The Mechanism of Skin Tumor Promotion Caused by Phorbol Esters: Possible Involvement of Arachidonic Acid Cascade/Lipoxygenase, Protein Kinase C and Calcium/Calmodulin Systems

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Accepted September 19, 1988

Abstract—12-O-Tetradecanoylphorbol-13-acetate (TPA) has been used as a potent tumor promoter in mouse skin. The mechanisms of TPA actions were studied by using several types of inhibitors. TPA-caused responses in mouse skin such as skin tumor promotion, epidermal ornithine decarboxylase (ODC) induction and skin inflammation were inhibited by various lipoxygenase inhibitors of the arachidonic acid cascade. Lipoxygenase inhibitors also inhibited TPA-caused ODC induction in isolated epidermal cells or cultured epidermal cells. Therefore, it is possible that these drugs inhibit TPA-caused ODC induction in mouse skin by directly acting on epidermal cells. TPA actions were also inhibited by either protein kinase C inhibitors, calmodulin antagonists or calcium blockers. These results suggest that arachidonic acid/lipoxygenase, protein kinase C and calcium-calmodulin systems play essential roles in the mechanism of tumor promotion by TPA.

The mechanism of carcinogenesis involves very complex processes that are induced by a variety of stimulants such as chemicals, viruses and radiation. However, this mechanism is still not completely understood. Chemical carcinogenesis can be subdivided at least into two stages, initiation and promotion, in a skin carcinogenesis model. In the initiation stage, a carcinogen is bound to DNA and forms a carcinogen-DNA adduct, leading to an alteration in the genome. In the promotion stage, repeated exposure to a promoter induces tumors by the formation of phenotypic expression of the initiated cells. The initiation stage is irreversible, whereas the promotion stage is essentially reversible. 12-O-Tetradecanoylphorbol-13-acetate (TPA) is well-known as a potent tumor promoter and frequently used experimentally for mouse skin carcinogenesis. In the present communication, the author has summarized and introduced the studies on the mechanism of TPA-caused tumor promotion in mouse skin using several types of inhibitors which might affect the arachidonic acid cascade, protein kinase C and calcium/calmodulin systems.

1. Tumor promotion and arachidonic acid cascade

Drugs inhibiting enzymes of the arachidonic acid cascade in epidermis: In skin, the epidermis is the main target for the various initiators and promoters. Arachidonic acid is released from the skin by a variety of stimuli including phorbol ester tumor promoters (1). The microsomal fraction from mouse epidermis which contains cyclooxygenase activity converts [14C]arachidonic acid to prostaglandins (PG) (2–7), and the main product is PGE₂ (3–5). Lipoxygenase activity has also been detected in the epidermal homogenates.
from humans (2), guinea pigs (8) and mice (2, 6) or in cultured neonatal mouse keratinocytes (9), and the main product is 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (7). Epidermal lipoxygenase and cyclooxygenase activities can be determined by the formation of $[^{14}C]12$-HETE and $[^{14}C]PGE_2$ from $[^{14}C]$arachidonic acid, respectively. Effects of various inhibitors on the lipoxygenase and cyclooxygenase activities in mouse epidermis have been reported (4, 5). Nordihydroguaiaretic acid (NDGA) and 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861) inhibit epidermal lipoxygenase, but do not inhibit epidermal cyclooxygenase activity. Indomethacin fails to inhibit lipoxygenase activity, but potently inhibits cyclooxygenase activity. Tetra- or pentahydroxyflavones, i.e., quercetin, morin, fisetin and kaempferol, potently inhibit mouse epidermal 12-lipoxygenase (10, 11). The potencies of these flavonoids are similar to or greater than those of well-known lipoxygenase inhibitors such as NDGA and phenidone. (+)Catechin and (−)epicatechin, which are structurally related to quercetin but have a dihydrobenzopyran structure instead of the 1,4-benzopyrone structure of flavonoids, are not able to inhibit epidermal lipoxygenase. Esculetin, which contains 1,2-benzopyrone and catechol structures, also inhibits mouse epidermal lipoxygenase, but its potency is less than that of the above-mentioned flavonoids (11). n-Propyl gallate, an antioxidant, is also a potent lipoxygenase inhibitor in mouse epidermis (11). Another widely used phenolic antioxidant, butylated hydroxyanisole (BHA), also inhibits epidermal lipoxygenase activity moderately. Some of the chalcone derivatives, which are opened-chain analogs of flavone and are involved in the biosynthesis of flavonoids, also inhibit lipoxygenase and cyclooxygenase activities in mouse epidermis at relatively low concentrations (3). A structure-activity relationship of chalcone derivatives for the inhibition of lipoxygenase and cyclooxygenase has been reported (3). Among these chalcone derivatives, 3,4,2′,4′-tetrahydroxychalcone is considered to be a potent and relatively specific lipoxygenase inhibitor in mouse epidermis (3). These inhibitors of arachidonate metabolism have been used for understanding the role of the arachidonic acid cascade in skin carcinogenesis.

**Skin tumor promotion:** The application of TPA to mouse skin or certain cells leads to a number of biochemical alterations and changes in cellular functions and skin tumor promotion. Effects of various inhibitors of the arachidonic acid cascade on the TPA-induced skin tumor promotion have been examined. p-Bromophenacylbromide (BPB), a phospholipase A2 inhibitor, inhibits TPA-induced skin tumor promotion in CD-1 mice (12) and SENCAR mice (13). The effect of indomethacin on TPA-induced tumor promotion is controversial. In CD-1 mice, Verma et al. (14) reported that indomethacin suppresses tumor promotion, but we (T. Nakadate et al., unpublished data) and Viaje et al. (15) demonstrated that indomethacin has only a slight inhibitory effect on tumor promotion. In NMRI mice, tumor promotion is inhibited by indomethacin (16, 17), and the inhibition can be reversed by PGE$_2$α (16, 17). However, Fischer et al. (18) reported that indomethacin rather enhances TPA-induced tumor promotion in SENCAR mice. Although the reason for the different responses to indomethacin in different strains of mice is unknown, Fischer et al. (16) suggested that the difference between NMRI and SENCAR mice appears to lie in the degree of involvement of the lipoxygenase pathway of the arachidonic acid cascade in skin tumor promotion. The lipoxygenase pathway appears to be more important in SENCAR mice, and the cyclooxygenase pathway is essential in NMRI mice (16).

We (12) have found that a lipoxygenase inhibitor, NDGA, also suppresses the tumor promotion by TPA. TPA-induced skin tumor promotion is inhibited by certain flavonoids and related compounds (11). Quercetin (10), morin (11) and 3,4,2′,4′-tetrahydroxychalcone (19) are potent inhibitors of skin tumor promotion. 3,4,2′,4′-Tetrahydroxychalcone (3), quercetin (10) and morin (11) have been revealed to be potent lipoxygenase inhibitors. Inhibitory effects of these compounds on tumor promotion are roughly parallel with their potency of lipoxygenase inhibition (10, 11, 19). A new lipoxygenase inhibitor, AA861, also potently inhibits TPA-induced skin tumor promotion.
promotion (19). These results suggest that a lipoxygenase product(s) plays an important role in TPA-induced skin tumor promotion. In SENCAR mice, Fischer et al. (13) also showed the inhibitory effects of lipoxygenase inhibitors on skin tumor promotion. Phenidone and 5,8,11,14-eicosatetraenoic acid (ETYA) inhibit the TPA-induced tumor promotion (13), while indomethacin enhances it (18). In NMRI mice, ETYA also inhibits tumor promotion (20). These findings also suggest the role of lipoxygenase product(s) in skin tumor promotion by TPA.

Epidermal ODC: Induction of epidermal ODC is one of the typical and prominent biochemical alterations elicited by TPA, and it has been thought to be a representative biochemical parameter of potent tumor promoters. Verma et al. (14) reported that pretreatment of CD-1 mouse skin with cyclooxygenase inhibitors such as indomethacin, naproxen, flufenamic acid or acetylsalicylic acid inhibits ODC induction by TPA. Inhibition of TPA-caused ODC induction by indomethacin is completely overcome by concurrent application of PGE2 with TPA. In contrast, PGF2α or arachidonic acid are ineffective. Therefore, PGE2 may play a crucial role in TPA-induced ODC activity, although PGE2 alone cannot induce ODC activity (14).

Treatment of CD-1 mice with phospholipase A2 inhibitors such as mepacrine, BPB and tetracaine inhibit the induction of ODC by TPA (21). The inhibition of TPA-caused ODC induction by mepacrine and BPB is not reversed by PGE2 (21). Inhibition either by BPB or mepacrine, however, is partially overcome by arachidonic acid (21). Arachidonic acid alone does not induce epidermal ODC activity (21). The results indicate that not only the cyclooxygenase pathway but also the lipoxygenase pathway of arachidonic acid are involved in the mechanism of epidermal ODC induction by TPA in CD-1 mice. Moreover, our findings (21, 22) clearly showed that TPA-caused ODC induction is inhibited by treatment of mice with well-known lipoxygenase inhibitors, i.e., NDGA, phenidone and 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline (BW755C). A potent lipoxygenase inhibitor, quercetin, also inhibits TPA-caused ODC induction (10). Morin, fisetin, kaempferol and n-propyl gallate markedly inhibit TPA-caused epidermal ODC induction (11). Effects of other flavonoids, antioxidants and related compounds, such as (+)-catechin, (-)-epicatechin, esculetin and BHA, on TPA-caused ODC induction have also been examined (11). The order of the potency of these compounds is closely related to the potency of lipoxygenase inhibition. These findings support the above contention that the lipoxygenase pathway is involved in the TPA-caused ODC induction.

It has been shown that protein kinase C is the receptor for the phorbol ester tumor promoters, and this enzyme is activated by TPA (23-25). Therefore, it is highly possible that the activation of protein kinase C is involved in the mechanisms of TPA-caused ODC induction. Recently, Gschwendt et al. (26) showed the inhibitory effect of quercetin on protein kinase C. In addition, several flavonoids, such as morin and kaempferol, also have an inhibitory effect on protein kinase C as determined by the histone H-1 phosphorylation (T. Nakadate et al., unpublished data). NDGA also inhibits protein kinase C activity (26 and T. Nakadate et al., unpublished data). However, the inhibitory effects of these compounds on protein kinase C are far less potent than the inhibitory effects on lipoxygenase. Other lipoxygenase inhibitors such as phenidone and BW755C have no inhibitory effects on protein kinase C (T. Nakadate et al., unpublished data). The recently introduced lipoxygenase inhibitors AA861 and 3,4,2',4'-tetrahydroxychalcone also have no inhibitory effect on protein kinase C (19). They are considered to be specific lipoxygenase inhibitors in the epidermis because they hardly inhibit cyclooxygenase activity in the epidermis (19). These lipoxygenase inhibitors, i.e., AA861 and 3,4,2',4'-tetrahydroxychalcone, potently inhibit TPA-caused tumor promotion and epidermal ODC induction (19). These findings further support the contention that lipoxygenase products play an important role in the mechanism of TPA actions.

In freshly isolated epidermal cells from newborn SENCAR or CD-1 mice, TPA causes ODC induction (27, 28). However, in isolated epidermal cells, indomethacin unexpectedly fails to inhibit TPA-caused ODC induction.
On the other hand, lipoxygenase inhibitors, i.e., NDGA, BW755C, phenidone, quercetin and AA861, inhibit TPA-caused ODC induction in isolated epidermal cells (28). Therefore, it is possible that lipoxygenase inhibitors inhibit TPA-caused ODC induction in mouse skin at least partly by directly acting on epidermal cells, while a cyclooxygenase inhibitor inhibits it indirectly by acting on cells other than epidermal cells.

**DNA synthesis:** Topical application of TPA stimulates epidermal DNA synthesis. Although epidermal hyperplasia is a necessary but not a sufficient condition for tumor promotion, the stimulation of DNA synthesis by TPA is one of the indexes for the hyperproliferative action of TPA and is obligatory for TPA-induced tumor promotion. DNA synthesis is generally measured by the [3H]thymidine incorporation into DNA. Furstenberger et al. (29, 30) reported that indomethacin inhibits the TPA-induced DNA synthesis of NMRI mice epidermis and that PGE₂ restores the DNA synthesis inhibited by indomethacin. However, indomethacin fails to inhibit TPA-induced DNA synthesis (20) in cultured mouse epidermal cells as well as in murine epidermal cell line HEL-30 (20). Fischer (31, 32) showed that DNA synthesis is enhanced by indomethacin both in the primary culture of newborn mice and in the adult mouse skin epidermis of SENCAR mice. In CD-1 mice indomethacin neither inhibits nor stimulates TPA-induced DNA synthesis (14).

At present, the effects of lipoxygenase inhibitors on TPA-induced DNA synthesis are complicated and controversial. The inhibitory effect of ETYA and phenidone on DNA synthesis has been reported (16, 31, 32). However, Furstenberger and Marks (20) and we (10, 11) have reported that lipoxygenase inhibitors such as ETYA (20), quercetin (10), NDGA (10) and morin (11), at doses which actually inhibit TPA-caused ODC induction and tumor promotion, cannot inhibit TPA-induced epidermal DNA synthesis in mouse skin.

**Skin inflammation:** Topical application of TPA induces skin inflammatory reactions as well as skin tumor promotion. The essential role of inflammation in tumor promotion has been investigated but still remains unclear, because not all irritants are promoters, while most promoters appear to be irritants that induce inflammation (24). Painting of the dorsal skin of CD-1 mice with TPA induces a dose-related increase in vascular permeability (4), which is determined by pontamine sky blue exudation into the skin. Indomethacin tends to inhibit the TPA-induced dye exudation, but the inhibition is not statistically significant (4). It has been also reported that indomethacin weakly inhibits (4, 33) or fails to inhibit (15, 30) TPA- or croton oil-induced inflammatory responses. On the other hand, treatment of mouse skin with NDGA, AA861 and quercetin inhibits the TPA-induced dye exudation (4). AA861 also inhibits the TPA-induced neutrophil infiltration (4). Gschwendt et al. (34) reported that lipoxygenase inhibitors such as ETYA, NDGA, esculetin and quercetin inhibit TPA-induced ear edema in NMRI mice. Young et al. (33) have also shown that NDGA and phenidone partially inhibit TPA-induced ear edema in Swiss Webster mice. These findings suggest the involvement of a lipoxygenase product(s) in the TPA-induced inflammatory reactions.

### 2. Tumor promotion and protein kinase C

**Possible involvement of protein kinase C in tumor promotion:** It has been established that protein kinase C is a receptor for phorbol ester tumor promoters (23–25). Diacylglycerol (DG), which is generated from phospholipids by phospholipase C activation, has been thought to be an endogenous activator of protein kinase C (23). It has been shown that DG mimics TPA action in many systems, although DG does not always mimic TPA actions (35). Treatment of epidermal cells or mouse skin with phospholipase C or DG results in ODC induction (27, 36–38). In addition, very recently, DG has been shown to be a stage II tumor promoter (39). These findings suggest the possible involvement of protein kinase C activation in the mechanism of skin tumor promotion by phorbol esters.

Bryostatin 1, like TPA, activates protein kinase C. However, bryostatin 1 induces only some of the effects in cultured epidermal cells that result from phorbol ester treatment, whereas it blocks other responses to the phorbol esters (40). In mouse skin in vivo, Hennings et al. (41) reported that bryostatin 1
was ineffective as a complete tumor promoter and displayed very weak activity as a second stage promoter. In addition, they showed that bryostatin 1 inhibited TPA-induced tumor promotion (41). These findings also support the idea that all classes of protein kinase C activators are not functionally equivalent. The role of protein kinase C activation in skin tumor promotion should be further elucidated.

Effects of protein kinase C inhibitors: Palmitoylcarnitine has been reported to be an inhibitor of protein kinase C (42). We have reported the inhibitory effect of palmitoylcarnitine on TPA-caused epidermal ODC induction (43), skin tumor promotion (43), TPA-caused refractoriness for epidermal ODC induction (44) and TPA-induced differentiation of HL60 cells (45). However, palmitoylcarnitine fails to inhibit protein kinase C activity which is activated by TPA, although the drug can inhibit the enzyme activated by calcium and phosphatidylinerine but in the absence of TPA (43). These findings are further confirmed using a highly purified protein kinase C (46). Therefore, it seems unlikely that the potent anti-tumor-promoting action of palmitoylcarnitine is due solely to its inhibitory effect on protein kinase C (43).

Staurosporine has been reported to be the most potent inhibitor of protein kinase C acting in the nM range (47). Staurosporine inhibits TPA-caused tumor promotion but fails to inhibit or rather enhances TPA-caused epidermal ODC induction (48). Staurosporine also fails to suppress TPA-induced mouse ear edema (48). In isolated epidermal cells, staurosporine by itself causes ODC induction and enhances TPA-induced ODC activity (49). Since staurosporine inhibits the TPA-induced 34K Da protein phosphorylation in

Table 1. Effects of inhibitors of arachidonic acid cascade, protein kinase C or calcium-calmodulin systems on TPA-caused mouse skin tumor promotion

| Inhibitors                             | TPA-caused skin tumor promotion | Strain of mouse | Ref.       |
|----------------------------------------|---------------------------------|-----------------|------------|
| (Phospholipase A₂)                     |                                 |                 |            |
| BPB                                    | Inhibition                      | CD-1, SENCAR    | 12, 13     |
| (Cyclooxygenase)                       |                                 |                 |            |
| Indomethacin                           | Inhibition or slight inhibition  | NMRI, CD-1     | 14, 16, 17|
|                                        | Enhancement                     | SENCAR          | 18         |
| (Lipoxygenase)                         |                                 |                 |            |
| NDGA                                   | Inhibition                      | CD-1            | 12         |
| Quercetin                              | Inhibition                      | CD-1            | 10         |
| Morin                                  | Inhibition                      | CD-1            | 11         |
| Esculetin                              | Slight inhibition               | CD-1            | 11         |
| AA861                                  | Inhibition                      | CD-1            | 19         |
| 3,4,2',4'-Tetrahydroxychalcone         | Inhibition                      | CD-1            | 19         |
| (Lipoxygenase and cyclooxygenase)      |                                 |                 |            |
| E TY A                                 | Inhibition                      | SENCAR, NMRI    | 13, 20     |
| Phenidone                              | Inhibition                      | SENCAR          | 13         |
|                                        | Inactive or slight enhancement  | CD-1            | 12         |
| (Protein kinase C)                     |                                 |                 |            |
| Palmitoylcarnitine                     | Inhibition                      | CD-1            | 43         |
| Staurosporine                          | Inhibition                      | CD-1            | 48         |
| (Calcium/calmodulin)                   |                                 |                 |            |
| W-7                                    | Inhibition                      | CD-1            | 51         |

*Effects of these drugs on skin tumor promotion cannot be explained solely by its protein kinase C inhibition.*
intact epidermal cells (48), it is plausible that protein kinase C is actually inhibited by staurosporine in epidermal cells. Differential inhibition by staurosporine of protein kinase C isozymes and/or substrate protein-dependent inhibition by staurosporine may explain the above controversial results. Alternatively, staurosporine may exert its effect not only through protein kinase C but also through protein kinase C-independent mechanisms.

A protein kinase C inhibitor, 1-(5-quinolinylsulfonyl)-2-methylpiperazine (H-7), inhibits TPA-caused ODC induction in vivo (T. Nakadate et al., unpublished data) and in vitro (49). H-7 also inhibits DG-caused ODC induction in isolated epidermal cells (49). These findings support the involvement of protein kinase C activation for TPA actions in skin tumor promotion.

3. Role of Ca\(^{2+}\) and calmodulin system in tumor promotion

The role of the Ca\(^{2+}\)-calmodulin system in TPA action has also been suggested. Verma et al. (50) showed that TPA-caused ODC induction is dependent on the extracellular Ca\(^{2+}\) using adult mouse skin pieces. We obtained similar results using the isolated epidermal cells (K. Ishii et al., unpublished data). The calmodulin antagonist trifluoperazine inhibits TPA-caused ODC induction (50). A more specific calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) also inhibits TPA-caused ODC induction (51). In addition, W-7 suppresses TPA- or teleocidin-induced tumor promotion in mouse skin (51, 52). A calcium channel blocker, verapamil, also inhibits ODC induction by TPA in isolated epidermal cells (K. Ishii et al., unpublished data). These findings suggest that the calcium-calmodulin mediating process is also possibly involved in the mechanisms of TPA actions.

4. Conclusion

Table 1 shows the effects of various inhibitors on TPA-caused mouse skin tumor promotion. At present, the roles of the arachidonic acid cascade, protein kinase C, and calcium/calmodulin systems in the mechanism of tumor promotion caused by TPA are not fully understood. However, various inhibitors of these systems can modulate tumor responses, suggesting the possible involvement of these systems in the mechanism of tumor promotion (Fig. 1). The pharmacological methods shown in this communication are not only powerful tools to elucidate the
mechanism of carcinogenesis but also have potential use for finding drugs for the prevention of carcinogenesis.

Acknowledgments: The author is most grateful to Professor Ryuichi Kato (Keio University) for pertinent suggestions and excellent support. I also wish to thank Dr. Satoshi Yamamoto and all collaborators at the Keio University School of Medicine for their kind cooperation. These studies were supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

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