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THE ROLE OF SECONDARY MESSENGERS IN THE REGULATION OF LIPID PEROXIDATION IN RAT LIVER MICROSOMES

G.N. BALDENKOV*, E.A. SERBINOVA**, R.A. BAKALOVA**, V.A. TKACHUK*, V.E. KAGAN** and TS.S. STOYTCHEV**

**National Research Center for Cardiology, Academy of Medical Sciences USSR, Moscow 121552, USSR; **Institute of Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. 23, Sofia 1113, Bulgaria*

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The effect of phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C (PK-C) on lipid peroxidation (LPO) in rat liver homogenates and microsomes was studied. PMA (10^{-10} to 10^{-6} M) produced a concentration-dependent inhibition of LPO, which was greatly decreased by polymyxin B (Px B) (an inhibitor of PK-C). The non-active analogue of PMA, 4α-phorbol-12,13-didecanoate (4α-PDD) exerted no inhibitory effect. The adenylate cyclase activator, forskolin (FK) (10^{-6} M) abolished the inhibitory effect of PMA on LPO. PMA and FK did not inhibit LPO in liposomes. It is suggested that LPO in biomembranes could be regulated by PK-C, whose inhibitory effect might be prevented by cAMP-dependent protein kinases.

KEY WORDS: Lipid peroxidation, protein kinase C, phorbol-12-myristate-13-acetate, forskolin.

1. INTRODUCTION

The efficiency of many processes in the cell is controlled by the system of secondary messengers generated by specific receptor-enzyme complexes, which are localized in the cell membrane. The activity of these complexes (adenylate cyclase, guanylate cyclase, phosphodiesterase C) depends on external (hormones, growth factors, xenobiotics) and internal (substrates, metabolites) factors as well as on the physico-chemical state of biomembranes.[1–4] One of the processes determining the properties of membranes is lipid peroxidation (LPO). It is known that LPO could change the functional activity of the systems which generate secondary messengers, exerting both stimulant and inhibitory effects.[1,2,5–7] We, however, failed to find data about the action of secondary messengers on LPO.

The present work was undertaken to study the possibility of LPO regulation by secondary messengers. To this end, we examined the effects of the adenylate cyclase activator forskolin (FK), the calcium-phospholipid-dependent protein kinase (PK-C) activator PMA (a structural analogue of the secondary messenger diacylglycerol (DAG), and of the PK-C inhibitor polymyxin B (Px B) on LPO in rat liver microsomes and homogenates as well as in liposomes from rat liver lipids.

Correspondence should be addressed to V.E. Kagan.

2. MATERIALS AND METHODS

Male Wistar rats, weighing 150–180 g, were used. The liver microsomal fraction was isolated by differential centrifugation (10 000 g supernatant, 100 000 g × 60 min) after preliminary perfusion of the liver with 1.15% (w/v) ice-cold KCl and subsequent homogenization. In some cases, microsomes were isolated after preincubation of the homogenate with the substances tested (10 min at 30°C) and subsequent centrifugation. The fraction of total lipids was isolated by the method of Folch *et al.*[8] Liposomes were prepared by sonication of the lipid suspension in Tris-HCl buffer, 20 mM (pH = 7.4) (MSE 150 W) at 12 W up to clarification of the suspension.[9] Protein was measured by the biuret test, using bovine serum albumin as a standard. LPO was induced in the medium containing: $FeSO_4$ (20 μM), ascorbate (0.5 mM), Tris-HCl buffer (20 mM), pH = 7.4; the protein (lipid) concentration was 1 mg/ml. Incubation was conducted with constant stirring at 37°C. The LPO products were estimated spectrophotometrically by the TBA method (λ = 535 nm), the molar extinction coefficient (ε) being $1.56 \times 10^5\,M^{-1}\,cm^{-1}$ (10). PMA, FK, 4α-PDD and α-tocopherol (α-T) were dissolved in ethanol (the final concentration of solvent in the incubation medium was $\leqslant 1\%$, which has no influence on (Fe^{2+} + ascorbate)-induced LPO in rat liver microsomes.[11]

The reagents used were: Tris-HCl buffer, albumin (Reanal); ascorbate, α-tocopherol (Serva); 2-thiobarbituric acid (TBA) (Sigma); PMA, 4α-PDD, FK, Px B (Merck).

3. RESULTS AND DISCUSSION

Figure 1 shows the concentration-dependent inhibition of the non-enzymic (Fe^{2+} + ascorbate)-induced LPO by PMA in microsomes and homogenates of rat liver as well as in liposomes from liver lipids. At concentrations as low as 10^{-9} to 10^{-8} M the inhibitory effect of PMA on LPO was 25–45% in microsomes and 10–20% in homogenates. The weaker effects of PMA in the homogenates might be associated both with its hydrolysis and with the high dilution of PMA in all membrane fractions of the homogenate.[12]. The inhibitory effect of PMA was much more pronounced as compared to that of many well-known direct antioxidants. Thus, α-T at concentrations of 10^{-8} to 10^{-9} M produced no LPO inhibition in both liver homogenates and microsomes.[13,14] The inhibitory effect of α-T in microsomes isolated from homogenates preincubated with α-T (10^{-4} M) for 10 min at 30°C rapidly decreased after LPO induction and completely disappeared after 20-min induction (Table I). In contrast, the inhibitory effect of PMA on LPO remained unchanged for 10 min and even after 20 min 40% of the inhibition was still retained.

In liposomes PMA even at a concentration of 10^{-5} M gave no inhibitory effect on LPO. Under the same conditions α-T possesses a relatively high efficiency of LPO inhibition.[14] This suggests that the mechanisms of inhibitory action of PMA and α-T are quite different and that the inhibitory effect of PMA in rat liver homogenates and microsomes is not due to its direct antioxidant action.

It is known that PMA at concentrations of 10^{-8} to 10^{-9} M exerts a stimulant effect on PK-C. This leads to the suggestion that the effects of PMA on LPO are mediated by PK-C activation and subsequent phosphorylation of specific proteins able to inhibit LPO.

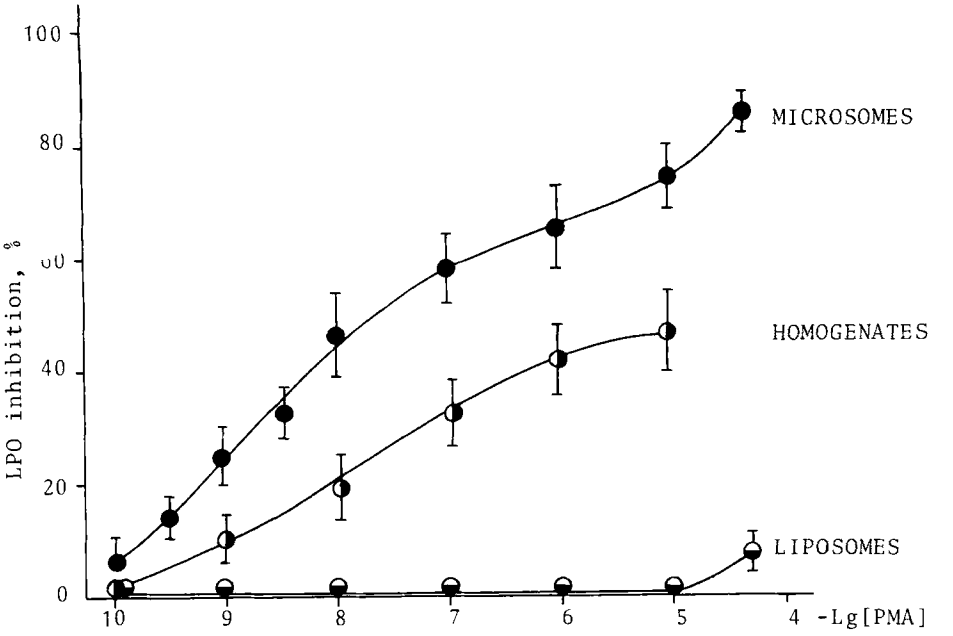

FIGURE 1 Inhibition of (Fe^{2+} + ascorbate)-dependent LPO (time of LPO induction — 10 min) by PMA in rat liver homogenates, microsomes and liposomes from total liver lipids (n* = 8). *n — number of separate experiments.

The participation of PK-C in the inhibitory effect of PMA was substantiated in the experiments using the analogue of PMA, 4α-PDD, which only slightly activates PK-C. When the liver homogenate was incubated with 4α-PDD at a concentration of 10^{-7} M its inhibitory effect on LPO was only 8.7% of the average of the effect of PMA at the same concentration and under the same conditions (Figure 2). The participation of PK-C in the PMA effects on LPO was confirmed by the experiments with Px B, a known inhibitor of PK-C.[15] The inhibitory effect of PMA (10^{-7} M) was much decreased in the presence of Px B (10^{-4} M) (Figure 2). Px B did not exert a direct antioxidant effect in microsomes or in liposomes.

The effects of the PK-C- and cAMP-dependent protein kinases on some physiological and biochemical processes as cell proliferation,[16,17] contractility,[18,19] activity of

TABLE I
Inhibition of non-enzymic LPO in rat liver microsomes isolated from homogenates preincubated with PMA and α-T

Time of LPO induction	LPO inhibition, %		
	PMA (10^{-7} M) n* = 8	α-T (10^{-7} M) n = 6	α-T (10^{-4} M) n = 6
5 min	72.0 ± 3.0	8.0 ± 0.5	53.0 ± 0.5
10 min	69.0 ± 2.0	0.0	48.0 ± 5.0
20 min	31.0 ± 0.5	0.0	0.0

*n — number of separate experiments.

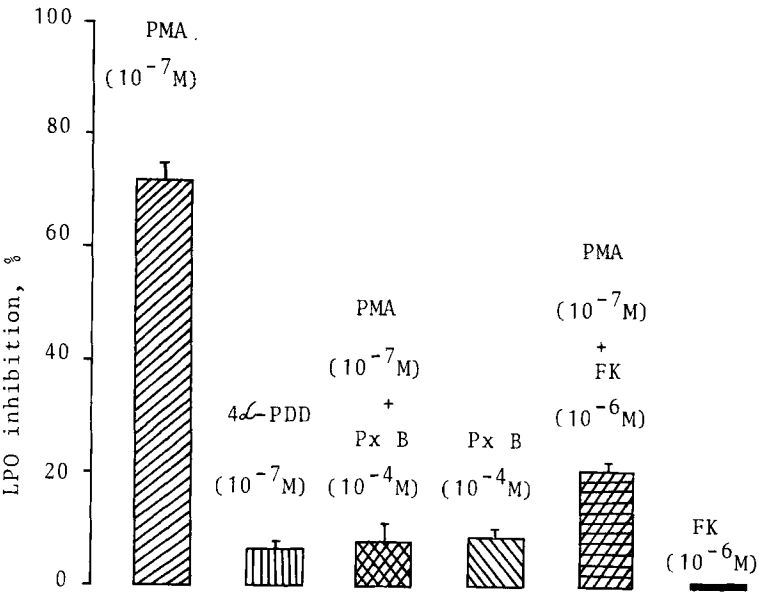

FIGURE 2 Inhibition of (Fe^{2+} + ascorbate)-dependent LPO (time of LPO induction − 10 min) in rat liver microsomes isolated from homogenates preincubated with the compounds tested for 10 min at 30°C (number of separate experiments = 8).

Ca^{2+} channels[20–22] and activation of neutrophils are opposite. Is this true also for the LPO regulation? To answer this question, we compared the effect of PMA with that of FK, an activator of adenylate cyclase. It turned out that FK at concentrations of 10^{-9} to 10^{-6} M did not change the LPO activity in homogenates and microsomes (isolated both with and without preincubation of the homogenates with FK). The addition of FK (10^{-6} M) and PMA (10^{-7} M) to the liver homogenate and subsequent preincubation for 10 min at 30°C (which is sufficient for adenylate cyclase activation) led to a great decrease of the inhibitory effect of PMA in microsomes isolated from the preincubated homogenate (Figure 2). It might be suggested that the stimulation of cAMP synthesis and the subsequent activation of cAMP-dependent protein kinases prevent the inhibitory effect of PMA on LPO in microsomes.

The results obtained strongly suggest that it is a matter of a new effect manifested in PK-C-mediated inhibition of LPO in biomembranes. This means that the LPO activity in the cell is probably controlled by the system of secondary messengers. PMA, an analogue of DAG, activating PK-C, inhibits LPO, while FK, stimulating adenylate cyclase and the cAMP-dependent reactions, prevents the inhibitory effect of PMA.

The effects observed are consistent with the data about participation of secondary messengers and antioxiodants in the regulation of the proliferative activity of the cells.[23,24] Phorbol esters, activating PK-C stimulate cell proliferation.[16,17,25] Furthermore, the present results showed that PMA could prevent the accumulation of LPO products, which are toxic for the dividing cells.[23,24]

There is evidence that cAMP inhibits the proliferative activity of the cells due to an activation of cAMP-dependent protein kinases, i.e. cAMP appears to be a natural

antagonist of the phorbol esters in this process. Our data suggest that this antagonism also exists with respect to the inhibitory effect of PMA on LPO in biomembranes. This should mean that under the conditions favoring the production of cAMP in the cell, cAMP would prevent the inhibitory effect of PMA on LPO. This could contribute to the increase of the inhibitory effect of the cAMP-dependent regulation on the cell proliferation. Thus the described effects of the secondary messengers on LPO enrich our knowledge of the mechanisms of regulation of the proliferative activity as well as of the control over the processes largely dependent on LPO, e.g. malignant transformation of the cells, atherosclerosis-related impairment of the vessels, microsomal metabolism of xenobiotics, etc.[26]

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