Inhibition of 12-O-Tetradecanoylphorbol-13-Acetate-Induced Increase in Vascular Permeability in Mouse Skin by Lipoxygenase Inhibitors

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Abstract—The painting of mouse dorsal skin with 12-O-tetradecanoylphorbol-13-acetate (TPA) (0.2-2.5 nmol/mouse) induced a dose-related increase in vascular permeability, which was determined by pontamine sky blue exudation into the skin 5 hr after the TPA treatment. Marked infiltration of neutrophils in the dermal interstitium was also observed 5 hr after TPA treatment. Treatment of mice with nordihydroguaiaretic acid (NDGA) (10 µmol/mouse), 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861) (10 µmol/mouse) and quercetin (3 µmol/mouse) significantly inhibited the TPA-induced dye exudation. However, indomethacin (250-1000 nmol/mouse) tended to inhibit the TPA-induced dye exudation, but the inhibition was not statistically significant. Treatment with AA861 (10 µmol/mouse) also caused a marked inhibition of TPA-induced neutrophil infiltration. Quercetin, NDGA and AA861 inhibited epidermal lipoxygenase activity, but indomethacin failed to inhibit it. On the other hand, indomethacin inhibited epidermal cyclooxygenase, but quercetin, NDGA and AA861 failed to inhibit it. The present study suggests involvement of a lipoxygenase product(s) in the mechanism of the TPA-induced increase in vascular permeability in the dorsal skin of mice.

Materials and Methods

Chemicals: TPA, indomethacin and nordihydroguaiaretic acid (NDGA) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; quercetin and pontamine sky blue.
blue 6B were from Tokyo Chemical Industry Co., Tokyo, Japan. 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861) was kindly supplied by Dr. S. Terao, Takeda Chemical Industries, Ltd., Osaka, Japan. [1-14C]Arachidonic acid (52.9 mCi/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A.

**Vascular permeability:** Female CD-1 mice (Charles River, Atsugi, Kanagawa Pref., Japan), 7–8 weeks of age were used. Mice were housed in an air-conditioned room (22–23°C) with illumination from 6 a.m. to 6 p.m. Food and water were available ad libitum. The dorsal hair of each mouse was shaved with clippers at least 2 days before use, and only the mice in a resting phase of the hair cycle were used. All chemicals were dissolved in reagent grade acetone and painted on the shaved area (about 10 cm²) in a volume of 0.2 ml using a micropipette. Mice were topically painted with vehicle or inhibitors 40 min prior to the application of TPA. The same amounts of inhibitors were painted again on the mice concurrently with TPA. Mice were killed 5 hr after the painting with TPA except in the cases indicated. Pontamine sky blue solution (in saline) was injected intravenously (250 mg/kg) 30 min before sacrifice. The constant area (5.3 cm²) of dorsal skin was punched out from each mouse, and the dermis and the epidermis were isolated by a brief heat treatment (17). Extraction of the dye extravasated into the epidermis was performed by the method of Harada et al. (18). In brief, the dermis or the epidermis obtained was mixed with a medium composed of 1.4 ml of acetone and 0.6 ml of a 0.5% aqueous solution of sodium sulphate in a test tube. The tubes were sealed firmly with stoppers and left to stand for 24 hr at room temperature. The amount of dye in the medium was calculated by comparing the absorbance of the medium at 630 nm with those of standard samples. The inhibitors recovered from the dermis or the epidermis did not interfere with the colorimetric determination of pontamine sky blue.

**Epidermal lipoxygenase and cyclooxygenase activities:** Epidermal lipoxygenase activity was determined as described in our previous report (14). Epidermal cyclooxygenase activity was determined by measuring the formation of [14C]PGE₂ from [14C]-arachidonic acid. Isolated epidermis was homogenized in 100 mM Tris HCl buffer (pH 8.0) containing 1 mM EDTA, 2 mM glutathione and 2 μM hemoglobin in a Polytron PT-10 homogenizer at 4°C, and centrifuged for 10 min at 12,000 × g. The supernatant was re-centrifuged for 60 min at 105,000 × g. The 105,000 × g particulate fraction of the epidermal homogenate was used for the cyclooxygenase assay. [14C]Arachidonic acid (final 3.6 μM) was added to the reaction tube containing an aliquot (500 μl) of the 105,000 × g particulate fraction resuspended in the same buffer, which was preincubated for 5 min with inhibitors to initiate the reaction. The reaction was carried out for 10 min at 37°C and terminated by the addition of 2.0 ml of a mixture of solvents consisting of 15 parts ethyl acetate, 2 parts methanol and 1 part 0.4 M citric acid. The products from [14C]arachidonic acid were analyzed by silica gel thin-layer chromatography (TLC) with the following solvent system: ethyl acetate/n-hexane/acetic acid/water (57:26:6:60, by vol., upper layer). Radioactivity of the products was determined with an automatic TLC-linear analyzer system (Berthold, LB2832, West Germany).

**Histological study:** Acetone (vehicle), TPA, and TPA plus AA861 were applied to mouse skin as described above. Five hours after TPA application, the skin was removed, fixed with 10% (vol./vol.) formalin, sectioned in paraffin, and stained with hematoxylin and eosin.

**Statistical analyses:** Statistical analyses were done by the t-test or Cochran-Cox’s method.

**Results**

Painting of mice with 2.5 nmol TPA caused an increase in pontamine sky blue exudation into the skin, as shown in Fig. 1. The dye exudation reached a peak 5 hr after TPA application and remained at a high level until 18 hr after TPA application. The increase in dye exudation determined 5 hr after TPA treatment was dose-related and reached the maximal point with 2.5 nmol TPA/mouse (Fig. 2). The level of dye exudation induced...
by 20 nmol of TPA was significantly less than the maximum response (2.5–10 nmol/mouse). Therefore, in the following experiments, the dose of TPA was fixed at 2.5 nmol/mouse and the dye exudation was determined 5 hr after TPA application to investigate the effects of various inhibitors of arachidonic acid metabolism on the TPA-induced vascular permeability.

Table 1 shows the effects of several lipoxygenase inhibitors, i.e., NDGA (19), AA861 (20) and quercetin (14, 21), and indomethacin, a selective cyclooxygenase inhibitor, on the TPA-induced dye exudation into the skin. Treatment of mice with NDGA (10 μmol/mouse), AA861 (10 μmol/mouse) and quercetin (3 μmol/mouse) significantly inhibited the TPA-induced dye exudation. Indomethacin (250–1,000 nmol/mouse) tended to inhibit the dye exudation, but the inhibition was not statistically significant. These inhibitors are capable of inhibiting TPA-induced epidermal ODC at the doses used in the present study (11, 12, 14, 22 and T. Nakadate et al., unpublished data). The effects of the inhibitors used in the present study on arachidonic acid metabolism of mouse skin were examined by determining epidermal lipoxygenase and cyclooxygenase activities in the presence of the above inhibitors because it was reported that activities of both enzymes were higher in the epidermis than in the dermis (23). Epidermal lipoxygenase activity and cyclooxygenase activity were determined by the formation of [14C]12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and [14C]PGE2 from [14C]arachidonic acid, respectively. When arachidonic acid is incubated with the 105,000×g supernatant fraction or the 105,000×g particulate fraction
of epidermal homogenate, 12-HETE or PGE$_2$ is the major metabolite, respectively. Quercetin, NDGA and AA861 potently inhibited epidermal lipoxygenase activity (Table 2 and ref. 14), but did not inhibit epidermal cyclooxygenase activity (Table 2).

However, indomethacin failed to inhibit lipoxygenase activity, but potently inhibited cyclooxygenase activity (Table 2).

Histological changes in mouse skin induced by topical application of TPA were also examined. Figures 3A and 3B show the

| Expt. Treatment | Dye exuded* |
|-----------------|-------------|
|                 | Epidermis | Dermis |
|                 | A        | B  | A    | B  |
| I Vehicle       | 1.6±0.2  | 0  | 1.6±0.1 | 0  |
| TPA (2.5 nmol)  | 18.3±3.2 | 100| 12.3±2.0 | 100|
| TPA+indomethacin (0.25 µmol) | 11.8±1.5**** | 61 | 7.1±1.0**** | 51 |
| TPA+indomethacin (1 µmol) | 12.0±2.9**** | 62 | 5.8±2.0**** | 39 |
| II Vehicle      | 2.1±0.2  | 0  | 1.8±0.3 | 0  |
| TPA (2.5 nmol)  | 12.5±2.5 | 100| 8.5±1.7 | 100|
| TPA+NDGA (10 µmol) | 5.9±1.2**** | 37 | 2.9±0.4*** | 16 |
| TPA+AA861 (10 µmol) | 5.1±1.3** | 29 | 2.2±0.2**** | 6  |
| III Vehicle     | 1.7±0.1  | 0  | 1.6±0.1 | 0  |
| TPA (2.5 nmol)  | 12.8±2.3 | 100| 7.1±0.9 | 100|
| TPA+quercetin (1 µmol) | 8.7±1.6**** | 63 | 5.9±0.6**** | 78 |
| TPA+quercetin (3 µmol) | 2.0±0.3** | 3  | 2.6±0.3** | 18 |

Mice were topically treated with vehicle or inhibitors 40 min prior to the application of TPA. The same amounts of inhibitors were applied again to the mouse concurrently with TPA. Mice were killed 5 hr after TPA application. Pontamin sky blue was injected intravenously (250 mg/kg) 30 min before sacrifice. The quantity of leaked dye extracted from epidermis and dermis was determined as shown in Materials and Methods. * Each result represents (A) mean±S.E. (nmol/30 min/cm$^2$) from 5 mice and (B) % stimulation. **P<0.01 vs. TPA. ***P<0.05 vs. TPA. ****not significantly different from TPA.

Table 2. Inhibition of epidermal lipoxygenase and cyclooxygenase activities by indomethacin, NDGA, AA861 and quercetin

| Inhibitor | Lipoxygenase IC$\text{_{50}}$ (µM) | Cyclooxygenase |
|-----------|---------------------------------|---------------|
| Indomethacin | >300                           | 0.1           |
| NDGA      | 5.5                             | >100          |
| AA861     | 2.0                             | >100          |
| Quercetin | 1.4                             | >100          |

Lipoxygenase and cyclooxygenase activities were determined by the formation of [$^{14}$C]12-HETE and [$^{14}$C]PGE$_2$ from [$^{14}$C]arachidonic acid, respectively. [$^{14}$C]arachidonic acid was incubated with the 105,000xg supernatant fraction or the 105,000xg particulate fraction of the epidermal homogenate in order to determine the respective lipoxygenase and cyclooxygenase activities. *Concentration of an inhibitor which inhibits the enzyme activity by 50%.
skin after acetone (vehicle) treatment and
the skin after TPA treatment, respectively.
Marked infiltration of neutrophils in the
dermal interstitium was observed in TPA
treated skin consistent with the previous
report (9). Figure 3C shows the skin treated
with TPA plus AA861. Neutrophil infiltration
was not observed in this case.

Fig. 3. Inhibitory effect of AA861 on the TPA-induced neutrophil infiltration in dorsal skin of mice. Mice were treated with (A and B) vehicle or (C) 10 μmol AA861. Forty minutes later, the mice were treated again with (A) vehicle, (B) 2.5 nmol TPA or (C) 2.5 nmol TPA plus 10 μmol AA861. Mice were killed 5 hr after TPA treatment and the skins stained with hematoxylin and eosin were examined with a light microscope (×200).

Discussion
Painting the dorsal skin of CD-1 mice
with TPA induced an acute increase in
vascular permeability and neutrophil infil-
tration in the dorsal skin. The time period of
dye exudation after TPA treatment (i.e.,
increase in vascular permeability) was
similar to that of mouse ear edema formation
induced by TPA or croton oil (7, 8, 10). In
addition to inducing skin inflammation,
topical application of TPA to dorsal skin of
the same strain of mouse also causes the
induction of epidermal ODC in a dose-
dependent manner (17). In order to obtain
the maximal ODC induction, 20 μmol/mouse
or a larger amount of TPA is needed (17, 24).
On the other hand, maximum dye exudation
was observed with 2.5 nmol TPA/mouse.
Since ODC induction caused by TPA was
observed even in isolated or cultured
epidermal cells (25), TPA-induced epidermal
ODC is solely attributable to its direct action
on epidermal cells. On the other hand many
cellular systems in addition to epidermis such
as leukocytes (26, 27) and platelets (27,
28), etc., which are capable of responding to TPA stimulation, may also be involved in the TPA-induced inflammatory reactions. Differences in the dose-response relation between ODC induction and the inflammatory reaction may at least in part reflect the participation of multiple cellular systems in inflammatory reactions induced by TPA.

It has been well-established that TPA stimulates phospholipase A2 and as a consequence, augments the release of arachidonic acid and prostaglandins (29, 30). The effect of indomethacin on TPA-induced inflammation is controversial. It has been reported that indomethacin weakly inhibits (6, 7, 10) or fails to inhibit (31) TPA- or croton oil-induced inflammatory responses. In our present study, indomethacin tended to inhibit TPA-induced increases in vascular permeability, but the inhibition was not statistically significant. Although the inhibitory action of indomethacin is not so potent, we cannot deny the possible involvement of prostaglandins in TPA-induced inflammatory responses. Moreover, since higher doses of indomethacin have been reported to inhibit phospholipase A2 (32) and leukocyte migration (33), other mechanisms, excepting cyclooxygenase inhibition, may also be involved in the inhibitory effect of indomethacin on TPA-induced inflammatory responses.

Since lipoxygenase activities are also detected in mouse skin (14, 15, 34), part of the arachidonic acid released after TPA treatment is oxidized through the lipoxygenase pathway as well as the cyclooxygenase pathway. The present results show that the lipoxygenase inhibitors, i.e., NDGA, AA861 and quercetin, markedly inhibit the TPA-induced augmentation of vascular permeability and that AA861 also inhibits TPA-induced neutrophil infiltration. Therefore, these results suggest the possible involvement of a lipoxygenase product(s) in the mechanism of these TPA actions in mouse skin. However, quercetin is known to inhibit various enzymes and cell functions (35) including histamine release (36), calmodulin function (37) and protein kinase C (38), and NDGA has an anti-oxidant's character. Therefore, we cannot exclude the possibility that the inhibitory effect of the present inhibitors on TPA-induced increase in vascular permeability in mouse skin is due to the other inhibitory actions except lipoxygenase inhibition. However, the common action of the three different inhibitors which we used in the present study is the potent inhibition of lipoxygenase, and these lipoxygenase inhibitors actually inhibited epidermal lipoxygenase without inhibiting cyclooxygenase. Therefore, it seems highly likely that a lipoxygenase product(s) is involved in the mechanism of TPA-induced inflammatory reactions in mouse skin. Very recently, Young et al. (10) have also shown that NDGA and phenidone partially inhibited TPA-induced ear edema.

It is generally believed that lipoxygenase products participate in various inflammatory responses (16). Various hydroxyeicosatetraenoic acids (HETEs), hydroperoxyeicosatetraenoic acids (HPETEs) and leukotriene B4 are chemotactic and increase leukocyte infiltration (39), and some leukotrienes cause an increase in vascular permeability (4, 40). It was also reported that 12-HETE or 12-HPETE, the main lipoxygenase product of mouse epidermis, stimulates leukotriene biosynthesis by activating the 5-lipoxygenase of the leukocyte (41) and also acts as a potent chemotactic agent for leukocytes (42). Although such a causal relationship is not established by our present study, it is plausible that the production of 12-HETE or 12-HPETE by TPA in epidermis could initiate the chain of inflammatory responses which may then be amplified by some lipoxygenase products or other chemical mediators produced in inner skin tissues or blood cells. Alternatively, it is also possible that a direct interaction of TPA with cells other than epidermal cells is the major initial event of TPA-induced inflammatory responses. Further investigation is now in progress.

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