Increased mouse epidermal ornithine decarboxylase activity by the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate involves increased amounts of both enzyme protein and messenger RNA

Ajit K. VERMA,* Deborah ERICKSON and Bruce J. DOLNICK

Department of Human Oncology, Wisconsin Clinical Cancer Center, University of Wisconsin Center for Health Sciences, Madison, WI 53792, U.S.A.

Evidence was sought that the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse epidermal ornithine decarboxylase (ODC, EC 4.1.1.17) activity involves both increased ODC mRNA and ODC protein. Application of 10 nmol of TPA to mouse skin led to a dramatic increase in soluble epidermal ODC activity which paralleled an increase in amount of enzymically active ODC protein as determined by gel electrophoresis of immunoprecipitated difluoromethyl[³H]ornithine-bound ODC. Application of TPA to mouse skin also resulted in an increase in ODC mRNA measured by dot-blot analysis using a radiolabelled cDNA probe. ODC mRNA induction preceded the increase in ODC activity by TPA. TPA-increased ODC mRNA displayed a single major band of 2.1 kilobases in size identified by the Northern blotting procedure.

INTRODUCTION

ODC, which decarboxylates ornithine to putrescine, is the first enzyme in the pathway of mammalian polyamine biosynthesis [1,2]. ODC is characterized by its inducibility and rapid turnover rate (half-life, $t_{1/2}$ ~ 17 min) [2]. Among the numerous biochemical changes elicited after topical application of the tumour promoter TPA to mouse skin, the induction of epidermal ODC activity is prominent [3,4]. Available data indicate that TPA-induced ODC activity and the resultant accumulation of putrescine may play important roles in mouse skin tumour promotion by TPA [3–6].

Direct evidence is lacking that increased mouse epidermal ODC activity by TPA is the result of increases in mRNA and ODC protein. The availability of radiolabelled DFMO (a suicide inhibitor of ODC) [7] and a cDNA probe [8] has enabled us to obtain clues about the role of mRNA and enzyme protein in the TPA-increased epidermal ODC activity. The supporting data that TPA-induced ODC activity involves increases in both mRNA and ODC protein are summarized in this paper.

MATERIALS AND METHODS

DL- α -[5-³H]DFMO (11.1 Ci/mmol), DL-[1-¹⁴C]ornithine (49.9 mCi/mmol) and Gene Screen (hybridization transfer membrane) were purchased from New England Nuclear (Boston, MA, U.S.A.). [α -³²P]dCTP (3000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). DNA polymerase I was obtained from Promega Biotec, Madison, WI, U.S.A. Reagents for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Enzyme-grade (NH₄)₂SO₄ was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). TPA was purchased from

Life Systems (Newton, MA, U.S.A.). Female Charles River CD-1 mice (7–9 weeks old) were purchased from Charles River Breeding Laboratory, Wilmington, MA, U.S.A. Monoclonal antibody to rat liver ODC was generously given by Dr. Shin-ichi Hayashi, Jikei University School of Medicine, Japan [9]. Anti-mouse IgG (heavy- and light-chain-specific) was from Cooper Biomedical (Malvern, PA, U.S.A.). pOD48, a cDNA clone prepared from mouse ODC mRNA, was generously given by Dr. P. Coffino [8]. Poly(U)-agarose was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). Nitrocellulose (BA 85) was purchased from Schleicher and Schuell (Keene, NH, U.S.A.).

Mice were housed and treated as described previously [6]. The dorsal skin of the mice was shaved 3–4 days before treatment, and only those mice in the resting phase of the hair cycle were used for experimentation. Acetone or 10 nmol of TPA in acetone was applied to the shaved backs of mice, and mice were killed by cervical dislocation at the indicated times after treatment. Epidermis from individual mice was scraped and homogenized in 50 mM-Tris/HCl (pH 7.5) containing 0.1 mM-EDTA, 0.1 mM-pyridoxal phosphate, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-2-mercaptoethanol, 0.1% Tween 80 and 0.1 mM-dithiothreitol. Soluble epidermal ODC extract was partially purified [35–55% -satd.-(NH₄)₂SO₄ fraction] and used for the assays of [³H]DFMO binding [10] and ODC activity [11].

[³H]DFMO-bound ODC was immunoprecipitated with anti-ODC monoclonal antibody and the second antibody, rabbit anti-mouse IgG [9]. The immunoprecipitates were analysed for associated radioactivity by SDS/polyacrylamide-tube-gel electrophoresis [12].

For isolation of total RNA from mouse skin, skin was excised and immediately placed in liquid N₂ and pulverized in a mortar. Total cellular RNA was prepared

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, α -difluoromethylornithine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

* To whom reprint requests should be addressed.

from the ground skin by the urea-extraction and CsCl-gradient-centrifugation method of Ross [13] with minor modifications. RNA was washed four times with 3.0 M-sodium acetate (pH 5.0) containing 5 mM-EDTA, and twice with 100% ethanol. The RNA was then desiccated, dissolved in sterile distilled water, and stored at -70°C . In a typical experiment, yield of total mouse skin RNA from five mice (3 g of skin) was 2.0 mg as quantified spectrophotometrically by using $50\text{ }\mu\text{g}$ of RNA/ A_{260} unit.

Species of RNA containing regions of ODC mRNA homology were identified by a modification of the Northern blotting procedure [14]. Total mouse skin RNA was fractionated by electrophoresis in denaturing formaldehyde/agarose gel [15]. Transfers to Gene Screen were performed at 4°C for 72 h, which allowed complete transfer. The filter was baked for 2 h at 80°C and prehybridized for 24 h with prehybridization buffer containing 0.6% (w/v) polyvinylpyrrolidone [16]. The filter was then hybridized with nick-translated ^{32}P -labelled pOD48 [17]. Phage- λ DNA cut with endonuclease *Hind*III and labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ by using Klenow DNA polymerase was denatured as for RNA and was included in the gels as M_r standard and a marker for transfer efficiency during Northern-blotting procedures [14,18]. RNA bands containing ODC mRNA homology were made visible by exposing Kodak X-Omat AR film to the washed nitrocellulose filter at -70°C with intensifying screens.

ODC mRNA was quantified by dot-blotting [19], by using a ^{32}P -labelled cDNA probe. Briefly, total RNA from mouse skin was denatured [20] and was spotted on a sheet of nitrocellulose presoaked in 3 M-NaCl/0.15 M-sodium citrate (pH 7.0) by using a Bethesda Research Laboratories Hybri-Dot manifold to apply the RNA in 3 mm^2 circles. After application of the RNA samples, the nitrocellulose was baked *in vacuo* for 2 h at 80°C , prehybridized and then hybridized with ^{32}P -labelled

nick-translated pOD48 (1.5×10^9 c.p.m./ μg) [8]. The resulting autoradiogram was scanned by soft-laser densitometry.

RESULTS

A time course of the effect of TPA on mouse epidermal ODC protein is shown in Fig. 1 and Table 1. In this experiment, soluble epidermal extract was prepared at various times (0, 2, 4, 6, 8 and 12 h) after a single application of acetone or TPA to mouse skin; ODC activity and the capacity to bind $[\text{H}]\text{DFMO}$ were determined. As shown in Fig. 1, $[\text{H}]\text{DFMO}$ -bound ODC migrated as a single peak corresponding to an M_r of about 55000 [11], the M_r of the subunit of ODC reported by others [10]. At all times points, ODC activity

Fig. 1. $[\text{H}]\text{DFMO}$ binding to the soluble epidermal ODC prepared at various times after a single application of TPA to mouse skin

Table 1. TPA-induced ODC activity correlates with the amount of ODC protein

The data are from the experiment illustrated in Fig. 1. Soluble epidermal extract $[(\text{NH}_4)_2\text{SO}_4$ fraction], which was used for $[\text{H}]\text{DFMO}$ -binding assays (Fig. 1), was also used to assay ODC activity. Each $[\text{H}]\text{DFMO}$ value in the Table is the total $[\text{H}]\text{DFMO}$ radioactivity (d.p.m.; converted into fmol) associated with the $[\text{H}]\text{DFMO}$ peak, which was a single peak corresponding to M_r 55000. The amount of ODC protein was calculated from $[\text{H}]\text{DFMO}$ bound to ODC (e.g. $92.8 \times 55000 \times 10^{-6} = 5.1\text{ ng}$).

Time after application of TPA (h)	ODC activity (nmol of CO_2 /60 min per mg of protein)	$[\text{H}]\text{DFMO}$ bound (fmol/mg of protein)	Amount of ODC (ng)
0	0.0	0.0	0.0
2	3.5	92.8	5.1
4	23.6	553.0	30.4
6	27.0	653.0	35.9
8	7.0	209.0	11.5
12	0.5	13.5	0.74

Acetone (○) or 10 nmol of TPA in acetone (●) was applied to intact mouse skin *in vivo*. Epidermis from eight mice was scraped (without heat treatment), homogenized, centrifuged, and soluble epidermal ODC was partially purified after $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis. $[\text{H}]\text{DFMO}$ (26.5 Ci/mmol) binding was assayed at the indicated times after the treatment. Equal amounts of protein (0.38 mg) from epidermal extracts were used for $[\text{H}]\text{DFMO}$ -binding assays. $[\text{H}]\text{DFMO}$ -bound enzyme was immunoprecipitated. The immunoprecipitates were analysed by SDS/7.5%-polyacrylamide-tube-gel electrophoresis. The tube gels were sliced into 2.2 mm slices and the radioactivity associated with each gel slice was determined.

Fig. 2. RNA-dot analysis of RNAs from acetone- and TPA-treated mouse skin

Groups of five mice were killed at 2, 2.5, 3, 3.5, 4 and 6 h after application of 10 nmol of TPA to their shaved backs. Total RNA from skin was prepared as described in the Materials and methods section. The same solution of TPA was applied to two additional mice of the same groups, and soluble (30000 g) epidermal ODC activity was assayed at 2, 4 and 6 h after TPA treatment. Indicated amounts of total cellular RNA were spotted on the nitrocellulose filter. RNA bands containing ODC mRNA were detected by exposing Kodak X-Omat AR film to the washed nitrocellulose filter at -70°C with intensifying screens. The resulting autoradiogram was quantified densitometrically. The extent of induction of ODC mRNA (fold induction over control values) was calculated from the densitometric scans. Top, autoradiograms; bottom, kinetics of TPA-induced ODC activity (▲) and ODC mRNA (●).

correlated with [^3H]DFMO binding (Table 1). The amount of ODC protein can be calculated from [^3H]DFMO bound to soluble epidermal extract. The M_r of the subunit of ODC is 55000. It is assumed that one molecule of DFMO is needed to inactivate each 55 kDa subunit of ODC. Thus 3.5, 23.6, 27.0, 7.0 and 0.5 unit of ODC are equivalent to 5.1, 30.4, 35.9, 11.50 and 0.74 ng of ODC respectively.

The effect of TPA on the amount of mouse skin ODC mRNA was determined by hybridization of total cellular RNA from mouse skin against ^{32}P -labelled nick-translated pOD48. Dot-blot analysis (Fig. 2) indicates that a single application of 10 nmol of TPA to mouse skin led to about 17-fold increase in hybridizable ODC mRNA at 3.5 h after treatment; the increase in ODC mRNA preceded the TPA-induced increase in ODC activity (Fig. 2). We measured the size of ODC mRNA by the Northern blotting procedure [14]. In this

experiment, total RNA, prepared from TPA-treated mouse skin, was fractionated by denaturing formaldehyde/agarose-gel electrophoresis. The gel was blotted to Gene Screen and hybridized with nick-translated ^{32}P -labelled pOD48. Application of TPA to mouse skin resulted in an increase in hybridizable-RNA-species-containing regions of ODC mRNA, and RNA displayed a single major band of 2.1 kilobases in size. No change was observed in the size of ODC mRNA after TPA treatment of mouse skin (results not shown). The size of ODC mRNA was similar to that reported by other workers [8,21].

DISCUSSION

ODC activity is increased in various tissues within a few hours in response to many different stimuli and, in general, the increased ODC activity is rapid and

transient [1,2]. The molecular mechanism of rapid turnover of ODC activity is not clearly understood. However, it has been suggested that changes in ODC activity may be regulated at the level of synthesis [21] and degradation [22], and also by post-translational modifications [23] or interaction with macromolecules [24]. The data presented here provide evidence that application of the tumour promoter TPA to mouse skin, which leads to a dramatic increase (up to 200-fold above the control) in epidermal ODC activity, is the result of increases in both ODC protein (Fig. 1 and Table 1) and ODC mRNA (Fig. 2).

The amount of [^3H]DFMO binding was used to quantify the amount of ODC protein. DFMO is a potent irreversible inhibitor of mammalian ODC; it is enzymically decarboxylated, generating an intermediate carbanionic species which, with the loss of fluorine, alkylates a nucleophilic residue at or near the active site, thereby covalently binding the inhibitor to the enzyme [7]. The specificity of [^3H]DFMO binding to ODC is well established [10,11].

The use of radiolabelled DFMO to determine the amount of ODC is limited by the fact that DFMO binds only to the active ODC molecule; denatured or post-translationally modified ODC will not be quantified by this procedure. With this reservation, we found that application of TPA to mouse skin resulted in an increase in ODC activity which was paralleled by [^3H]DFMO binding, indicating an increased amount of enzymically active ODC (Fig. 1 and Table 1).

TPA-increased ODC protein may be the result of increased synthesis, decreased degradation and/or activation of ODC. To substantiate the conclusion that TPA may affect the synthesis of mouse epidermal ODC, we determined the effect of TPA on the amount of ODC mRNA. The results shown in Fig. 2 indicate that the increased amount of ODC protein caused by TPA may be the result of increases in ODC mRNA. Clearly, TPA treatment results in increases in RNA homologous to ODC cDNA. TPA increased the quantity, but did not alter the nature, of ODC mRNA (results not shown).

The mechanism by which TPA leads to the transcription of epidermal ODC is unclear. TPA binds specifically to mouse epidermal fractions, and [^3H]TPA-binding activity co-purifies with Ca^{2+} -activated phospholipid-dependent protein kinase (protein kinase C) [25–30]. Recently, we found, using primary culture of newborn-mouse epidermal cells, that the concentration of available Ca^{2+} may play a role in TPA induction of ODC. Also, diacylglycerol, an activator of protein kinase, and phospholipase C (which releases diacylglycerol from membrane phospholipids), induced ODC activity in cultured newborn-mouse epidermal cells. Diacylglycerol-, phospholipase-C- and TPA-induced ODC activity in newborn-mouse epidermal cells was accompanied by a concomitant increase in ODC mRNA. Similarly, palmitoylecarnitine, an inhibitor of protein kinase [30], inhibited both the induction of ODC and skin tumour promotion by TPA (A. K. Verma, unpublished work). The role of protein kinase in ODC induction has also been suggested by two other reports [31,32]. The mechanism of how TPA-receptor-protein kinase C activation transduces signals for ODC-gene transcription is unknown, and this warrants further investigation.

This work was supported by U.S. Public Health Service grants CA-35368 and CA-42585 awarded to A.K.V. by the National Cancer Institute. B. J. D. is the recipient of a Junior Faculty Research Award from the American Cancer Society.

REFERENCES

1. Raina, A. & Jänne, J. (1981) *Med. Biol.* **59**, 269–461
2. Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
3. O'Brien, T. G. (1976) *Cancer Res.* **36**, 2644–2653
4. Verma, A. K. (1983) in *Cancer: Etiology and Prevention* (Crispen, R. G., ed.), pp. 241–260, Elsevier, New York
5. Takigawa, M., Verma, A. K., Simsiman, R. C. & Boutwell, R. K. (1983) *Cancer Res.* **43**, 3732–3738
6. Verma, A. K., Shapas, B. G., Rice, H. M. & Boutwell, R. K. (1979) *Cancer Res.* **39**, 419–425
7. Metcalf, B. W., Bey, P., Danzin, D., Jung, M. J., Casara, P. & Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
8. McConlogue, L., Gupta, M., Wu, L. & Coffino, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 540–544
9. Matsufuji, S., Fujita, K., Kameji, T., Kanamoto, R., Murakami, Y. & Hayashi, S. (1984) *J. Biochem. (Tokyo)* **96**, 1525–1530
10. Pritchard, M. L., Seely, J. E., Pösö, H., Jefferson, L. S. & Pegg, A. E. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1597–1603
11. Verma, A. K. (1985) *Biochim. Biophys. Acta* **846**, 109–119
12. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
13. Ross, J. (1976) *J. Mol. Biol.* **106**, 403–420
14. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201–5205
15. Carmichael, G. G. & McMaster, F. K. (1980) *Methods Enzymol.* **65**, 380–390
16. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683–3687
17. Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1184–1188
18. Dolnick, B. J. & Bertino, J. R. (1981) *Arch. Biochem. Biophys.* **210**, 691–697
19. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. (1979) *Nucleic Acids Res.* **7**, 1541–1552
20. Cheley, S. & Anderson, R. (1984) *Anal. Biochem.* **137**, 15–19
21. Gilmour, S. K., Avdalovic, N., Madara, T. & O'Brien, T. G. (1985) *J. Biol. Chem.* **260**, 16439–16444
22. Seely, J. E., Pösö, H. & Pegg, A. E. (1982) *J. Biol. Chem.* **257**, 7549–7553
23. Russell, D. H. (1981) *Biochem. Biophys. Res. Commun.* **99**, 1167–1172
24. Heller, J. S., Fong, W. F. & Canellakis, E. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1858–1862
25. Ashendel, C. L., Staller, J. M. & Boutwell, R. K. (1983) *Cancer Res.* **43**, 4333–4337
26. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
27. Nidel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 36–40
28. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 11442–11445
29. Driedger, P. E. & Blumberg, P. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 567–571
30. Wise, B. C., Glass, D. B., Jen Chou, C. H., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. S., Kibler, R. F. & Kuo, J. F. (1982) *J. Biol. Chem.* **257**, 8489–8495
31. Jetten, A. M., Ganong, B. R., Vandenbark, G. R., Shirley, J. E. & Bell, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1941–1945
32. Sasakawa, N., Ishii, K., Yamamoto, S. & Kato, R. (1985) *Biochem. Biophys. Res. Commun.* **128**, 913–920