Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 9-15

INHIBITION OF PHORBOL ESTER-CAUSED INDUCTION OF ORNITHINE DECARBOXYLASE AND TUMOR PROMOTION IN MOUSE SKIN BY STAUROSPORINE, A POTENT INHIBITOR OF PROTEIN KINASE C*

Shuhei Yamada, Kenji Hirota, Kazuhiro Chida, and Toshio Kuroki[1]

Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

Received September 30, 1988

Summary We found that staurosporine, a potent inhibitor of protein kinase C, inhibits induction of ornithine decarboxylase (ODC) and tumor promotion caused by 12-O-tetradecanoylphorbol-13-acetate (TPA) in CD-1 mouse skin. When applied 5 min either before or after treatment with TPA, 1 μg of staurosporine caused about 56% inhibition of ODC-induction by 5 μg of TPA. However, staurosporine did not inhibit TPA-induced epidermal hyperplasia. In two-stage carcinogenesis, staurosporine at 1 μg was applied 5 min before application of 5 μg of TPA to the initiated skin: number of tumors was suppresed by about 40% although the incidence was not affected. No tumors developed when staurosporine alone was applied to the initiated skin.

Nishizuka and his colleagues have demonstrated in a series of the studies that protein kinase C (PKC) has a crucial role in signal transduction on cell membrane (1). Phorbol ester tumor promoters, e.g. 12-O-tetradecanoylphorbol-13-acetate (TPA) bind to and activate PKC, by which pleiotropic actions of phorbol esters on cell growth and differentiation are now interpreted. The hypothesis that PKC is causally involved in tumor promotion is largely based on this ability of phorbol ester tumor promoters. Up to the present time, however, no direct evidence for this hypothesis has been presented.

We have been investigating possible roles of PKC in tumor promotion by the use of tumor promotion systems in tissue culture and in mice. Previously we demonstrated that TPA activates PKC by its intracellular translocation from cytosol to membrane in mouse skin *in vivo* and also in BALB/3T3, C3H10T1/2 and FRSK cells in which two stage transformation occurs

*Supported in part by a Grant-in-Aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science and Culture of Japan.

[1]To whom all correspondence should be addressed.

Abbreviations: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[a]anthracene.

0006-291X/88 $1.50

(2-4). Target proteins which are specifically phosphorylated by PKC were identified in mouse epidermis in vivo and in BALB/3T3 and C3H10T1/2 cells in culture, but their significance in tumor promotion is not clear (2, 3). The mechanism and significance of down regulation of PKC were studied using FRSK cells and human epidermal keratinocytes in primary culture (4, 5). Furthermore, we screened 41 cell lines for their PKC activities and phorbol ester binding: in general, activities of PKC were high in normal or untransformed cells and low in malignant or transformed cells (accompanied paper).

In the present study, we used staurosporine, a potent inhibitor of PKC (6), to investigate the possible involvement of PKC in tumor promotion. We chose staurosporine among known inhibitors of PKC because of its potent and specific inhibitory effect on PKC and because of its solubility in acetone which facilitates topical application to mouse skin. We found that this inhibitor inhibited induction of ornithine decarboxylase (ODC) and tumor promotion caused by TPA in mouse skin.

Materials and Methods

Animals. Seven to 9-week old female CD-1 mice obtained from Charles River Co. (Atsugi, Japan) were used. The dorsal skin was shaved with electric clippers and treated with a depilatory agent (Kowa Co., Ltd., Nagoya, Japan) 2 to 3 days before commencement of experiments. Depilation itself affected neither epidermal ODC activity nor hyperplasia induced by TPA. The test chemical in 200 µl of acetone was applied to the depilated area.

Chemicals. TPA was purchased from Consolidated Midland Co. (Brewster, NY). Staurosporine was obtained from Kyowa Medex Co., Ltd., (Tokyo, Japan). Both agents were dissolved in acetone.

Assay of ODC activity. The activity of ODC was determined by the method reported elsewhere (5). In brief, the epidermis was scraped off with a razor blade that had been heated at 55°C for 30 sec. The epidermal preparation was sonicated at 0°C in 0.5 ml of extraction solution consisting of 50 mM sodium phosphate (pH 7.2), 0.1 mM pyridoxial phosphate and 0.1 mM EDTA and was then centrifuged at 30,000 x g for 20 min. The supernatant fraction was used as a source of the enzyme. The activity of ODC was determined by measuring release of $^{14}CO_2$ in a reaction mixture consisting of 50 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 0.5 mM dithiothreitol, 0.05 mM EDTA, 0.1 µCi DL-[1-^{14}C]ornithine, 0.1 mM L-ornithine and 100 µl of enzyme solution in a final volume of 1 ml. Enzyme activity was expressed as nmol CO_2 liberated in 1 h per mg protein. The protein content of the enzyme solution was determined by the method of Bradford (7).

Carcinogenesis experiments. Groups of 20 mice were used. As an initiator, 7,12-dimethylbenz[a]anthracene (DMBA) at a dose of 25 µg was applied to the dorsal skin 1 week before promotion. TPA at a dose of 5 µg was applied to the same area of the skin once a week for 20 weeks. Staurosporine at a dose of 1 µg was applied alone or 5 min before the application of TPA, once a week for 20 weeks. Animals were observed for a further 10 weeks after termination of promotion. Numbers of tumors were recorded once a week. At 30 weeks tumors were removed for histological examination.

Results

Inhibition of TPA-caused induction of ODC by staurosporine. As shown in Fig. 1, topical application of TPA at 5 µg to the dorsal skin of mice caused

a rapid and transient increase in epidermal ODC activity with the activity reaching a peak (0.99 nmol CO_2/h/mg protein) at 4 h and then rapidly decreasing. Staurosporine at a dose of 1 µg did not induce ODC activity under these conditions. When 1 µg of staurosporine was applied 5 min before treatment with 5 µg of TPA, a decrease in ODC activity was observed. Inhibition by staurosporine of ODC induction at 4 h was 56.2 ± 12.1% (mean ± S.D.) in 8 independent experiments. The time-course of ODC-induction by TPA was not changed by pretreatment with staurosporine.

Staurosporine inhibited ODC-induction by TPA in a dose-dependent manner with an estimated dose of 0.79 µg required for 50% inhibition (data not included).

We found that time of application was critical for staurosporine to exert its inhibitory effect on ODC-induction. As seen in Fig. 2, inhibition was observed only when staurosporine was applied 5 min, either before or

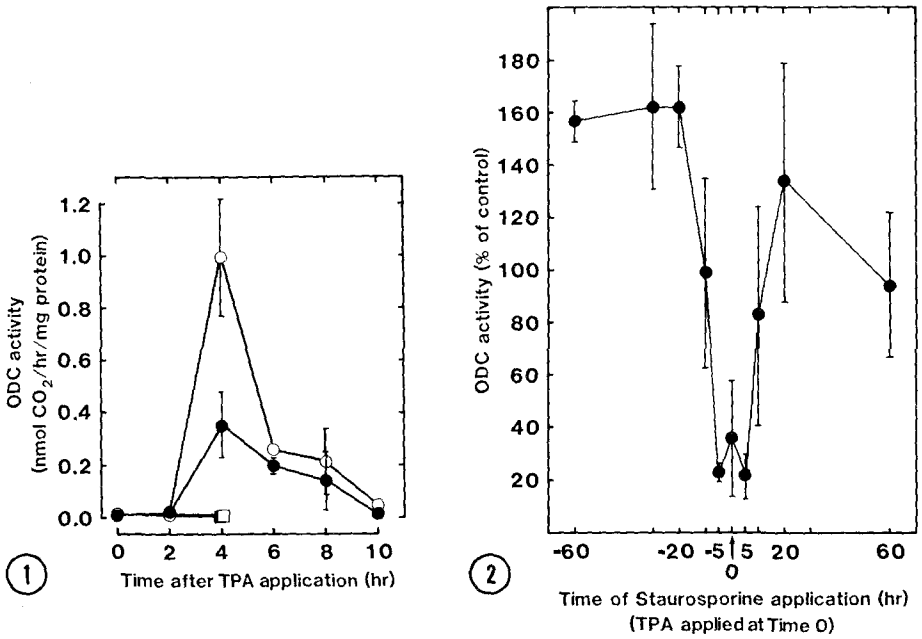

Fig. 1. Time course of induction of epidermal ODC by TPA in mouse skin with and without treatment with staurosporine. Mice (female CD-1) were treated topically with 1 µg of staurosporine followed by 5 µg of TPA administered 5 min later. Acetone was used as a solvent control. ○, TPA alone; ●, staurosporine-TPA; ■, staurosporine alone; □, acetone. Values are means of duplicate measurements; bars, SD: the absence of a bar indicates that the SD is within the symbol.

Fig. 2. Effect of time of application of staurosporine on ODC-induction by TPA. Mice were treated with 1 µg of staurosporine at the time indicated, either before or after application of 5 µg of TPA, and were killed 4 h after TPA-treatment. ODC activity is expressed as in Fig. 2. Values are means of 3 measurements. bars, SD.

after, treatment with TPA. At 10 min, either before or after, treatment with TPA, staurosporine did not inhibit ODC-induction. Moreover, when applied 30 - 60 min before TPA application, staurosporine stimulated ODC induction by about 60%. Thus, depending on time of application, staurosporine either stimulated or inhibited ODC-induction by TPA.

The possibility that staurosporine inhibits by direct interaction with the ODC molecule, with its substrate (i.e. ornithine) or with co-factors, was examined by adding staurosporine to the reaction mixture of the ODC assay. In no instance inhibition was observed.

Absence of inhibitory effect on TPA-induced epidermal hyperplasia. Most tumor promoters in mouse skin carcinogenesis induce epidermal hyperplasia on day 2 - 3. We examined histologically the effect of staurosporine on TPA-caused epidermal hyperplasia. When 1 μg of staurosporine was applied 5 min before TPA (5 μg), no inhibition of epidermal hyperplasia was observed. The skin treated with staurosporine alone kept the normal thickness of the skin. This observation is in good agreement with that obtained for other anti-tumor promoters such as retinoic acid and 1α,25-dihydroxyvitamin D_3 which inhibit induction of ODC but not epidermal hyperplasia caused by phorbol esters (8).

Inhibition of tumor promotion in two-stage carcinogenesis of mice. The above observations suggest an anti-promoting effect of staurosporine in two-stage carcinogenesis of mouse skin. We examined this possibility by applying staurosporine (1 μg) 5 min before application of TPA (5 μg) to DMBA(25 μg)-initiated skin of CD-1 female mice. Results are summarized in Table 1 and Fig. 3. Tumors developed in 90% of mice after 15-weeks of promotion irrespective of whether treated with TPA alone or with TPA plus staurosporine. Numbers of tumors, however, were significantly less in the group treated with TPA plus staurosporine. At 20 weeks of promotion,

Table 1. Inhibition of tumor promotion by staurosporine in two-stage carcinogenesis of mouse skin

Group	Promotion	Survival (%)		Incidence[a]		Tumors/mouse	
		20 wk	30 wk	20 wk	30 wk	20 wk	30 wk
1	Acetone	100	95	0	0	0	0
2	TPA	95	80	89.5	87.5	20.3	17.8
3	TPA + Staurosporine	100	95	90.0	89.5	12.1	10.2
4	Staurosporine	100	95	0	0	0	0

Twenty mice (female CD-1 mice) were used for each group. All mice were initiated with 25 μg of DMBA and, from 1 week later, were promoted with 5 μg of TPA once a week for 20 weeks. Staurosporine at a dose of 1 μg was applied alone (Group 4) or 5 min before TPA application (Group 3), once a week for 20 weeks. Acetone was applied as a solvent control (Group 1).

[a] % of mice bearing tumors among surviving mice.

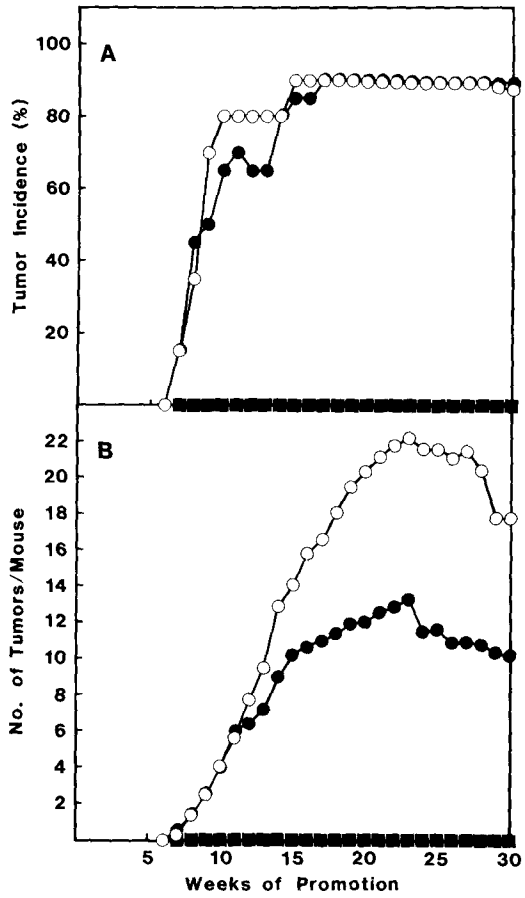

Fig. 3. Tumor formation (A, incidence; B, numbers of tumors per mouse) in mice promoted with TPA alone (o), TPA plus staurosporine (●) or staurosporine alone (■). Mice were initiated with 25 µg of DMBA and from 1 week later, promoted with 5 µg of TPA, once a week for 20 weeks. Staurosporine was applied alone or 5 min before TPA application.

staurosporine-treated mice had about 12 tumors/mouse which is about 40% of the number of tumors per mouse treated with TPA alone. A similar difference in the number of tumors per mouse was also noted at 30 weeks, i.e. 10 weeks after termination of tumor promotion. Histologically, all except one of the 299 tumors were papillomas. The exception was a squamous cell carcinoma.

No tumors developed when staurosporine alone was applied to DMBA-initiated skin, indicating that staurosporine itself has no tumor promoting activity under these conditions.

Discussion

Staurosporine, an alkaloid isolated from *Streptomyces SP.* (9, 10), is a potent and specific inhibitor of PKC (6). It inhibits PKC with an inhibition constant (Ki) of 0.7 nM, while Ki values for cAMP- and

cGMP-dependent kinases are 7.0 and 8.5 nM, respectively. The 50% inhibition concentration (IC50) of staurosporine for PKC isolated from rat brain has been reported to be 2.7 nM, while micromolar concentrations of other known inhibitors of PKC are required (6). A similar IC50 value of staurosporine has been reported for PKC of the particulate fraction of epidermis of mice (11). The inhibition of PKC by staurosporine is not competitive with phosphatidylserine or diacylglycerol, suggesting direct interaction of staurosporine with the PKC molecule (6). Nakadate et al. (12) recently demonstrated that staurosporine inhibits the catalytic fragment of the PKC molecule while having no effect on binding of a phorbol ester, suggesting the absence of interaction of it with the regulatory fragment. More recently, Wolf and Baggiolini (13) reported that staurosporine, like phorbol esters, induces association of purified PKC with inside-out vesicles from erythrocyte membranes, but kinase activity of PKC is inhibited under these circumstances. These results suggest that the site of action of phorbol esters and staurosporine is not the same.

We have recently reported that staurosporine inhibits the phosphorylation of target proteins of PKC in mouse skin (3). In this paper, we found that staurosporine inhibits the induction of ODC and tumor promotion by TPA in mouse skin. Our observations, however, do not seem to be consistent with those of others. Kiyoto et al. (11) found that staurosporine not only failed to inhibit, but markedly augmented, TPA-caused ODC induction in isolated mouse epidermal cells in vitro. Yoshizawa et al. (14, 15) reported dual effects of staurosporine on two-stage mouse skin carcinogenesis. According to them, treatment with 10 μg of staurosporine 15 min before each application of 2.5 μg teleocidin B, administered twice weekly, slightly inhibited the promoting activity of teleocidin B while treatment with staurosporine alone at 50 μg resulted in the development of skin tumors in DMBA-initiated mice. Furthermore, application of 200 μg of staurosporine alone caused irritation of mouse ear and induced histidine decarboxylase activity in mouse skin. These authors also reported that staurosporine inhibits induction of adhesion of human promyelocytic leukamia cells and of EB-virus early antigen in Raji cells by teleocidin B.

The discrepancy between these observations and ours may be due to differences in experimental procedure, e.g., time of application of staurosporine (5 min before the TPA application in the present study versus 15 min for Yoshizawa et al. (14)), dose of staurosporine (1 μg in the present study versus 10 or 50 μg for Yoshizawa et al. (14, 15)), tumor promoters used (TPA in the present study versus teleocidin B for Yoshizawa et al. (14)), doses of DMBA used for initiating carcinogenesis (25 μg in the present study versus 100 μg for Yoshizawa et al. (14, 15)) and the

experimental system employed for ODC-induction (mouse skin *in vivo* in the present study *versus* cell suspension *in vitro* for Kiyoto *et al.* (11)).

We found in this study that the time of application of staurosporine is very critical for exerting its inhibitory effect on the ODC-induction by TPA. Inhibition was observed only when staurosporine was applied 5 min either before or after application of TPA, and that when it was applied 20-60 min before application of TPA, stimulation, rather than inhibition of ODC-induction by TPA was observed. A similar critical time-course has been reported with W-7, a calmodulin antagonist which showed reduction of phorbol ester binding sites in mouse skin only within 5 min after TPA-treatment (16). Reasons for such a critical timing of application are not clear.

The present study demonstrates that an inhibitor of PKC inhibits inductin of ODC and tumor promotion by TPA in two-stage carcinogenesis in mouse skin. We are, however, cautious of taking this observation as evidence for the involvement of PKC in tumor promotion, because staurosporine may have pharmacological effects other than inhibition of PKC. Obviously, further experimentation is necessary if we are to determine the cellular and molecular mechanisms involved in tumor promotion.

References

1. Nishizuka, Y. (1984) Nature 308, 693-698.
2. Chida, K., Hashiba, H., Sasaki, K., and Kuroki, T. (1986) Cancer Res. 46, 1055-1062.
3. Chida, K., Yamada, S., Kato, N., and Kuroki, T. (1988) Cancer Res. 48, 4018-4023.
4. Chida, K., Kato, N., and Kuroki, T. (1986) J. Biol. Chem. 261, 13013-13018.
5. Chida, K., and Kuroki, T. (1984) Cancer Res. 44, 875-879.
6. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397-402.
7. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
8. Chida, K., Hashiba, H., Suda, T., and Kuroki, T. (1984) Cancer Res. 44, 1387-1391.
9. Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaya, J., Tsuchiya, H., Takahashi, Y., and Masuma, R. (1977) J. Antibiotics 30, 275-282.
10. Morioka, H., Ishihara, M., Shibai, H., and Suzuki, T. (1985) Agric. Biol. Chem. 49, 1959-1963.
11. Kiyoto, I., Yamamoto, S., Aizu, E., and Kato, R. (1987) Biochem. Biophys. Res. Commun. 148, 740-746.
12. Nakadate, T., Jeng, A. Y., and Blumberg, P. M. (1988) Biochemical Pharmacol. 37, 1541-1545.
13. Wolf, M., and Baggiolini, M. (1988) Biochem. Biophys. Res. Commun. 154, 1273-1279.
14. Yoshizawa, S., Fujiki, H., Suganuma, M., Suguri, H., Hirota, M., and Sugimura, T. (1987) Proceedings of the Japanese Biochemical Society, p. 684.
15. Yoshizawa, S., Fujiki, H., Suganuma, M., Suguri, H., Nakayasu, M., Ohuchi, K., and Sugimura, T. (1988) Proceedings of the Japanese Cancer Association, p. 83.
16. Nishino, H., Fujiki, H., Suganuma, M., Horiuchi, T., Iwashima, A., and Sugimura, T. (1984) Biochem. Biophys. Res. Commun. 124, 726-730.