Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 749-756

EARLY PROSTAGLANDIN E SYNTHESIS IS AN OBLIGATORY EVENT IN THE INDUCTION OF CELL PROLIFERATION IN MOUSE EPIDERMIS IN VIVO BY THE PHORBOL ESTER TPA

Gerhard Fürstenberger and Friedrich Marks

Deutsches Krebsforschungszentrum (German Cancer Research Center), Institut für Biochemie, D-6900 Heidelberg, F.R.G.

Received November 10, 1979

SUMMARY

Following the topical application of the phorbol ester TPA to mouse skin in vivo a rapid increase of the prostaglandin E content after 10 and 60 minutes was observed. Pretreatment of mouse skin with indomethacin abolished the first PGE peak as well as the cellular proliferation induced by TPA. Both effects could not be prevented when indomethacin was applied 30 to 60 minutes after TPA treatment, suggesting that the early increase in epidermal PGE is an obligatory event in the course of the induction of epidermal cell proliferation by TPA. A small increase of epidermal PGE was also seen after treatment with the TPA-analogue "Ti_8", whereas 4-O-methyl-TPA was inactive in this respect. "Ti_8"-induced epidermal cell proliferation could be partially inhibited by indomethacin, whereas 4-O-methyl-TPA-induced cell proliferation was insensitive to the drug.

INTRODUCTION

When topically applied to mouse epidermis the tumor-promoting phorbol ester TPA[+] stimulates epidermal ornithine decarboxylase activity (1) and cellular proliferation (2,3). Both effects are completely prevented by topical pretreatment of the skin with the non-steroidal anti-inflammatory drug indomethacin (4,5). This inhibition can be specifically reversed by applying PGE_2 simultaneously with TPA (4,5), indicating that TPA might exert its stimulatory effects through the intervention of PGE_2. Evidence that an early increase in the prostaglandin E content of mouse epidermis is an obligatory event in the induction by TPA of epidermal cellular proliferation and hyperplasia is presented.

[+]Abbreviation: TPA, 12-O-tetradecanoylphorbol-13-acetate; "Ti_8", 12-O-(2Z, 4E, 6,8-tetradecatetraenoyl)phorbol-13-acetate; 4-O-methyl-TPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate; PGE, prostaglandin E; PGF, prostaglandin F.

0006-291X/80/030749-08$01.00/0

MATERIALS AND METHODS

The mice used in all experiments (strain NMRI, female, age 7-8 weeks) were kept as described (5). The back skin of the animals was shaved with an electric clipper 5 days before the experiment. Only those animals which did not show regrowth of hair were used.

12-O-tetradecanoylphorbol-13-acetate (TPA), 12-O-(2Z, 4E, 6,8-tetradecatetraenoyl) phorbol-13-acetate ("Ti_8"), 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate (4-O-methyl-TPA) and indomethacin were dissolved in acetone (0.1 ml) and topically applied to the shaved area of the back skin with a micropipette.

The mice were killed by cervical dislocation at appropriate times after treatment. The back skin was dissected, flattened on a filter paper and immediately frozen on a cold table at -90°C. The average time between cervical dislocation and freezing was 30 seconds. The frozen epidermis of each specimen of skin was scraped off with a precooled scalpel and powdered in a mortar under liquid nitrogen. The powdered epidermis was extracted twice with 3 ml ethyl acetate for 20 minutes at 4°C. After centrifugation the sediment was suspended in 2 ml 10^{-3}M aqueous HCl and extracted twice with 2 ml ethyl acetate. The organic phases were combined and evaporated under reduced pressure. The residue was dissolved in methanol and then subjected to thin layer chromatography on precoated silica gel plates 60 F (Merck, Darmstadt, F.R.G.) with the upper phase of isooctane/water/ethyl acetate/acetic acid = 50/100/110/20 (v/v) as solvent (6). This chromatographic system separates PGE from all other prostaglandins and thromboxanes (6). The zones containing PGE and PGF were scraped off, and the scrapings were eluted four times each with 2 ml methanol. Aliquots of two different dilutions of the eluates were assayed for PGE and PGF by radioimmunoassay (7). The antibody from the Pasteur Institute has been checked to show an extremely low degree of cross-reactivity with other prostaglandins and their metabolites. After addition of 1 ml 1.2 M $HClO_4$ the DNA content of the aqueous phase was determined as described in reference 3. Using the described analytical procedure the percentage recoveries of PGE and PGF were 60-75% as determined by adding radioactively labeled prostaglandins to frozen epidermis powder.

The phorbol esters TPA and 4-O-methyl-TPA were kindly provided by Prof. Dr. E. Hecker, German Cancer Research Center, Heidelberg, F.R.G. Prostaglandins and indomethacin were purchased from Sigma, München, F.R.G. Radioactively labeled (5,6,8,11,12,14) (n) ^{3}H) PGE_2 (160 ci/mmole and (5,6,8,11, 12,14,15 (n) ^{3}H) $PGF_{2\alpha}$ (160 ci/mmole) were obtained from Amersham Buchler, Braunschweig, F.R.G. The rabbit antisera for PGE and PGF were obtained from the Pasteur Institute, Paris, France.

RESULTS

The prostaglandin E content of mouse epidermis was found to be 7.1 $\pm$ 2.5 pg PGE/µg DNA with the analytical procedure described at -90°C. Strict adherence to the experimental procedure was of critical importance, as shown by the fact that when the analysis was performed at 4°C a value of 104.9 $\pm$ 11.1 pg PGE/µg DNA was obtained.

A single topical application of 10 nmoles TPA to mouse skin *in vivo* resulted in a rapid biphasic increase of the epidermal PGE level (Figure 1).

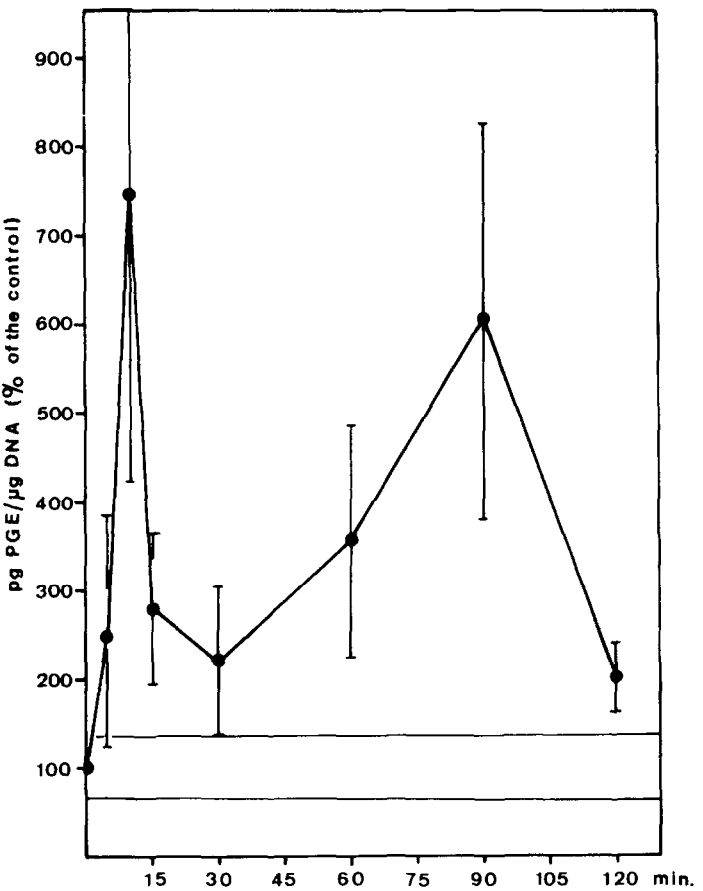

Figure 1. Effect of a single local application of TPA on the level of PGE in dorsal mouse epidermis in vivo.

Mice were locally treated with either 0.1 ml acetone or a solution of 10 nmoles TPA in 0.1 ml acetone and killed at the times indicated. PGE was isolated from frozen epidermis and determined by radioimmunoassay (see Methods). Each experimental point represents the mean ± S.D. for at least 12 mice. Control (acetone treated), indicated by horizontal lines: 7.1 ± 2.5 pg PGE/µg DNA, N = 24 (number of mice).

A first peak (700% of the control) was reached after 10 minutes, while a second peak was apparent after 90 minutes. The level of prostaglandin F was not significantly altered during this time (data not shown). The increase in PGE content was dependent on the dose of TPA, as shown in Figure 2, and could be prevented by applying the prostaglandin synthetase inhibitor (8) indomethacin one hour prior to TPA treatment (Table 1). Using the same experi mental protocol indomethacin was also found to inhibit the TPA-induced mitogenic response of epidermis (5). Neither the prompt increase in the PGE

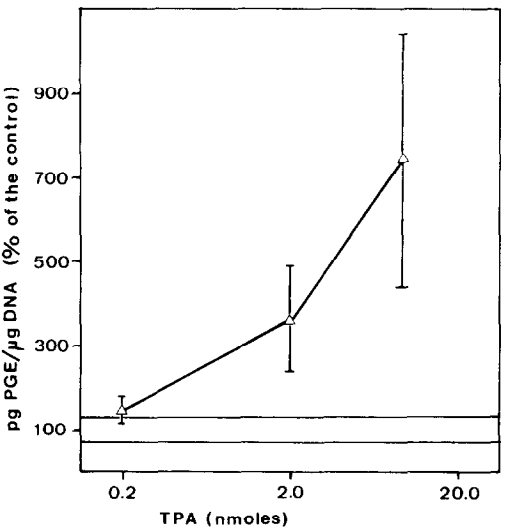

Figure 2. Increased PGE levels in mouse epidermis 10 minutes after topical application of various doses of TPA.

Mice were treated with either 0.1 ml acetone or with 0.2, 2.0 and 10 nmoles TPA in 0.1 ml acetone each. The mice were killed 10 minutes after TPA treatment. For details see Figure 1. N = 6 (± S.D.).

Table 1. Effect of indomethacin on TPA-induced PGE synthesis in mouse epidermis

Treatment	Epidermal PGE content pg PGE/µg DNA	
acetone	7,1 ± 2,5[a)]	7,1 ± 2,5[b)]
TPA	52,6 ± 21,6[a)]	45,1 ± 15,0[b)]
indomethacin, TPA	9,2 ± 2,7[a)]	24,4 ± 7,1[b)]

Mice were treated with acetone (0.1 ml) or 1.1 µmoles indomethacin in 0.1 ml acetone one hour before application of 0.1 ml acetone or 10 nmoles TPA in 0.1 ml acetone, and killed 10 or 90 minutes after the second treatment. For details see Figure 1. N = 12 ± S.D.

a) 10 minutes after treatment

b) 90 minutes after treatment

content (Figure 3) nor the stimulation of cellular proliferation could be prevented when indomethacin was applied 30-60 minutes after TPA treatment.

In contrast with the TPA-induced epidermal cell proliferation the proliferative response evoked by the unsaturated TPA analogue "Ti_8" and by

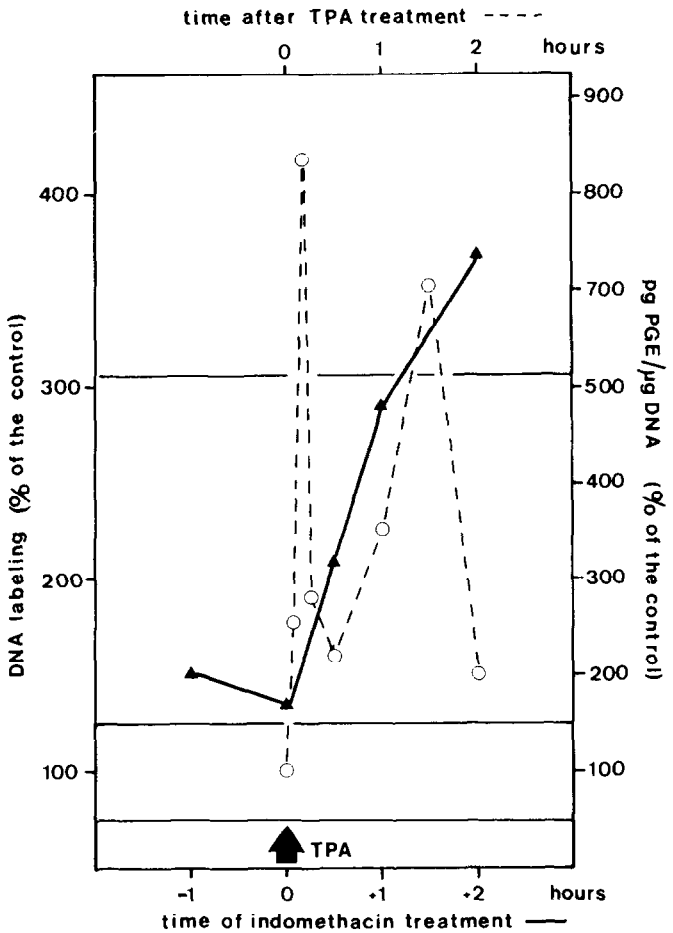

Figure 3. Relationship between the kinetics of epidermal PGE-synthesis induced by TPA (broken line) and the effect on epidermal DNA labeling of varying the time interval between indomethacin and TPA treatment (solid line)

Mice were treated with 0.1 ml acetone or 10 nmoles TPA dissolved in 0.1 ml acetone and killed at the times indicated for determination of the epidermal PGE content (see Figure 1 and Methods). To determine the effect of indomethacin on DNA labeling, mice were treated with 0.1 ml acetone or 1.1 umoles acetone (0.1 ml acetone) or TPA (10 nmoles in 0.1 ml acetone) and killed 18 hours after TPA treatment. Labeled thymidine was injected i.p. one hour prior to sacrifice (For details see ref. 5). The horizontal lines indicate either control (acetone-treated) DNA labeling: 51 ± 13 cpm/µg DNA, or DNA labeling 18 hours after TPA treatment: 201 ± 49 cpm/µg DNA, N = 10.

4-O-methyl-TPA were either only partially inhibited by indomethacin or not at all (9,10; Figure 4). Concomitantly, the increase in the epidermal PGE level found 10 minutes after application of "Ti_8" was less pronounced than after TPA, whereas no elevation was seen after treatment with 4-O-methyl-TPA (Figure 4).

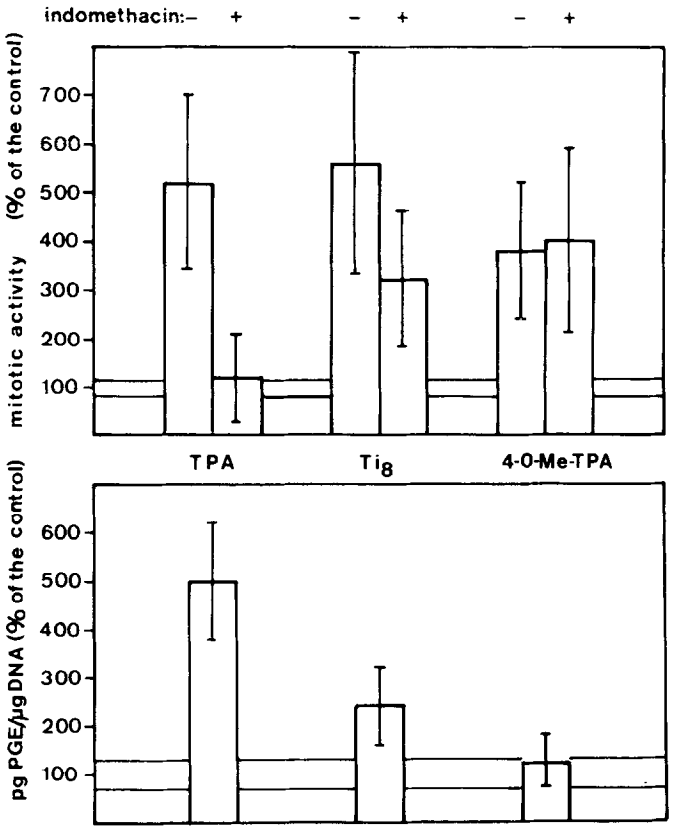

Figure 4. Effect of indomethacin on the mitotic activity of mouse epidermis stimulated by various phorbol esters compared with its ability to induce PGE synthesis.

Mice were treated with acetone or indomethacin (1.1 umoles) one hour before application of acetone TPA (10 nmoles), "Ti_8" (10 nmoles) or 4-O-methyl-TPA (400 nmoles). The mice were killed 27 hours after acetone or TPA treamtent and 24 hours after "Ti_8" or 4-O-methyl-TPA treatment. Vincristine was injected i.p. 4 hours prior to sacrifice (see ref. 5). Each value is the mean ± S.D. of at least 16 sections prepared from 5 mice (upper diagram). Mice were treated with acetone or TPA (10 nmoles) "Ti_8" or 4-O-methyl-TPA (400 nmoles) and killed 10 minutes after treatment for the determination of the epidermal PGE content (see Figure 1 and Methods). N = 12 ± S.D. (lower diagram). The volumes of acetone and of acetone solutions were standardized at 0.1 ml.

DISCUSSION

The results of these experiments show that within a few minutes of treatment with TPA PGE synthesis in mouse epidermis *in vivo* is strongly increased. This is the earliest known biochemical response of mouse skin to the phorbol ester *in vivo*. A stimulatory effect of TPA on epidermal prostaglandin synthesis at later times has been independently shown by Ashendel and Boutwell (11) and by Bresnick et al. (12). The PGE level of normal mouse

epidermis as reported by the latter authors is higher than the PGE levels recorded by Ashendel and Boutwell (11) and by us by a factor of about 30. We also found a 15fold higher PGE level using an analytical procedure similar to that of Bresnick (12). This value may reflect an artifactual synthesis of prostaglandins in unfrozen epidermis, probably caused by the trauma of excision (see also ref. 13).

Indomethacin prevents the early increase in PGE synthesis when applied prior to or simultaneously with TPA, as it inhibits the mitogenic response induced by TPA. The inhibition of epidermal mitosis can be reversed by PGE_2 (5). On the other hand indomethacin fails to inhibit cellular proliferation when applied 30-60 minutes after TPA treatment. It may be concluded, therefore, that the early increase in the epidermal PGE content (after 10 minutes) is an obligatory event in the course of the induction of epidermal cellular proliferation by TPA. In addition, TPA dramatically potentiates the sensitivity of the epidermis towards PGE_2 (5). Thus, propagation of the mitogenic stimulus by E-prostaglandins means that both the prostaglandin E content and the sensitivity to prostaglandin E are increased.

Nevertheless, mediation by prostaglandins is not an obligatory condition of epidermal cell proliferation and hyperplasia; whether or not prostaglandins are involved depends on the nature of the stimulus. Thus, the TPA analogue "Ti_8" evokes a proliferative response which can be only partially inhibited by indomethacin and which is accompanied by a less pronounced elevation of PGE as compared with that following TPA treatment. Finally, the hyperplastic reaction induced by 4-O-methyl-TPA is not preceded by any measureable increase in PGE synthesis and, concomitantly, cannot be prevented by indomethacin. Considering the close chemical relationship of the three phorbol esters, these differences may prompt fundamental questions regarding the existence of a proposed "phorbol receptor".

Phorbol esters of the TPA type induce an increase in prostaglandin synthesis not only in epidermis in vivo but also in vitro (14) as well as in several

other cell types such as canine kidney cells (15), macrophages (16) and bone organ culture (17). In the latter case TPA also stimulates bone resorption which may, therefore, be mediated by prostaglandins (17). Furthermore, TPA increases the synthesis of PGE in clones of Friend leukemia cells sensitive to TPA-induced inhibition of differentiation and adhesion, but not clones resistant to TPA, suggesting that TPA-induced PGE-synthesis is closely linked with its effect on differentiation and adhesion (18).

ACKNOWLEDGEMENT

The technical assistance of Eva Besemfelder is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft and the Wilhelm-und Maria-Meyenburg Foundation.

REFERENCES

1. O'Brien, T.G. (1976) Cancer Res. 36, 2644-2653.
2. Baird, W.M., Sedgwick, J.A., and Boutwell, R.K. (1971) Cancer Res. 31, 1434-1439.
3. Krieg, L., Kühlmann, I., and Marks, F. (1974) Cancer Res. 34, 3135-3146.
4. Verma, A.K., Rice, H.M., and Boutwell, R.K. (1977) Biochem.Biophys. Res.Commun. 79, 1160-1166.
5. Fürstenberger, G. and Marks, F. (1978) Biochem.Biophys.Res.Commun. 84, 1103-1111.
6. Flower, R.J., and Blackwell, G.J. (1976) Biochem.Pharmacol. 25, 285-291.
7. Jaffee, B.M., and Behrmann, H.R. (1978) Methods of Hormone Radioimmunoassay, edited by Jaffee, B.M., and Behrman, H.R., pp. 1942, Academic Press, New York.
8. Flower, R., Gryglewski, R., Cedro, K.H., and Vane, J.R. (1972) Nature New Biol. 238, 104-106.
9. Fürstenberger, G., de Bravo, M., Bertsch, S., and Marks, F. (1979) Res.Commun.Chem.Pathol.Pharmacol. 24, 533-541.
10. Fürstenberger, G., and Hecker, E. (1977) Experientia 33, 986-988.
11. Ashendel, C.L., and Boutwell, R.K. (1979) Biochem.Biophys.Res.Commun. 90, 623-627.
12. Bresnick, E., Meunier, P., and Lamden, M. (1979) Cancer Letters 7, 121-125.
13. Snyder, D.S. (1976) Prostaglandins 11, 631-643.
14. Hammarström, S., Lindgren, J.A., Marcelo, C., Duell, E.A., Anderson, Th.F., and Voorhees, J.J. (1979) J.Invest.Dermatol. 73, 180-183.
15. Levine, L., and Hassid, A. (1977) Biochem.Biophys.Res.Commun. 79, 477-484.
16. Brune, K., Kalin, H., Schmidt, R., and Hecker, E. (1978) Cancer Letters 4, 333-342.
17. Tashjian, Jr., A.H., Ivey, J.L., Delclos, B., and Levine, L. (1978) Prostaglandins 16, 221-232.
18. Yamasaki, H., Mufson, R.A., and Weinstein, I.B. (1979) Biochem.Biophys. Res.Commun. 89, 1018-1025.