Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 345-352

ACTIVITY AND DISTRIBUTION OF PROTEIN KINASE C IN LIVER DURING THE ACUTE-PHASE RESPONSE

Roberta PICCOLETTI, Maria Giovanna ALETTI, Paola BENDINELLI, Danilo ARIENTI and Aldo BERNELLI-ZAZZERA

Istituto di Patologia Generale dell'Università degli Studi di Milano; Centro di Studio sulla Patologia Cellulare del CNR, Via Mangiagalli 31, 20133 Milano, Italy

Received January 11, 1990

Activity and subcellular distribution of protein kinase C were estimated in liver cytosol and membrane fractions of rats carrying a turpentine-induced inflammation. Protein kinase C activity increases significantly 8 h after treatment in the membrane fraction, with concurrent reduction in the cytosol; 10 h after treatment the membrane-associated activity returns to normal, without concomitant recovery of that detected in the cytosol. The specific binding of phorbol dibutyrate to the liver membrane fraction increases but overall the effect is less evident and delayed in time. The changes are associated to alterations in the phosphorylation pattern of some liver proteins. Liver protein kinase C activity and intracellular distribution seem to be affected by a treatment which is known to induce an acute-phase response in the liver cells.

The systemic reaction to tissue injury caused by either inflammation, infection or trauma, is referred to as the acute-phase response (1) and includes consistent increases in plasma concentration of some proteins synthesized by the liver, known as acute-phase proteins (APP). This response, which helps the organism to face and overcome adverse conditions, represents a useful model to study gene regulation in the liver cells. Substances released from the focus of inflammation, such as Interleukin-1 (IL-1) and Interleukin-6 (IL-6), start the chain of events that will finally lead to activation of APP genes (2, 3) but the steps between these inflammatory products and gene activation have not been clearly identified. External signals detected by surface receptors are translated into a limited repertoire of intracellular second messengers (4). In a previous work we have demonstrated the activation of arachidonate metabolism in the liver of rats

0006-291X/90 $1.50

carrying a turpentine-induced inflammation (5): in the present paper, we investigate possible changes in activity and/or intracellular distribution of protein kinase C, Ca^{2+} -sensitive and phospholipid-dependent protein kinase (PK-C), in the liver of rats carrying a turpentine-induced inflammation. Although the in vivo substrates of PK-C in liver remain to be defined, phosphorylation of definite proteins could be involved in APP gene activation, as described for other cell systems (6,7).

MATERIALS AND METHODS

Male Wistar rats of 250 g were used throughout. Inflammation was induced by subcutaneous injection of sterile turpentine, 0.5 ml/100 g body wt. Injections were done so that animals of all groups were killed between 9 and 10 a.m. Normal untreated rats were taken as controls after preliminary experiments showed that saline-injected rats did not undergo changes in PK-C activity at any time after treatment.

Preparation of soluble and particulate fractions. The liver was homogenized in buffer containing: 0.25 M sucrose; 20 mM Tris/HCl (pH 7.5); 2 mM dithiothreitol; 5 mM EDTA; 2 mM EGTA; 1 mM phenylmethylsulfonylfluoride; 20 μg/ml leupeptin; 1 μg/ml pepstatin A. The post-nuclear supernatant was centrifuged at 105.000 x g for 60 min to obtain a final supernatant (cytosol) and a particulate fraction (membranes). For the measurement of PK-C activity the membranes were dissolved in the buffer described above containing 0.5% Triton X-100, incubated in ice for 60 min and centrifuged to remove the undissolved material. For the phorbol 12,13-dibutyrate (PDBu) binding experiments the pellet resulting from the first centrifugation was carefully resuspended in 20 mM Tris/HCl (pH 7.5). These preparations are defined as "particulate fraction". The amount of protein in these fractions was estimated by the Bradford method (8).

Protein kinase C assay. It was performed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus histone H1 (type III-S from Sigma) (9). The reaction mixture (200 μl) contained: 20 mM Tris/HCl (pH 7.5); 7.5 mM $MgCl_2$; $CaCl_2$ 500 μM in excess of chelator concentrations; 50 μg histone; 25 μg phosphatidylserine; 1 μg diolein; 10 nmol ATP; 0.2 μCi [γ-^{32}P]ATP and the enzyme preparation to be assayed (5 μg protein). Basal activity was obtained by replacing $CaCl_2$ and phospholipids with 5 mM EGTA. The mixture was incubated for 10 min at 30°C: under these conditions the reaction proceeded linearly for at least 20 min and the activity was proportional to the amount of the enzyme. The reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid and 200 μg of bovine serum albumin. The precipitates were collected on Whatman GF/A glass fiber filters and counted in a liquid scintillation counter.

[^3H] PDBu-binding assay. The PDBu binding assay was performed with 100 μg particulate fraction incubated in a final vol. of 500 μl, containing: 20 mM Tris/HCl (pH 7.5); 1 mM dithiothreitol; 5 mM $MgCl_2$; 150 μM $CaCl_2$; 1 mg γ-globulin; 25 nM [^3H]PDBu in the absence (total binding) or presence of 500-fold excess of radioinert PDBu (non-specific binding), as described by Moruzzi et al. (10). The incubation was carried out at 25°C for 20 min. Bound PDBu was recovered by addition of polyethylene glycol (15% final concentration) and centrifugation at 16.000 x g for 15 min in an Eppendorf Microfuge: the pellet was finally dissolved in Protosol (New England Nuclear) and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the difference between total and non-specific binding. Saturation and Scatchard analysis of PDBu receptors was performed in

liver membranes from control and 8 h turpentine-treated rats, at 2.5-50 nM [^{3}H]PDBu.

Protein phosphorylation. Phosphorylation of endogenous membrane proteins was carried out as described above, but in the absence of histone. EGTA or $CaCl_2$ or $CaCl_2$ plus phospholipids were added at the concentrations indicated above. The reaction was stopped by the addition of buffer to reach final concentration of 2% SDS; 62.5 mM Tris/HCl (pH 6.8); 10% glycerol; 5% β-mercaptoethanol and 0.001% bromophenol blue; samples were analyzed by 10% polyacrylamide SDS gel electrophoresis (11) and autoradiography.

Statistical analysis was performed by Student's t test.

RESULTS

Effect of turpentine treatment on intracellular partitioning of protein kinase C. The subcellular fractions were used immediately after isolation. PK-C activity of control animals was in agreement with other authors (12). Experiments at earlier times of treatment revealed no changes (data not shown) but 8 h after turpentine injection the activity of PK-C increases significantly in the membranes, with a concurrent reduction of the activity measured in the cytosol, that becomes 59% of the control value (Table 1, A). Later on (10 h) the membrane-associated activity returns to the normal level, without any concomitant recovery of the cytosol-associated activity. As protein content of the cells might change during the acute-phase response, PK-C activity was calculated x g of tissue (Table 1, A).

Effect of turpentine treatment on [^{3}H]PDBu binding activity. PK-C associated with cell membrane components can also be assayed by [^{3}H]PDBu binding in the presence of physiological concentration of Ca^{2+} (10). Turpentine treatment increases the specific binding of PDBu to the particulate fraction of rat liver (Table 1, B) but overall the effect is less evident and it does not show a strict correlation with the trend of PK-C activity; at 8 h in fact the increase in binding is small and the level of significance is reached later on (10 h). The binding activity declines at 12 h, but is still significantly higher than normal when calculated x g of liver. Saturation and Scatchard analysis of PDBu binding by membrane-associated PK-C 8 h after treatment show that apparent Kd is the same as the control (5.6 ± 0.3 and 5.8 ± 0.3 nM, respectively), whereas the

Table 1. Effect of turpentine treatment on protein kinase C of rat liver
A : enzyme activity of particulate and cytosolic fractions
B : [^{3}H] phorbol dibutyrate binding to membranes

A

a = pmol [^{32}P]/mg protein/min
b = pmol [^{32}P]/g of liver/min

	Particulate fraction		Cytosolic fraction	
Time of treatment	a	b	a	b
Controls (12)	209 ± 17	6539 ± 451	140 ± 11	9438 ± 554
8 h (8)	349 ± 33*	10642 ± 779*	82 ± 7*	5285 ± 714*
10 h (10)	171 ± 12	6190 ± 348	72 ± 7*	5517 ± 684*
12 h (5)	223 ± 14	7983 ± 437	169 ± 13	11010 ± 812

B

a = pmol [^{3}H]PDBu/mg of protein
b = pmol [^{3}H]PDBu/g of liver

Time of treatment	a	b
Controls (7)	2.8 ± 0.16	86 ± 6.9
8 h (5)	3.52 ± 0.38	105 ± 13.4
10 h (6)	4.27 ± 0.41*	141 ± 13.7*
12 h (5)	3.25 ± 0.20	117 ± 6.7*

Activity and binding assayed as described under Materials and Methods. Means ± SEM, number of animals in parentheses. Negative results ($P > 0.05$) obtained 1.5, 3 and 6 hours after treatment are not reported in the table.
* significantly different vs. control: $P < 0.01$.

number of specific binding sites is higher (5.5 ± 0.4 and 4.6 ± 0.4 pmol/mg protein, respectively).

Effect of turpentine treatment on membrane protein phosphorylation. Addition of Ca^{2+} and phospholipids to the incubation medium results in an increased phosphorylation of several membrane proteins (Fig. 1). A similar pattern is observed for control and 8 h turpentine-treated rats, but in the latter the extent of phosphorylation is greater than in the former, in particular in the 60-80 kDa range. Proteins relevant to the present investigation, such as PK-C itself, which is capable of autophosphorylation (9), and receptors for IL-1

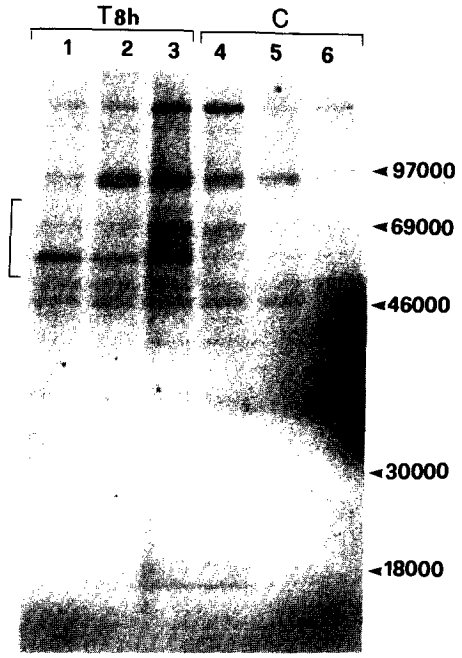

Fig. 1. Autoradiograph of SDS PAGE showing endogenous membrane protein phosphorylation from control (C) and 8 h turpentine-treated (T 8 h) animals. Assays were performed as described in Materials and Methods section and 12.5 μg of solubilized membrane protein were electrophoresed in each lane.

Lane 1,6: in the presence of EGTA
Lane 2,5: in the presence of Ca^{2+}
Lane 3,4: in the presence of Ca^{2+} + phospholipids

Mol. wt. markers are indicated on the right side: **bracket** on the left indicates the bands relevant to the present investigation.

(13) and IL-6 (14), are known to band in this range: it would be tempting, though entirely speculative, to relate the increased phosphorylation of these proteins to the changes of PK-C observed in the present experiments.

DISCUSSION

Our results provide direct evidence for an increase in PK-C activity after turpentine treatment of the animal that induces an acute-phase response in the liver cell (1). PK-C has been detected in both membranous and soluble phases of the hepatocytes and a number of extracellular signals, whose mechanisms of action appear to involve phosphatidyl-4,5-bisphosphate hydrolysis (4, 15), are known to cause translocation of PK-C to membranes

(15). Our results show that turpentine-induced inflammation operates according to this scheme, with an increase in the membrane-associated activity at the expense of the enzyme in the cytosol. We advance the hypothesis that the enzyme, once associated to membranes, can undergo a partial proteolytic cleavage, with retention on the membrane of the PDBu-receptive regulatory site (16), and concurrent release to the cytosol of the fragment containing the catalytic site, that would lose its Ca^{2+} and phospholipid dependence (17). This possibility has already been described (18). Our interpretation is supported by: 1) the discrepancy in time between increase of PK-C activity (8 h after treatment) and increase in PDBu binding (10 h after treatment); 2) the decrease in cytosolic Ca^{2+} and phospholipid-dependent protein kinase activity which persists at 10 h, while Ca^{2+} and phospholipid-independent kinase increases by 17% (data not reported).

The functional significance of PK-C intracellular relocation is difficult to define and the results of experiments in vivo hardly allow to identify any specific mediator of this effect. The cytokine most likely involved in the hepatic acute phase response, IL-6, has been recently shown to trigger the association of its receptor with a new possible signal transducer, (14). However, PK-C activation could be related to multi-hormone changes (19) occurring in an organism carrying a turpentine-induced inflammation: catecholamines, through α_1 receptors (15), and insulin, by de novo phosphatidic acid synthesis (20), could well be responsible for the increase in diacylglycerol, leading in turn to the activation of the enzyme. A role of PK-C in the synthesis of acute-phase proteins is suggested by the observation that the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA), as well as a synthetic diacylglycerol, can induce the synthesis of fibrinogen mRNA in a liver cell line (21). But PK-C cannot be envisioned as a trigger, because activation of acute-phase genes starts before the changes observed in the present experiments (22), although it may be involved in the maintenance and in the fine tuning of the response. Indeed, it has been reported that TPA

participates in the stimulation of acute-phase genes and it is crucial to the regulation of responsiveness of liver cells to IL-6 and IL-1, in part by modulating available cell surface receptor activity (23). Activation of PK-C might explain other phenomena which accompany the turpentine-induced hepatic acute-phase reaction, such as increased amino acid influx (24), which can be stimulated by PK-C (25); activation of the whole protein synthetic machinery (26), some steps of which can be influenced by PK-C (27); enhanced protein glycosylation (28) and secretion (1), phenomena which can be both potentiated by PK-C activation (29).

Although PK-C phosphorylates many proteins in vitro, the in vivo substrates in liver remain to be defined (15). But the alterations in the phosphorylation pattern detected in this research-work suggest that the changes in PK-C are functionally meaningful. Only when the targets of PK-C are defined it will be possible to assess more precisely the role of the activation and intracellular relocation of this enzyme in the response of liver to inflammation.

ACKNOWLEDGMENTS

We thank Dr. Maria G. MONTI for helpful advice on PDBu binding and Ms. M.Grazia BOMBONATO for typing the manuscript. The work was supported by grants from Ministero della Pubblica Istruzione.

REFERENCES

1. Koj,A. (1985) in The Acute-phase Response to Injury and Infection(Gordon, A.H. and Koj,A. eds) pp.145-151, Elsevier Scientific Publishing Co., Amsterdam.
2. Morrone,G., Ciliberto,G., Oliviero,S., Arcone,R., Dante,L.,Content,J. and Cortese,R. (1988) J. Biol. Chem. 263,12554-12558.
3. Andus,T., Geiger,T., Hirano,T., Kishimoto,T. and Heinrich,P.C.(1988) Eur. J. Immunol. 18, 739-746.
4. Berridge,M.J. (1987) Ann. Rev. Biochem. 159, 154-193.
5. Piccoletti,R., Aletti,M.G., Ferioli,M.E., Scalabrino,G. and Bernelli-Zazzera,A. (1987) Res. Commun. Chem. Pathol. Pharm.56, 291-300.
6. Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) Cell 49, 729-739.
7. Andrews,W.V.,Maurer,R.A.and Conn,P.M.(1988) J.Biol.Chem. 263,13755-13761.
8. Bradford,M.M. (1976) Anal. Biochem. 72, 248-254.
9. Kikkawa,U., Takai,Y., Minakuchi,R., Inohara,S. and Nishizuka,Y. (1982) J. Biol. Chem. 257, 13341-13348.
10. Moruzzi,M., Barbiroli,B., Monti,M.G., Tadolini,B., Hakim,G. and Mezzetti,G. (1987) Biochem. J. 247, 175-180.
11. Laemmli,U.K. (1970) Nature 227, 680-685.

12. Azhar,S., Butte,J. and Reaven,E. (1989) Int. J. Biochem. 21, 209-218.
13. Stern,A.S., Pan,Y.E., Hellamann,R.S., Parker,K.P.,Mueller,D.,Hulmes,J.D., Kilian,P. and Chizzonite,R. (1989) Arch. Biochem.Biophys. 274, 26-36.
14. Taga,T., Hibi,M., Hirata,Y., Yamasaki,K., Yasukawa,K., Matsuda,T., Hirano,T. and Kishimoto,T. (1989) Cell 58,573-581.
15. Exton,J.H. (1988) Hepatology 8, 152-166.
16. Hoshijma,M., Kikuchi,A., Tanimoto,T., Kaibuchi,K. and Takai,J. (1986) Cancer. Res. 46,3000-3004.
17. Inoue,M., Kishimoto,A., Takai,Y. and Nishizuka,Y. (1977) J. Biol. Chem. 252, 7610-7616.
18. Melloni,E., Pontremoli,S., Michetti,M., Sacco,O., Sparatore,B. and Horecker,B.L. (1986) J. Biol. Chem. 261, 4101-4105.
19. Beisel,W.R. (1981) in Infection, The Physiologic and Metabolic responses of the Host (Powanda,M.C. and Canonico,P.G., eds.) pp. 147-172, Elsevier/North-Holland Biomedical Press, Amsterdam.
20. Farese,R.V., Suman Konda,T., Davis,J.S., Standaert,M.L., Pollet,R.J. and Cooper,D.R. (1987) Science 236, 586-589.
21. Evans,E., Courtois,G.M., Kilian,P.L., Fuller,G.M. and Crabtree,G.R. (1987) J. Biol. Chem. 262, 10850-10854.
22. Birch,H.E. and Schreiber,G. (1986) J. Biol. Chem. 261, 8077-8080.
23. Baumann,H., Isseroff,H., Latimer,J.J. and Jahreis,G.P. (1988) J. Biol. Chem. 263, 17390-17396.
24. Wannemacher,R.W.,Pekarek,R.S., Thompson,W.L., Cournow,R.T., Beall,F.A., Zenser,T.V., De Rubertis,F.R. and Beisel,W.R. (1975) Endocrinology 96, 651-661.
25. Kitagawa,K., Nishino,H. and Iwashima,A.(1986) Experientia 42, 1038-1040.
26. Piccoletti,R., Aletti,M.G., Cajone,F. and Bernelli-Zazzera,A. (1984) Br. J. Exp. Pathol. 65, 419-430.
27. Morley,S.J. and Traugh,J.A. (1989) J. Biol. Chem. 264, 2401-2404.
28. Lombart,C., Sturgess,J. and Schachter,H. (1980) Biochim. Biophys. Acta 629, 1-12.
29. Liu,T.C. and Jackson,G.L. (1987) Endocrinology 121, 1589-1595.