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LACK OF CORRELATION BETWEEN TPA-INDUCED PROSTAGLANDIN BIOSYNTHESIS AND ORNITHINE DECARBOXYLASE ACTIVITY IN BALB/c MOUSE 3T3 FIBROBLASTS

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12-O-tetradecanoylphorbol-13-acetate (TPA) induced in Balb/c 3T3 cells an earliest prostaglandin biosynthesis and an ornithine decarboxylase activation, this time-relation being more evident if serum was added to incubation medium in low concentration (0,2%). However the two TPA-induced events can be almost totally dissociated by pharmacological means, such as indomethacin and calcium-ionophore A23187 which affected PG response to TPA, but did not influence ODC induction.

Tumor promotion has been studied extensively in non-human systems mostly with mice or mouse cell cultures.(1)

TPA, the most active derivative among the phorbol esters tumor promoters, affects a large number of biological systems, where it elicits a variety of cellular responses.(1)

One of the pleiotropic biochemical effects of TPA is increased prostaglandin biosynthesis; in fact TPA is a potent inducer of in vivo and in vitro arachidonic acid release from cellular phospholipids and of cycloxygenase and lipoxygenase metabolites synthesis in mouse skin (2) and in epidermal cells (3) (4) as well as in non-epidermal cells such as fibroblasts (5), cultured rat liver cells (6) and Madin-Darby canine kidney cells (7).

Also the activity of ornithine decarboxylase, the first and rate-limiting enzyme in polyamine biosynthesis, is in general dramatically increased by growth stimuli, like tumor promoters, serum etc. (8) (9); this hyperplastic response depends on a short pulse of prostaglandin E_2 synthesis occurring in epidermis within 10 minutes after TPA topical exposure (10).

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The most compelling evidence that prostaglandin production is involved in the process of tumor formation has come from inhibition of tumor formation in vivo. Several cycloxygenase inhibitors like indomethacin, are capable of inhibiting tumor promotion by TPA, as well as TPA-induced ODC activity and DNA synthesis (11) (12). This inhibition can be overcome by PGE_2 (12).

Furthermore PGF_2 was reported to induce the initiation of DNA synthesis either by itself, as in quiescent 3T6 and Swiss 3T3 cells, or synergistically with growth factor such as epidermal growth factor (EGF) (13).

In order to study the importance of inflammation-related cell proliferation in tumor promotion, we have determined whether the in vivo postulated biochemical correlation between TPA-induced prostaglandin biosynthesis and ODC activity, a well known tumor promoter-related event, occurs in vitro in Balb/c mouse 3T3 fibroblasts.

METHODS

Chemicals

TPA, indomethacin, calcium ionophore A23187, pyridoxal-5'-phosphate, di-thiothreitol, L-ornithine, prostaglandin E_2, $F_{2\alpha}$, 6-oxo-prostaglandin $F_{1\alpha}$ and thromboxane B_2 were purchased from Sigma (Sigma Chemical Co St. Louis, Mo, USA), D,L-1-^{14}C -ornithine (60 mCi/mmol) from Amersham (Bucks. UK). Other chemicals were analytical or reagent grade.

Cell cultures

Balb/c mouse 3T3 (HG3 clone) cells were cultured in minimum Eagle's essential medium (MEM), supplemented with 10% (v:v) fetal calf serum (FCS; Gibco Laboratories Grand Island, N.Y.), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM tricine buffer and 1% (v:v) non essential aminoacid solution.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cells for experimental purposes were harvested with trypsin/EDTA and seeded at a density of about 4 X 10^5 cells in 35 mm plastic culture dishes (Corning, Glassworks Amedfield, MA, USA) and grown in 1,5 ml of MEM containing 10% FCS at 37°C for 3 days until confluency was reached (2 X 10^6 cells/dish). The medium was changed daily.

For all experiments the medium was replaced by 1 ml of MEM added FCS in different percentage and containing either 0,5% acetone (as control) or a solution of TPA in acetone and indomethacin or A23187 dissolved in saline or ethanol 0.5% respectively.

Determination of prostaglandin release

Prostaglandin E_2 and I_2 (as stable hydration metabolite 6-oxo-prostaglandin- $F_{2\alpha}$) were measured directly in the medium by a specific radioimmunoassay described by Pomerantz (16). Prostaglandin E_2, $F_{2\alpha}$, thromboxane B_2 and 6-oxo-prostaglandin-$F_{1\alpha}$ were assayed with gaschromatography mass spectrometry as described by Sautebin (17).

Determination of ornithine decarboxylase activity

ODC activity was assayed in intact living cells as described by Roseeuw (18). The results of the ODC determination are expressed as nmol $^{14}CO_2$/mg protein per 60 minutes.

Statistical evaluation

Statistical differences were calculated using one way analysis of variance (ANOVA) with the Dunnett's or Student's test.

RESULTS

The time-course of TPA (10^{-7} M) induction of ornithine decarboxylase activity in Balb/o 3T3 cells is shown in fig. 1. Peak activity of enzyme was found 4-6 h after TPA treatment. In the same experiment the medium was collected and the time-dependent PGs release was determined. Except for prostaglandin E_2, arachidonic acid metabolites (prostaglandin $F_{2\alpha}$, 6-oxo-prostaglandin $F_{1\alpha}$ and thromboxane B_2) were not detectable with GC-MS.

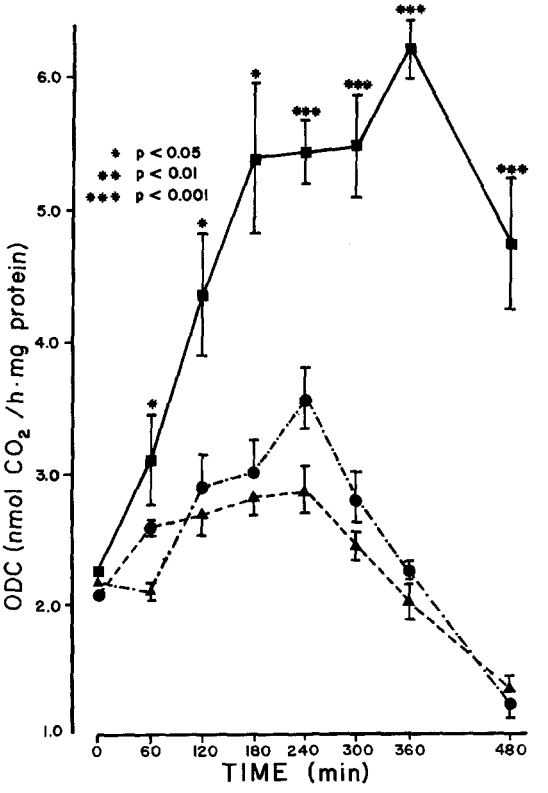

Fig 1. Effect of TPA on ornithine decarboxylase activity in 3T3 cells at different times after treatment.
All values are the average $\pm_{=7}$ S.E. of three determinations, (●) control; (▲) acetone 0,5%; (■) TPA 10^{-7} M.

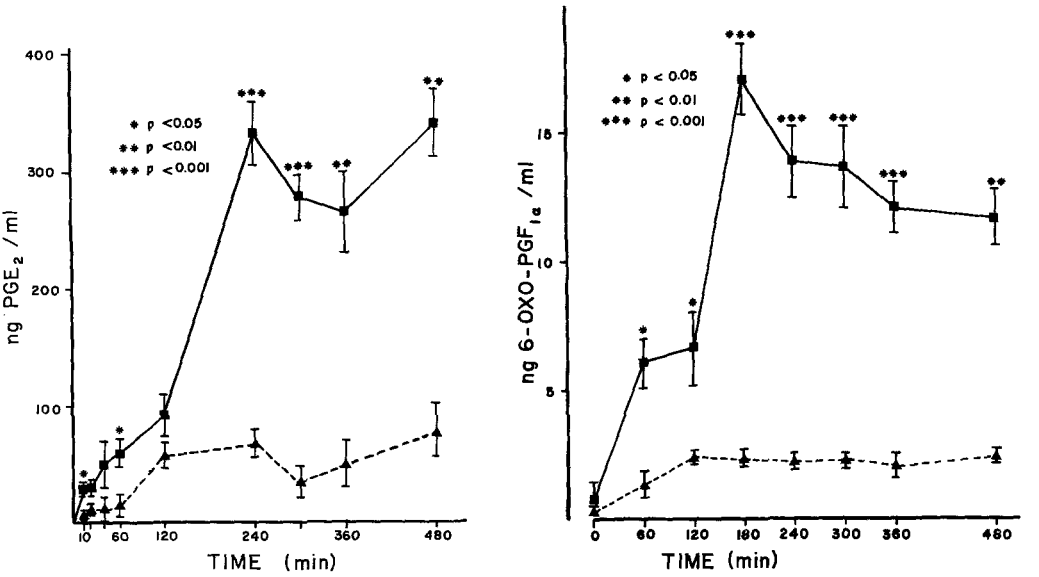

Fig 2. Effect of TPA on PGI_2 and PGE_2 synthesis in 3T3 cells at different times after treatment.
All values are the average ± S.E. of three determinations (▲) acetone 0,5%; (■) TPA 10^{-7} M.

In fig. 2 the profile of PGE_2 and PGI_2 release in the medium is presented as a function of time. After a lag-phase of 10-20 min. PGE_2 began to increase in TPA-treated cultures, both reaching a level of 400-700% of acetone control within 240 min.

TPA at concentration of 1 nM to 0,5 uM produced a dose-dependent induction of selected prostaglandin I_2 synthesis and ODC activity at 240 min. (fig. 3).

The influence of serum added to the medium on basal and induced enzyme activities can be seen in fig. 4 which shows that 0.2% FCS is sufficient to increase TPA stimulation of both activities.

Fig. 5 demonstrates the time-course of TPA stimulation on both systems in cultures incubated with MEM to which only 0.2% FCS had been added.

As compared with usual experimental conditions (10% FCS), a shift in the time of increased ODC activity was evident concomitantly with a similar lag-phase for PGI_2 synthesis.

Treatment of the cells, in these conditions (0,2% FCS) with indomethacin (5×10^{-7} M) caused a strong inhibition of basal and TPA-induced release of PGI_2 and PGE_2 in the medium, but only slightly affected ODC induction, measured

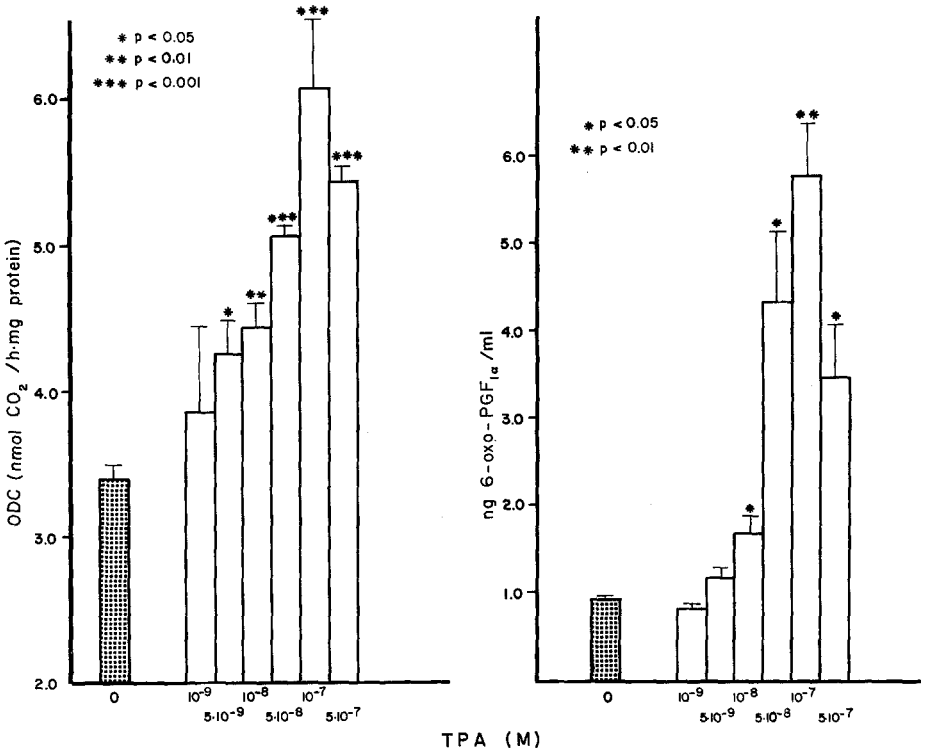

Fig 3. Ornithine decarboxylase activity and PGI_2 synthesis induced by different doses of TPA in 3T3 cells, 240 minutes after treatment. All values are the average ± S.E. of three determinations.

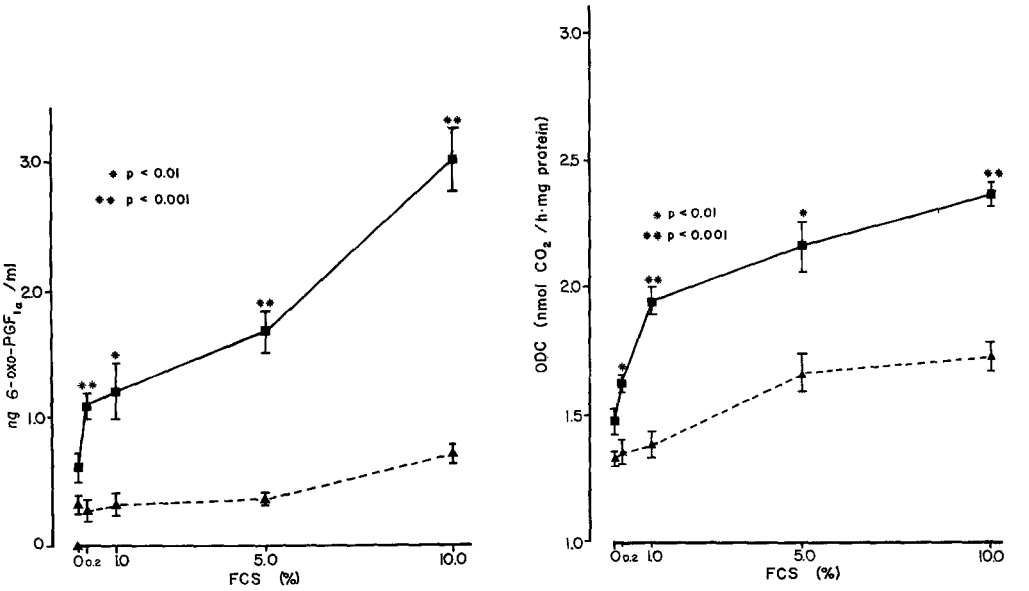

Fig 4. Effect of serum (FCS) added to the incubation medium on TPA-induced ornithine decarboxylase and PGI_2 synthesis in 3T3 cells. All values are the average ± S.E. of three determinations (▲) acetone 0,5%; (■) TPA 10^{-7} M.

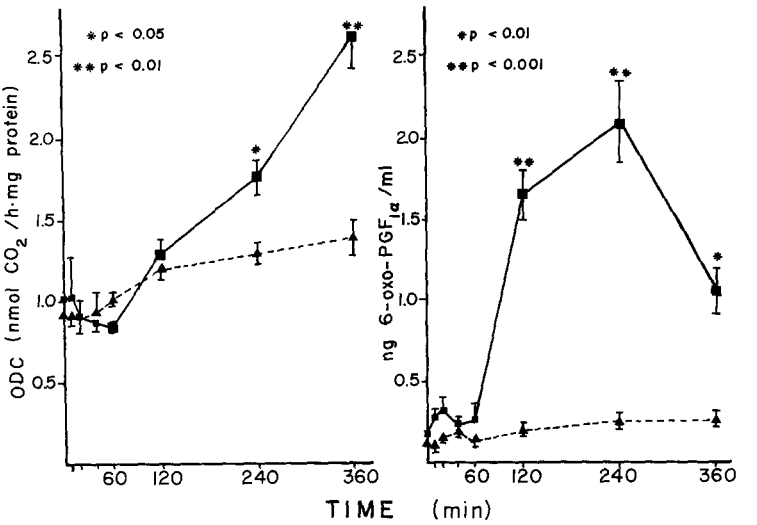

Fig 5. Time-course of ornithine decarboxylase activity and PGI_2 synthesis induced by TPA in 3T3 cell in presence of 0.2% serum in the medium. All values are the average ± S.E. of three determinations (▲) acetone 0.5%; (■) TPA 10^{-7} M.

in lysate cell supernate fractions, only 6 h after TPA exposure (Table 1). Also the ionophore A23187 (10^{-6} M) did not also influence ODC activity in control and TPA exposed cells, although PGE_2 and I_2 biosynthesis was considerably increased in both cultures (Table 1).

DISCUSSION

In the skin, prostaglandins and other arachidonic acid derived compounds have been shown to be involved in inflammatory process (14), to influence epidermal growth with respect to wound repair (15) and to play a critical role in the induction of epidermal proliferative processes by means of a variety of mechanical and chemical stimuli (2).

Induction of arachidonic acid release and prostaglandin synthesis in response to promoters has been postulated to be causal in several mitogen responses (2).

A variety of growth factors enhances PG synthesis in cultured cells; these include epidermal growth factor, platelet-derived growth factor (19) and serum factors in Swiss 3T3 cells (9).

FCS added to the medium potentiates TPA effects, probably owing to its content of growth factors; this might be explained bya synergism between the promoter and the serum.

Table 1: **Effects of indomethacin and Calcium-ionophore A23187 of TPA-induced ornithine decarboxylase activity and PGI_2 and PGE_2 biosynthesis in 3T3 cells**

TIME (min)	TREATMENT ng/ml	6-oxo-$PGF_{1\alpha}$ ng/ml	PGE_2 ng/ml	ODC activity nmoli CO_2/h.mg prot.
0	Control[a]	N.D.[b]	0.27±0.11	0.19±0.02
240	Control	0.35 ± 0.01	0.70 ± 0.05	0.37 ± 0.02
	Indomethacin[c]	0.18 ± 0.01	0.23 ± 0.02	0.33 ±.0.06
	A 23187[d]	0.94 ± 0.01	2.35 ± 0.87	0.38 ± 0.03
	TPA[e]	1.27 ± 0.06	22.10 ± 1.36	1.02 ± 0.06
	TPA + Ind.	0.14 ± 0.02	0.23 ± 0.02	1.07 ± 0.13
	TPA + A23187	9.99 ± 3.0	52.63 ± 5.11	1.08 ± 0.11
360	Control	0.63 ± 0.18	0.73 ± 0.08	0.23 ± 0.03
	Indomethacin[c]	0.14 ± 0.01	0.06 ± 0.01	0.20 ± 0.03
	A23187[d]	1.39 ± 0.11	1.40 ± 0.24	0.22 ± 0.02
	TPA[e]	1.38 ± 0.39	21.33 ± 0.62	1.45 ± 0.13
	TPA + Ind	0.16 ± 0.03	0.10 ± 0.04	0.92 ± 0.06
	TPA + A23187	4.49 ± 0.79	47.53 ± 3.07	1.53 ± 0.16

All values are the average ± S.E. of at least three determinations. (+) p 0.05; (++) p 0.01; (+++) p 0.001 V.S control; (*) p 0.05; (**) p 0.01; (***) p 0.001 V.S. TPA.
a) acetone 0.5%, (b) non-detectable, (c) indomethacin $5x10^{-7}$ M, (d) calcium-ionophore 10^{-6} M, (e) TPA 10^{-7} M.

Our studies demonstrate that TPA triggers a cascade of events in Balb/c 3T3 cells, involving an earliest prostaglandin biosynthesis and an ornithine decarboxylase activation.

The time-relation between these two biochemical events is more evident if the experiments are performed using only 0,2% FCS in the medium in order to minimize serum influence on these systems.

However, in these particular conditions, treatment of cells with indomethacin and A23187 which affected TPA-induced PG biosynthesis, did not influence ODC induction.

These results show that the two TPA-induced events can be almost totally dissociated by pharmacological means.

A similar uncoupling of phorbol-stimulated prostaglandin release from ODC induction, as well as DNA synthesis, has been previously observed in epidermal cell cultures (3,4).

This is however in contrast with the situation observed in mouse skin in vivo (21).

Possible explanations for this lack of correlation between the biochemical events are that other messengers, other than prostaglandins which are unable to stimulate cell division in 3T3 cells (22), trigger cell proliferation subsequent to TPA administration, and/or that prostaglandins could be only amplifiers rather than mediators of TPA actions in vivo (23).

Tumor promoting phorbol diesters directly interact with many receptor systems which have been implicated as regulatory elements in cellular control of growth and proliferation: phospholipid and Ca^{2+}-dependent protein kinase C (24), adenylate cyclase (25), glucocorticoid (26) and growth factor (27) receptors. TPA also influences G_1-chalone functionality (28).

Cyclic AMP, glucocorticoids and G_1-chalone generally act as antiproliferative hormonal factors on mouse epidermis (29). A concomitant tissue refractiveness to these endogenous agents can create a favorable situation for the TPA induced hyperplastic response, including enhanced ODC activity.

On the other hand phospholipases A_2 activity induction, a Ca^{2+}-dependent event (30), could be related to a rapid association of protein kinase C with the plasma membrane induced by TPA treatment producing an increased arachidonic acid release from membrane phospholipids, followed by a rise in its metabolites.

Thus prostaglandin synthesis and ODC activity could constitute separate mitogenic signals which are elicited by different second messengers, probably phosphorylated proteins.

Further studies are in progress to determine whether the lipoxygenase derivated compounds of arachidonic acid in vitro behave in the same way as the cycloxygenase metabolites in relation to TPA-induced hyperproliferative events.

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