Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 103-110

THE TUMOR PROMOTER 12-O-TETRADECANOYLPHORBOL-13-ACETATE STIMULATES LACTATE PRODUCTION IN BALB/c 3T3 PREADIPOSE CELLS

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Received March 2,1979

SUMMARY

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), which reversibly inhibits the adipose conversion of BALB/c 3T3 preadipose cells, increases lactate production by these cells. The stimulation of lactate production requires 4-7 days for optimal effect. Once TPA is removed from the cultures, the rate of lactate production falls to control levels. The concentration dependence for the TPA-mediated stimulation of lactate production is similar to that for its inhibitory effect on adipose conversion. Exogenous lactate in the absence of TPA also inhibits adipose conversion. These results suggest that the ability of TPA to interfere with the normal pattern of glucose metabolism may be important in the inhibitory effect of TPA on triglyceride accumulation in these cells.

INTRODUCTION

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)* has been shown to inhibit the terminal differentiation of several cell types *in vitro*, including Friend erythroleukemia cells (1, 2), chicken myoblasts and chondroblasts (3, 4), and murine neuroblastoma cells (5). We have previously reported (6) that TPA and other phorbol diester tumor promoters also reversibly inhibit the "adipose conversion" of a clone (A31T) of BALB/c 3T3 fibroblasts. These cells, and similar lines of Swiss 3T3 cells developed by Howard Green and colleagues (7), cease to divide upon reaching confluence and begin to accumulate triglyceride in their cytoplasm. They eventually acquire the morphological and biochemical characteristics of adipose cells *in vivo* (8-10).

This report documents a specific biochemical change induced in these cells by TPA: the stimulation of lactate production. The preadipose cells after reaching confluence are characterized by a low and stable saturation

*Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate.

0006-291X/79/090103-08$01.00/0

density and a low rate of lactate production. The ability of TPA to stimulate lactate production in these cells may be another example of how the promoter can induce untransformed cells to assume phenotypic properties usually associated with transformed cells (11, 12). However, in this system glucose in the culture medium presumably serves as a precursor of both lactate and the triglyceride accumulated by the cells, and so we have begun to explore the possibility that the change in the pattern of glucose metabolism caused by TPA could be involved in its inhibitory effect on this type of cell differentiation *in vitro*.

MATERIALS AND METHODS

BALB/c 3T3 (clone A31T) cells (6) were grown in Auto-Pow minimum essential medium (Flow Laboratories, Rockville, Md.) supplemented with vitamins as formulated for Eagle's basal medium and 10% heat-inactivated fetal bovine serum (Reheis Chemical Co., Chicago, Ill.). Stock cultures were passaged by trypsinization when subconfluent and were reconstituted from frozen stocks approximately every 10 passages. For experiments, cells were inoculated at a density of 2.4×10^4 cells/cm^2 in 60 mm dishes and refed with the same medium supplemented with insulin (1 μg/ml) every 3-4 days.

Lactate was determined on deproteinized samples of culture medium by the enzymatic procedure of Hohorst (13). In some experiments, medium containing fetal bovine serum exhaustively dialyzed against phosphate-buffered saline was used to eliminate the lactate contributed by this source.

The number of triglyceride-containing cells per 4 mm^2 was scored by microscopic observation without staining at regular intervals and expressed as a percentage of total cells after trypsinizing the cells and counting them in an electronic cell counter.

RESULTS

The pattern of lactate production, as measured by its concentration in the medium, during adipose conversion of control cultures and of cultures treated continuously with TPA beginning at various times after reaching confluence is shown in Fig. 1. From a level of 5 mM produced during the growth of the cells to confluence, the lactate concentration in the control cultures gradually fell to very low levels as the cells converted to adipocytes. The addition of TPA (1.6×10^{-7} M) to control cultures at any time always resulted in an increased medium concentration of lactate, but it is clear that the greatest stimulation occurred when TPA was added at the first or second medium change. TPA was also most effective at inhibiting

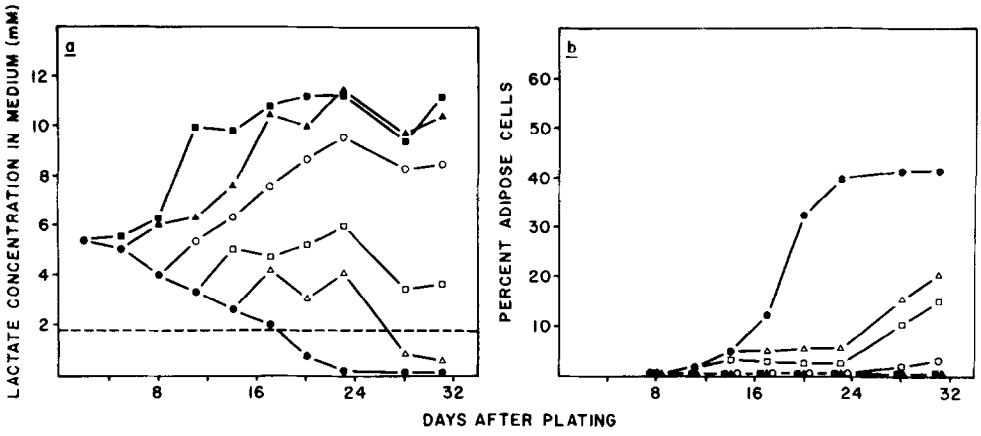

Figure 1: Lactate concentration of the culture medium during adipose conversion of BALB/c 3T3 cells in the presence and absence of TPA. Cells were plated at 5 x 10^5 cells/60 mm dish in 5 ml medium. On day 2, all cells were refed with control medium (●). TPA (1.6 x 10^{-7} M) was added at refeeding on day 2 (■), day 5 (▲), day 8 (o), day 11 (□), or day 14 (Δ) and continued for the duration of the experiment. The lactate concentration (a) was measured in the spent media removed at each refeeding from duplicate cultures as described in Materials and Methods. The same cultures were also scored for the percentage of adipose cells (b). The dashed line in (a) represents the medium concentration of lactate (from fetal bovine serum).

the adipocyte conversion when it was added at these times (Fig. 1b). Eventually some of the promoter-treated cultures will "break through" the inhibition caused by TPA and begin to differentiate (6); in the experiment shown in Fig. 1, this occurred only when TPA was added at later times after plating.

If cultures were exposed to 1.6 x 10^{-8} M TPA instead of the standard concentration of 1.6 x 10^{-7} M, the stimulation of lactate production was not as great nor as long lasting and adipose conversion was only slightly delayed, while a concentration of 1.6 x 10^{-9} M had no effect on either lactate production or adipose conversion (data not shown).

The increased production of lactate caused by TPA is reversible (Fig. 2a); if cultures treated for various lengths of time are washed and incubated in TPA-free medium, the lactate concentration in the medium gradually falls, and the number of triglyceride-containing cells increases to the level of control cultures never exposed to TPA (Fig. 2b). TPA is thus not caus-

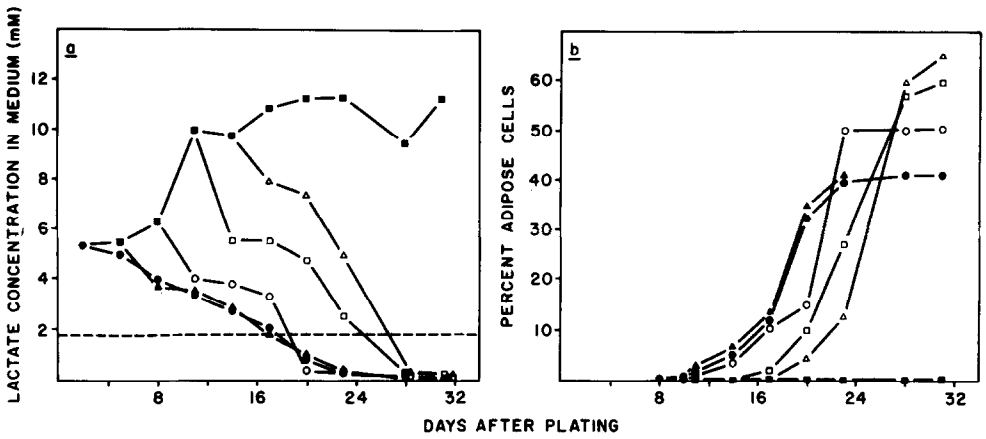

Figure 2: Reversibility of the TPA-mediated stimulation of lactate production and inhibition of adipose conversion. Some of the cultures treated with TPA beginning at day 2 in the experiment shown in Fig. 1 were washed and incubated in TPA-free medium after 3 (▲), 6 (o), 9 (□), and 12 (Δ) days in TPA-containing medium. The lactate concentration of media samples from duplicate dishes (a) and the percentage of adipose cells (b) were determined.

ing a permanent alteration or "transformation" of these cells.

Several points about the data depicted in Figs. 1 and 2 should be emphasized. 1) When TPA is most effective at stimulating lactate production, the conversion of medium glucose to lactate is very efficient. The glucose concentration of the medium is 1 g/L or 5.5 mM. If each glucose molecule is converted to two molecules of lactate, the theoretical limit for the lactate concentration in the medium = 11.0 mM + 1.8 mM (serum contribution) or 12.8 mM. Since the lactate concentration can reach 11.5 mM in TPA-treated cultures, this represents a 90% conversion of glucose to lactate. 2) TPA stimulates appreciable lactate production only when adipose conversion is inhibited. Once the cells have "broken through" the inhibitory effect of TPA, the lactate concentration of the medium falls to low levels (Fig. 1 and unpublished observations). 3) When large numbers of fat cells are present, there is a net utilization of lactate by these cultures, for the medium concentration falls below the level contributed by the serum. More than 75% of the lactate utilized by the cells under these conditions

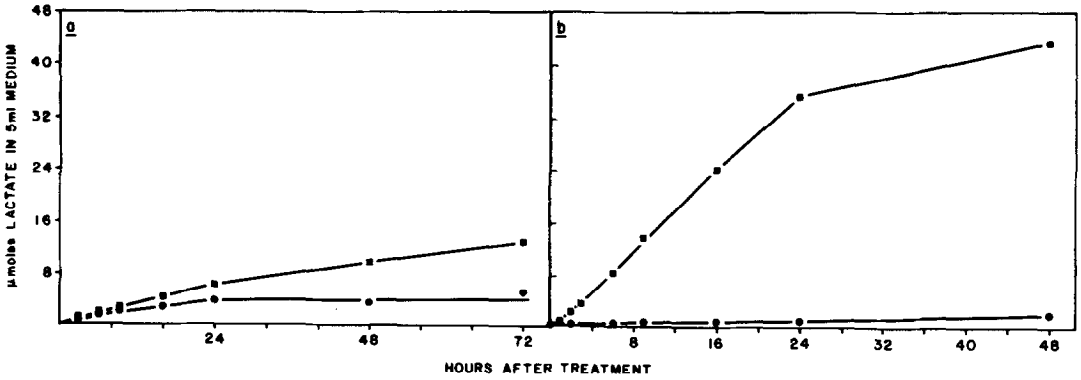

Figure 3: The effect of TPA on lactate production in BALB/c 3T3 cells following the (a) first and (b) fourth exposures to TPA. Cells were plated at 5×10^5 cells/60 mm dish in 5 ml standard medium and (a) refed 4 days later without TPA or (b) refed 2, 4 and 7 days later with standard medium with or without TPA (1.6×10^{-7} M). After an additional 3 days in both cases, the cultures were washed twice with warm phosphate-buffered saline and refed with 5 ml Auto-Pow minimum essential medium containing 10% dialyzed fetal bovine serum with (■) or without (●) TPA (1.6×10^{-7} M). Media was harvested from duplicate dishes at the indicated times and lactate concentration determined.

is converted to triglyceride, as estimated from the incorporation of [^{14}C]lactate following a 24-hr pulse (data not shown).

The kinetics of lactate production following the first and fourth refeeding with TPA are illustrated in Fig. 3. To increase the sensitivity of the lactate determinations, these experiments were done in medium containing dialyzed fetal calf serum. TPA added for the first time to confluent cultures containing few, if any, fully differentiated cells increased only slightly the rate of lactate production above that of control cultures during a 3 day period (Fig. 3a). The pattern of lactate production in cultures that had been treated with the promoter for 8 days is shown in Fig. 3b. At the time of the measurements, the control cultures had not started to accumulate large amounts of triglyceride. With only a slight lag period, TPA caused a dramatic increase in the rate of lactate production. Assuming that 1 µmole of glucose can be converted to 2 µmoles of lactate, in the presence of TPA 64% and 79% of the glucose in the medium was converted to lactate after 24 and 48 hr, respectively. In the control cultures never exposed to TPA, the corresponding values were 1.5 and 3.8%.

TABLE 1

EFFECT OF L (+) LACTIC ACID ON THE ADIPOSE CONVERSION OF BALB/c 3T3 CELLS

	Adipose cells/4 mm^2 [a,b]			
Treatment	Day 6	Day 8[c]	Day 10	Day 14
Control	15 ± 2	97 ± 22 (2.4)	131 ± 33	292 ± 52
10 mM Lactate	3 ± 1	7 ± 4 (15.7)	8 ± 1	5 ± 2
5 mM Lactate	3 ± 1	16 ± 2 (9.2)	37 ± 9	160 ± 25
1 mM Lactate	11 ± 1	26 ± 7	70 ± 3	275 ± 20
0.1 mM Lactate	15 ± 4	48 ± 6	111 ± 27	304 ± 40
TPA (1.6×10^{-7} M)	0	0 (7.3)	0	6 ± 2

Cells were plated at 5×10^5 cells/60 mm dish in 5 ml standard medium and the experiment was started 4 days after plating when the cultures had just reached confluence and no adipose cells were present. The cultures were refed with medium containing insulin (1 µg/ml) and the indicated additions at 3-4 day intervals and scored for the number of adipose cells at the indicated times after the start of the experiment.

[a]Numbers represent mean ± S.D. of three different fields.

[b]Cell density in TPA-treated cultures was approximately twice that in control and lactate groups, which had similar cell densities.

[c]Numbers in parenthesis are the lactate concentrations in the medium (in mM) as measured enzymatically.

The effect of exogenous lactate on the differentiation program of these cells was tested to determine if this compound *per se* could inhibit adipose conversion in the absence of TPA (Table 1). An exogenous lactate concentration of 10 mM, approximately the concentration attained in the presence of TPA, did inhibit adipose conversion; lower concentrations were progressively less effective. This inhibitory action of lactate does not appear to be related simply to the extracellular pH since buffering the culture medium at various pHs including pH 6.9, the approximate pH of medium containing 10 mM lactate, had no apparent effect on adipose conversion (data not shown).

DISCUSSION

These studies were undertaken to characterize in detail an incidental observation of ours that the tumor promoter TPA caused an increase in the

acidity of the medium of BALB/c 3T3 cell cultures whenever it was an effective inhibitor of their conversion to adipocytes. In addition, when on rare occasions an odd culture in an experiment exhibited a high spontaneous rate of lactate production in the absence of TPA, adipose conversion was greatly delayed or absent. These observations suggested that the ability of TPA to stimulate lactate production might be involved in its inhibitory effect on triglyceride accumulation in this system.

The stimulation of lactate production by TPA has these features: (i) it is not immediate, but requires 4-7 days for optimal effect (see Figure 3); (ii) it is reversible; (iii) the stimulation of lactate production is of lower magnitude and shorter duration when the addition of TPA is delayed until the cultures have undergone partial differentiation; and (iv) under optimal conditions, TPA-treated cells convert as much as 90% of the glucose in the medium to lactate over a 3-day period.

Glucose is the major precursor of the lactate produced by cells in culture (14, 15). 3T3 cells produce significant amounts of lactate during growth to confluence, but thereafter production falls as the cells cease to divide and begin to accumulate triglyceride (Fig. 1). TPA increases the saturation density of these cells (6) but once this higher plateau density is reached, the cells regain their density-dependent growth control. After the initial exposure to TPA and the increase in cell number 3-4 days later, there is no further cell number increase but the high rate of lactate production continues until either TPA treatment is discontinued (Fig. 2) or the cells "break through" the inhibition of differentiation. The greatly increased rate of lactate production in the presence of TPA cannot be explained, therefore, by a continuous growth stimulation by this agent.

Glucose is probably a major precursor of at least the glycerol moiety of triglyceride as well as lactate in these cells. In contrast, adipose tissues *in vivo* obtain significant amounts of fatty acids from blood lipoproteins (such as very low-density lipoproteins) in a reaction catalyzed

by the enzyme lipoprotein lipase (16). Under certain circumstances some preadipose cells in culture can utilize medium triglycerides as precursors for intracellular triglyceride (17, 18), but under the culture conditions described in this report this probably does not represent a major pathway. Thus, glycolysis is probably required for the generation of both lactate and the glycerol and fatty acid moieties of the triglyceride accumulated by these cells. The mechanism by which TPA alters the pattern of glucose catabolism so that triglyceride synthesis is inhibited and lactate production is increased is not known. An understanding of how this is accomplished should provide clues about the biochemical basis for the inhibition of differentiation by TPA in this system.

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