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering $EP2^{-/-}$ mice with the EP4 antagonist and found that the administration of the EP4 antagonist further decreased the ear swelling of $EP2^{-/-}$ mice ($51+5.6\,\mu$m). This extent of reduced ear swelling level was not as same as indomethacin-treated group, but quite similar. It might be interesting to investigate the role of other PGs, such as

PGI_2, in the development of inflammation. In human, both PGD_2 and 6-keto $PGF_{1\alpha}$, stable metabolites of PGI_2, were detected in the skin after UVB irradiation.[21] The differences may exist in the usage of PGs for the UV-induced skin response between mice and human. Nonetheless, the importance of PGE_2, as documented in this study, is particularly notable. As cAMP is the downstream signaling in both EP2 and EP4, we expected that the differences in cAMP production are involved in the synergistic effect of EP2 deficiency and the EP4 antagonist treatment. However, the cAMP level in these mice were barely detected in the skin, and not elevated by UV exposure (data not shown). Moreover, there was no significant difference in cAPM production between these groups. We presume that cAMP is difficult to be detected in the skin en bloc even though it is induced in the blood vessels after UV exposure. One possibility is that PGE2 is produced consistently after UV exposure and may not elevate cAMP levels robust enough to be detected *in vivo*. The local cAMP production around the vessels may be elevated, but it is difficult to detect this slight increase.

Keratinocytes are the major target of UV radiation and play a central role in the inflammatory and immune modulatory changes observed after UV exposure, at least partly via the UV-induced release of cytokines (IL-1, IL-6, IL-8, IL-10, GM-CSF, TNF-α)[22] and COX products.[23] IL-6 induces fever and the acute phase response and stimulates leukocytes infiltration in the skin.[24] PGE_2 plays a role in vasodilatation and in the erythemal response of the skin after solar exposure.[25] It has been reported that nitric oxide is involved in the induction of vascular dilatation after UVB exposure.[3,13,26] As indomethacin treatment did not completely inhibit the UV-induced ear swelling response, there still remains the possibility that other factors are involved in this process. IL-6 and nitric oxide are the candidate in this process at present. So far, using normal human keratinocytes, Pupe *et al*[27] demonstrated that eicosaepentaenoic acid decreased both PGE_2 and IL-6 secretion induced by UV-irradiation.[27] However, the relationship between PGE_2, IL-6 and nitric oxide *in vivo* events remains unknown and is an interesting issue to be pursued in the future. In this study, we focused on the acute phase of UV-induced skin inflammation. During the acute process of fluid exudation, the role of histamine release from mast cell is important by increasing the vascular permeability.[28] Although PGE_2 reportedly suppresses mediator release by some mast cell subtypes *in vitro,*[29] it also enhances mediator release from mouse mast cells.[30] The mechanisms and EP receptor subtypes responsible for PGE_2-mediated inhibition of mast cell activation in UV-induced skin inflammation are incompletely understood.

At cellular levels, UV radiation triggers cytokine production,[22] regulates surface expression of adhesion molecules,[31] affects cellular mitosis,[32] and induces apoptotic cell death of skin components.[33] The UV-induced keratinocyte apoptosis is an important factor for preventing from skin cancer formation. EP2 and EP4 signaling blockade can be prophylactic for the acute sunburn reaction in which vasodilatation, exudation, and inflammation participate. On the other hand, it has been already reported that PGE_2-induced skin cancer is mediated by EP1 or EP2 receptor signaling,[34,35] and there still remains the possibility that other PGE_2 receptor signaling may be involved in this process. The present study clearly demonstrated the deep relationship between PGE_2 and acute UV reactions. The involvement of PGE_2 and its receptors in more chronic UV-induced conditions, such as cancer development, is an issue to be elucidated.

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