Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 110-117

THE STIMULATION OF PROSTAGLANDIN PRODUCTION BY TRANSFORMING GROWTH FACTOR-α AND 12-O-TETRADECANOYL-PHORBOL-13-ACETATE OR 1-OLEOYL-2-ACETYL-GLYCEROL IS SYNERGISTIC

Lawrence Levine, Dian-mo Xiao, Nancy Worth and Whitman E. Lilley

Department of Biochemistry
Brandeis University
Waltham, Massachusetts 02254

Received May 24, 1985

Stimulation of prostaglandin production in C-9 rat liver cells by transforming growth factor (TGF)-α was synergistic with that of the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). TGF-α and TPA synergized the release of radiolabelled compounds from [^{3}H]arachidonic acid prelabelled C-9 cells. 1-Oleoyl-2-acetyl-glycerol (OAG) and TGF-α also synergized prostaglandin production in the C-9 cells suggesting that the tumor promoter was mimicking the physiological activator of protein kinase C, 1,2-diacylglycerol. TGF-α and TPA also synergistically stimulated arachidonic acid metabolism by NRK-49F rat kidney cells. In A-549 human lung carcinoma cells, TGF-β, but not TGF-α, stimulated arachidonic acid metabolism synergistically with TPA.

Several growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), interleukin-1, as well as sarcoma, kidney, and bladder carcinoma transforming growth factors (1,2), stimulate arachidonic acid metabolism by cells in culture (3-11). The transforming growth factors probably contained transforming growth factor (TGF)-α- or β-like polypeptides or both (2). We show here that both recombinant human TGF-α and human TGF-β, independently, can synergistically stimulate arachidonic acid metabolism in the presence of 12-O-tetradecanoyl-13-acetate (TPA) and that TGF-α can synergistically stimulate arachidonic acid metabolism in the presence of 1-oleoyl-2-acetyl-glycerol (OAG). The synergistic stimulation of arachidonic acid

Abbreviations: EGF, epidermal growth factor; MEM, minimal essential media; OAG, 1-oleoyl-2-acetyl-glycerol; PDGF, platelet derived growth factor; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TxB_2, thromboxane B_2.

0006-291X/85 $1.50

metabolism by TGF-α and TPA in C-9 cells appears to reflect increased deesterification of arachidonic acid from its cellular lipids.

MATERIALS AND METHODS

Cells were grown as monolayers using MEM containing 2 mM L-glutamine and supplemented with 10% (v/v) fetal bovine serum. Cells treated with trypsin-EDTA (0.25%) were seeded at 0.4 x 10^6 cells/35-mm tissue culture dishes (Falcon Plastics, Oxnard, CA) in 1 ml of the serum-supplemented medium. After 1 day of growth, the medium was removed, and each dish was washed twice with 1 ml of MEM and then incubated in 1 ml of MEM containing the indicated substances at 37° in an atmosphere of 95% air-5% CO_2 for 24 hours unless specified otherwise. The culture media were collected and, after centrifugation to remove any floating cells, analyzed by radioimmunoassay. For radiolabelling of the cells, [^{3}H]arachidonic acid (5,6,8,9,11,12,14, 15-[^{3}H(N)], 87.4 Ci/mmol) (New England Nuclear, Boston, MA) was added to cells at the time of seeding. The seeding for these radiolabelling experiments was at a density of 2.0 x 10^6 cells per 60 mm dish. After incubation for 24 hours, the unincorporated [^{3}H]arachidonic acid was removed by washing the cells 5 times with MEM. In the radiolabelling experiments, fatty acid-free albumin, 5 mgs per ml (Sigma Chemical Co., St. Louis, MO) was present in all culture media.

The phorbol ester (TPA) was purchased from CMC Cancer Research Chemicals, Inc. (Brewster, NY). 1-Oleoyl-2-acetylglycerol (OAG) was purchased from Molecular Probes, Inc., Junction City, OR. The rat liver cells (the C-9 cell line) and the A-549 cell line were obtained from the American Type Culture Collection (Rockville, MD). The NRK-49F cell line was obtained from Dr. Michael Newman, Department of Biochemistry, Brandeis University, Waltham, MA. The TGF-α and TGF-β were generously supplied by Dr. Rik Derynck, Genentech, Inc. (South San Francisco, CA) and Dr. Anita B. Roberts, National Cancer Institute (Bethesda, MD), respectively.

Transforming growth factor-β (TGF-β) was purified from human platelets as described by Assoian et al. (12) except that urea was removed by desalting on C18 high performance liquid chromatography. The preparation used was free of contaminants as analyzed by SDS-polyacrylamide gel electrophoresis and silver staining. In particular, there was no evidence for contamination with platelet-derived growth factor. There was no evidence for contamination with EGF. TGF-α was a recombinant product of 50 amino acid residues produced by expression of the appropriate human coding sequence in E. coli as described by Derynck et al. (13). The final product (Lot No. 8/31 TGF1) was homogeneous by analysis on HPLC and by amino acid composition and its concentration, analyzed by inhibition of [^{125}I]EGF binding, is given as EGF equivalents.

Measurement of Arachidonic Acid Metabolites. Products of arachidonic acid metabolism are released into the culture medium. The concentrations of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and the stable, hydrolytic product of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in medium were determined by radioimmunoassay using antisera of known specificities. The antibodies directed against PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ are highly specific.

Statistical Analysis. Results of each experiment were subjected to analysis of variance, and the standard errors (SE) were calculated from the residual error term of that analysis.

RESULTS

The C-9 cell line of rat liver cells metabolizes arachidonic acid via the cyclooxygenase, not the lipoxygenase, pathway. About 95% of the cyclooxy-

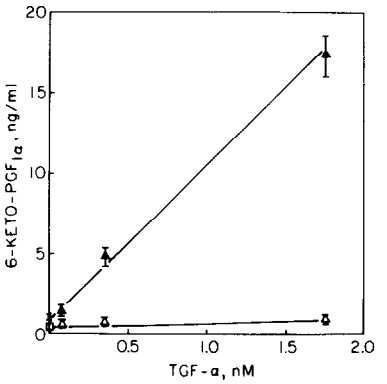

Fig. 1. Synergistic stimulation of 6-keto-$PGF_{1\alpha}$ production by increments of TGF-α in presence (▲) and absence (Δ) of TPA. Data represent the means and SEM for analyses of 3 to 6 dishes. Production of 6-keto-$PGF_{1\alpha}$ (ng/ml) in MEM, 0.42 ± 0.057 (□); in TPA (8.5 nM), 1.05 ± 0.060 (X). $PGF_{2\alpha}$ (ng/ml), with TPA, 0.036; with TPA and TGF-α, 0.168. PGE_2 (ng/ml), with TPA 0.030; with TPA and TGF-α, 0.088.

genase products is prostaglandin I_2 (PGI_2), measured as its stable hydrolytic product 6-keto-$PGF_{1\alpha}$; PGE_2 and $PGF_{2\alpha}$ are the other products. EGF as well as PDGF stimulates arachidonic acid metabolism by C-9 cells (14). TPA also stimulates metabolism of arachidonic acid in many cells, including the C-9 cells (15). TGF-α in the presence of TPA dose dependently synergized arachidonic acid metabolism (Fig. 1). TGF-β, at least at the highest level used (0.08 nM), did not statistically significantly synergize the stimulation of arachidonic acid metabolism with TPA. All of the cyclooxygenase products of this cell ($PGF_{2\alpha}$, PGE_2, as well as the 6-keto-$PGF_{1\alpha}$) were increased synergistically during incubation of the cells with TPA and TGF-α. The stimulation must have occurred at or before the formation of the endoperoxide intermediate. The C-9 cells, when prelabelled with [^{3}H]arachidonic acid, released radiolabel and 6-keto-$PGF_{1\alpha}$ into the conditioned media synergistically ($P < 0.01$) when treated with both TGF-α and TPA (Table 1). Deesterification, most likely a phospholipase A_2 reaction, was being stimulated. However, the possibility that reesterification of enzymatically liberated arachidonic acid was inhibited directly or indirectly by the TGF-α cannot be ruled out.

Normal rat kidney cells (the NRK-49F cell line) produce about equal amounts of PGE_2, $PGF_{2\alpha}$ and PGI_2 (if TxB_2 and PGD_2 or lipoxygenase products are

TABLE 1. SYNERGISTIC STIMULATION OF RELEASE OF RADIOLABELLED MATERIAL FROM [^{3}H]ARACHIDONIC ACID LABELLED CELLS AND 6-KETO-$PGF_{1\alpha}$ PRODUCTION IN RAT LIVER (C-9) CELLS BY TGF-α IN THE PRESENCE OF TPA[a,b,h]

Agonist	Concentration nM	Release of [^{3}H]-labelled compounds from [^{3}H]arachidonic acid prelabelled cells cpm/ml[f]	6-keto-$PGF_{1\alpha}$ ng/ml
MEM[c]		1479 ± 48 (4)[d]	2.53 ± 0.222 (4)[d,g]
TPA	1.7	1255 ± 17 (4)	1.78 ± 0.094 (4)
TGF-α	1.8	1459 ± 41 (4)	3.03 ± 0.309 (4)
TPA and TGF-α	1.7 1.8	2031 ± 131 (4)[e]	5.65 ± 0.589 (4)[e]

[a] 2.0 x 10^6 cells per 60 mm dish.
[b] After a 60 minute incubation.
[c] MEM contained fatty acid-free albumin (5 mg/ml).
[d] Data represent the means ± SEM for the number of dishes in parentheses.
[e] Significantly different from MEM ($P < 0.01$).
[f] About 10% of the radiolabelled compounds chromatographed with authentic 6-keto-$PGF_{1\alpha}$ and 90% with authentic arachidonic acid.
[g] This level of 6-keto-$PGF_{1\alpha}$ is much higher than that seen in Fig. 1, even though the incubation was 60 minutes instead of 24 hours. However, more cells are used on these experiments (2 x 10^6 compared to 4 x 10^5). In addition, the MEM contains fatty acid-free bovine albumin (5 mg/ml) so that any liberated arachidonic acid would be bound and reesterification would be inhibited.
[h] For this experiment, the TPA concentration was 5 fold less than that used in Fig. 1. With this low level of TPA, release of [^{3}H]label is not stimulated and the synergistic response with TGF-α is not as large as that seen with higher concentrations of TPA (Fig. 1).

produced, they are below the limits of the sensitivities of our radioimmunoassays). PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the NRK-49F cell line also was synergistically stimulated ($P < 0.01$) by TGF-α and TPA (Table 2) [the conditioned media were not analyzed for $PGF_{2\alpha}$].

Human lung carcinoma cells (the A-549 cell line) do not metabolize arachidonic acid to a great extent, but when incubated in the presence of TPA, PGE_2 can be measured in the conditioned media. In contrast to the response of the C-9 rat liver cells and the NRK-49F rat kidney cells, TGF-β, but not TGF-α, synergized PGE_2 production ($P < 0.01$) in the A-549 cells (Table 3).

The cellular target of the tumor promoting phorbol esters is protein kinase C (16). Its physiological counterpart in the activation of protein kinase C is 1,2-diacylglycerol (17), one of two putative second messengers produced during phosphatidylinositoltriphosphate hydrolysis (18). 1-Oleoyl-2-acetyl-glycerol (OAG), a derivative of diacylglycerol, also activates

TABLE 2. SYNERGISTIC STIMULATION OF PGE_2 AND 6-KETO-$PGF_{1\alpha}$ PRODUCTION IN NRK-49F CELLS BY TGF-α AND TPA

Agonist	Concentration nM	PGE_2 ng/ml[a]	6-keto-$PGF_{1\alpha}$ ng/ml[a]
MEM	---	0.19 ± 0.030 (4)	0.15 ± 0.024 (4)
TGF-α	1.8	0.36 ± 0.051 (4)	0.44 ± 0.012 (4)
TPA	c	0.40 ± 0.002 (6)	0.60 ± 0.029 (6)
TPA[c] + TGF-α	1.8	3.06 ± 0.126 (4)[b]	5.22 ± 0.714 (4)[b]

[a] Data represent the means ± SEM for the number of dishes in parentheses.
[b] Significantly different from TPA or TGF-α ($P < 0.01$).
[c] TPA, 8.4 nM.

protein kinase C (19). TGF-α synergized prostaglandin production ($P < 0.01$) in the rat liver cells in the presence of OAG (Table 4).

DISCUSSION

The response of the cells to TGF-α and TGF-β was dependent on the cell type. TGF-α synergistically stimulated arachidonic acid metabolism in the presence of TPA in rat liver and rat kidney cells in culture; TGF-β, at least at the highest concentration used, did not. On the other hand, TGF-β synergistically stimulated prostaglandin production in the presence of TPA in human lung carcinoma cells more effectively than did TGF-α. Both TGF-α and TGF-β have been found to stimulate bone resorption as assessed by ^{40}Ca release from and prostaglandin production by mouse calvaria (11). The synergistic stimulations of arachidonic acid metabolism by the C-9 and NRK-49F cells are occurring at or before the formation of the cyclooxygenase catalyzed intermediate,

TABLE 3. SYNERGISTIC STIMULATION OF PGE_2 PRODUCTION IN HUMAN LUNG CARCINOMA (A-549) CELLS BY TGF-β AND TPA

Agonist	Concentration nM	PGE_2[a] (ng/ml)
MEM	---	<0.01
TGF-α	1.8	<0.01
TGF-β	0.1	<0.01
TPA	c	0.66 ± 0.059 (6)
TPA + TGF-α	1.8	0.72 ± 0.035 (3)
TPA[c] + TGF-β	0.1	3.20 ± 0.000 (3)[b]
TPA[c] + TGF-β	0.02	1.17 ± 0.024 (3)[b]

[a] Data represent the means ± SEM for the number of dishes in parentheses.
[b] Significantly different from TPA or TGF-β ($P < 0.01$).
[c] TPA, 17 nM.

TABLE 4. STIMULATION OF 6-KETO-$PGF_{1\alpha}$ AND $PGF_{2\alpha}$ PRODUCTION IN RAT LIVER CELLS BY TGF-α IN THE PRESENCE AND ABSENCE OF OAG

Agonist	Concentration nM	6-keto-$PGF_{1\alpha}$[a] ng/ml	$PGF_{2\alpha}$[b]
MEM	---	0.08 ± 0.013 (3)	<0.014
TGF-α	1.8	0.21 ± 0.031 (3)	<0.014
TGF-α	0.4	0.16 ± 0.016 (3)	---
OAG	c	0.24 ± 0.015 (3)	0.017
OAG[c] + TGF-α	1.8	2.32 ± 0.208 (3)[d]	0.080
OAG[c] + TGF-α	0.4	0.86 ± 0.039 (3)[d]	---

[a] Data represent means ± SEM for the number of dishes in parentheses.
[b] Data are means of duplicate analyses of pools of 3 dishes. Analyses agreed within 10%.
[c] OAG, 62 μM.
[d] Significantly different from OAG or TGF-α ($P < 0.01$).

the endoperoxide, as all products of the endoperoxide metabolism were stimulated in a similar manner. In the rat liver C-9 cells, the increased endoperoxide formation reflects stimulated liberation of arachidonic acid (Table 1), probably resulting from increased phospholipase A_2 activity. However, the possibility that cyclooxygenase activity also is being stimulated cannot be excluded. The increased phospholipase A_2 activity appears to be mediated by TPA's activation of protein kinase C, since the TGF-α's synergistic stimulation of prostaglandin production was also seen with OAG, a derivative of the physiologically activated diacylglycerol that activates protein kinase C (19). Thus, the prostaglandin-stimulating activities of the TGFs may be important physiologically since their activity can accompany generation of the putative second messenger, diacylglycerol, an intermediate in cellular signal transmission pathways (18).

Many of the pleiotropic effects of TPA are thought to result from perturbation of membrane structure (20). By binding to their specific receptors, TGF-α and the TGF-β not only are perturbing the membrane structure but also are regulating cellular processes and also stimulating deesterification of cellular lipids. We are not postulating any role for the pharmacologically-active products of the arachidonic acid metabolic cascade in the regulatory processes, just altered membrane properties that may accompany stimulation of the deesterification pathway. Drugs that block cyclooxygenase or lipoxygenase activities may have no effect on processes that accompany altered membrane

properties (possibly transformation) but drugs that effect deesterification pathways, e.g. corticosteroids (21), may.

Several observations have encouraged considerable speculation on the relationship between prostaglandins and cancer (8). Increased prostaglandin levels have been found in blood and/or urine of animals carrying neoplasms, as well as in conditioned media of transformed cells growing in tissue culture. In addition, it has been shown that arachidonic acid metabolism is associated with tumor promotion. Nevertheless, a causal relationship has never been demonstrated. Such associations could have resulted from increased growth factor activities. Recent studies have demonstrated sequence homologies between growth factors, growth factor-receptors and certain oncogene products (22-23). Increased production of cellular oncogenes that regulate, directly or indirectly, growth processes could have led to greater arachidonic acid metabolism.

ACKNOWLEDGEMENTS

We would like to thank Dr. Rik Derynck, Genentech, Inc., South San Francisco, CA, and Dr. Anita B. Roberts, National Cancer Institute, Bethesda, MD, for the preparations of TGF-α and TGF-β. This work was supported by Grant GM 27256 from the National Institutes of Health. L.L. is an American Cancer Society Research Professor of Biochemistry (Award PRP-21). Publication 1555 of the Department of Biochemistry, Brandeis University, Waltham, MA 02254.

REFERENCES

1. Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B. De Larco, J.E., and Todaro, G.J. (1980) Proc. Natl. Acad. Sci. USA 77, 3494-3498.
2. Roberts, A.B., Anzano, M.A., Meyers, C.A., Wideman, J., Blacher, R., Pan, Y.E., Stein, S., Lehman, S.R., Smith, J.M., Lamb, L.C., and Sporn, M.B. (1983) Biochemistry 22, 5692-5698.
3. Coughlin, S.R., Moskowitz, M.A., Antoniades, H.N., and Levine, L. (1981) Proc. Natl. Acad. Sci. USA 78, 7134-7138.
4. Dayer, J-M., Robinson, D.R., and Krane, S.M. (1977) J. Exp. Med. 145, 1399-1404.
5. Horowitz, A.D., and Weinstein, I.B. (1983) in Growth and Maturation Factors (Guroff, G., ed.) pp. 151-191, John Wiley and Sons, New York.
6. Levine, L., and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 76, 1181-1187.

7. Krane, S.M., Goldring, S.R., and Dayer, J-M. (1982) in Lymphokines, Vol. 7 (Pick, E., and Landy, M., eds.) pp. 75-136, Academic Press, New York.
8. Levine, L. (1981) Adv. Cancer Res. 35, 49-79.
9. Levine, L. (1982) in Carcinogenesis Vol. 7 (Hecker, E. et al., eds.) pp. 477-494, Raven Press, New York.
10. Tashjian, A.H., Jr., and Levine, L. (1978) Biochem. Biophys. Res. Commun. 85, 966-975.
11. Tashjian, A.H., Jr., Voelkel, E.F., Lazzaro, M., Singer, F.R., Derynck, R., Winkler, M.E., and Levine, L. Proc. Natl. Acad. Sci. USA, in press.
12. Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M., and Sporn, M.B. (1983) J. Biol. Chem. 258, 7155-7160.
13. Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y., and Goeddel, D.V. (1984) Cell 38, 287-297.
14. Rigas, A., and Levine, L. (1984) J. Pharmacol. Exp. Therap. 231, 230-235.
15. Snoek, G.T., and Levine, L. (1983) Cancer Res. 43, 4743-4746.
16. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikawa, U., and Nishizuka, Y. (1982) J. Biol Chem. 257, 7847-7851.
17. Nishizuka, Y. (1983) Trends Biochem. Sci. 8, 13-16.
18. Berridge, M.J. (1984) Biochem. J. 220, 345-360.
19. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701-6704.
20. Weinstein, I.B., Mufson, R.A., Lee, L.S., Fisher, P.B., Laskin, J., Horowitz, A, and Ivanovic, V. (1980) in Carcinogenesis: Fundamental Mechanisms and Environmental Effects (Pullman, B., Ts'o, P.O.P., and Gelboin, H., eds.) R. Reidel Publishing Co., pp. 543-563, Amsterdam, The Netherlands.
21. Hong, S.L., and Levine, L. (1976) Proc. Natl. Acad. Sci. USA 73, 1730-1734.
22. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M.D. (1984) Nature (Lond.) 307, 521-527.
23. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J.S., and Deuel, T.F. (1983) Nature (Lond.) 304, 35-39.