Ann. Ist. Super. Sanità
Vol. 24, N. 1 (1988), pp. 137-142

MOLECULAR MECHANISM OF REGULATION OF PHOSPHOLIPID METABOLISM IN MEMBRANES

F. HIRATA

Department of Environmental Health Sciences, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland, USA

Summary. - *Glucocorticoids induce the synthesis of a family of phospholipase inhibitory proteins, lipocortins. This family of lipocortins includes inhibitory proteins on phospholipase A_2, phospholipase C and phosphatidylinositol phospholipase C, enzymes that degrade membrane phospholipids to release arachidonic acid, a precursor of leukotrienes and prostaglandins. Hence, glucocorticoids can reduce the formation of arachidonate metabolites, inflammatory compounds by inhibiting cellular phospholipases. Induction of the synthesis of lipocortin by glucocorticoids requires 1 h for mRNA and 5 h for protein in various tissues and cells. However, glucocorticoids often exert their suppressive effect on arachidonic acid release before induction of the synthesis of lipocortin takes place. This is attributable to the nonenzymic formation of the adduct between glucocorticoids and lipocortin. This adduct is easily inserted into the membranes and more resistant to digestion by proteases, thus becoming more biologically potent with respect to suppressive effect on release of arachidonic acid.*

Riassunto (Meccanismo molecolare della regolazione del metabolismo fosfolipidico nelle membrane). - *I glicorticoidi inducono la sintesi di lipocortine che è una famiglia di proteine che inibiscono la fosfolipasi. Questa famiglia di lipocortine include proteine inibitrici sulla fosfolipasi A_2, fosfolipasi C e fosfolipasi C del fosfatidilinositolo, enzimi che degradano i fosfolipidi di membrana e quindi rilasciano l'acido arachidonico, un precursore di leucotrieni e prostaglandine. I glucocorticoidi, quindi, possono ridurre la formazione di metaboliti dell'arachidonato e composti infiammatori attraverso l'inibizione della fosfolipasi cellulare. L'induzione della sintesi di lipocortine da parte di glicocorticoidi in svariati tessuti e cellule richiede un'ora per l'RNA e 5 ore per le proteine. Comunque, i glucocorticoidi spesso esercitano il loro effetto soppressivo sul rilascio dell'acido arachidonico prima che avvenga l'induzione della sintesi di lipocortina. Questo è attribuibile alla formazione non enzimatica dell'addotto tra glucocorticoidi o lipocortine. Questo addotto è facilmente inserito nelle membrane e più resistente alla digestione da parte di proteasi, perciò più potente biologicamente riguardo all'effetto soppressorio sul rilascio dell'acido arachidonico.*

Introduction

Glucocorticoids are clinically used as therapeutic agents effective for many inflammatory and immunological diseases [1]. Although these steroids can affect a variety of metabolisms at many levels [1, 2], it has been proposed that the anti-inflammatory action of glucocorticoids is attributable to reduction in the formation of prostaglandins, leukotrienes and other inflammatory compounds derived from arachidonic acid [3]. Glucocorticoids, however, cannot suppress the formation of those inflammatory compounds, when arachidonic acid is given to intact cells. Furthermore, actinomycin D and cycloheximide, inhibitors of mRNA and protein synthesis, respectively, can block the action of glucocorticoids on the formation of prostaglandins and thus, diminish the anti-inflammatory action of glucocorticoids. Hence, it has been proposed that glucocorticoids block the release of arachidonic acid by inducing the synthesis of proteins that inhibit cellular phospholipases [4]. Such proteins were partially purified and characterized by several laboratories and termed "lipocortins" [5, 7]. In this communication, we would like to describe the mechanism of regulation of cellular phospholipid metabolism by lipocortins.

Properties of lipocortins

Lipocortins are a family of phospholipase inhibitory proteins whose synthesis is induced in a variety of tissues and cells by glucocorticoids [5, 6]. Recently, two different cDNA clones which encode human lipocortins have been isolated and their predicted amino acid sequences have been reported [8, 9]. Their molecular weights calculated from the amino acid compositions are 36,000 and 37,000, respectively. They are composed of 4 repeats which have 41% homology on average. Each repeat has the 17 amino acid consensus sequence, which

are common to 4 related proteins, p36, pII, calectrin and endonexin [10]. In addition, the amino acids 78 to 236 of lipocortin have a strong homology with the amino acids 22 to 171 of c-K-ras 2a, a mammalian ras-onc gene product, which is thought to interact with phosphatidylinositol phospholipase C in a G protein like fashion [11]. However, isolated lipocortin is more specific for phospholipase A_2 rather than for phosphatidylinositol phospholipase C [12]. Nevertheless, the C-MT peptide whose sequence is homologous to the N-terminal amino acids of the consensus amino acid sequence, is an inhibitor against phosphatidylinositol phospholipase C [13]. Recently, we highly purified a phosphatidylcholine phospholipase inhibitory protein from human peripheral lymphocytes. This protein has an apparent molecular weight of 38,000. Some of monoclonal antibodies raised against a phospholipase A_2 inhibitory protein crossreact with this phospholipase C inhibitory protein, suggesting that they have structural resemblance. In addition to these inhibitory protein, we found an inhibitory protein specific for phosphatidylinositol phospholipase C. How specificity of these inhibitory proteins for phospholipases is determined should wait for further investigation. Since inhibitory proteins on other classes of phospholipases have been reported in bacteria and other sources, we proposed the names of members in a family of lipocortin, phospholipase inhibitory protein, as shown in Table 1. In this chapter, I focus mainly on the action of beta-lipocortin.

Table 1. - *Various phospholipase inhibitory proteins in cells* [30]

Phospholipases	Lipocortins	Molecular weight
A_1	alpha	28,000 *
A_2	beta	38,000 ± 2,000 ** 11,000 ± 2,000 *
C	gamma	36,000 ± 2,000 ** 14,000 ± 2,000 *
D	delta	—
Phosphatidylinositol Phospholipase C	epsilon	30,000 ± 2,000 *

* Bacterial origins
** Mammalian origins

Induction of lipocortin synthesis by glucocorticoids

When rabbit peritonial neutrophils were treated with flucinolone acetonide, a potent synthetic glucocorticoids, the release of arachidonic acid elecited with fMetLeuPhe, a synthetic chemoattractant, was suppressed in time and dose dependent manners [14]. The suppression of arachidonate release was first observed in 4 h and gradually increased to attain the maximal 10 h after the treatment was initiated (Table 2). The level of mRNA for lipocortins was quantified by incorporation of ^{35}S-methionine into the immunoprecipitates by the anti-lipocortin antibody after poly A$^+$-mRNA was translated by the rabbit reticulocyte lysates. The major protein in the immunoprecipitates had an apparent molecular weight of 37,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The increase of mRNA for lipocortin preceded the increase in lipocortin. Furthermore, this increase in mRNA levels could not be blocked by 5 µg/ml cycloheximide, suggesting that glucocorticoids directly enhance the synthesis of lipocortin through the mechanism called "genomic effects" [2]. The degree of suppression of arachidonic acid release did not necessarily parallel the cellular levels of lipocortin as measured by the radioimmunoassay [15]. This might be partly due to the existence of isoforms of lipocortin, and partly due to the post-translational modification of lipocortin. It is highly possible that there are some isoforms of beta-lipocortin, since isoforms of phospholipase A_2 have been reported [16], and lipocortin makes a complex with each isoform of phospholipase A_2.

Post-translational modification of lipocortins

Lipocortin was first discovered in the culture media in which macrophages, neutrophils and kidney interstitial cells were cultured together with glucocorticoids [5-7]. As being often the case with other secretory proteins, it was characterized as glycoproteins. However, this family of proteins has been shown to be identical with p35 and p36 in placenta, proteins which serve as substrate proteins for epidermal growth factor (EGF) receptor and retrovirus tyrosine protein kinases [9]. These substrate proteins for tyrosine kinases have been demonstrated by the immunological methods to be associated with cytoskeletal elements such as actins, and their cDNA clones revealed that these proteins have no signal peptide sequences. To determine the mechanism of how these

Table 2. - *Induction of mRNA and lipocortins by flucinolone acetonide* [31]

Incubation time (h)	mRNA (cpm/0.1 µg polyA$^+$ mRNA)	Lipocortins (ng/mg protein)	fMetLeuPhe induced arachidonic acid release (cpm/10^6 cells)
0	594 ± 50	18 ± 2	1954 ± 10
1	822 ± 62	16 ± 2	1840 ± 60
2	1082 ± 84	15 ± 2	1620 ± 30
5	1416 ± 91	22 ± 2	1450 ± 87
10	1270 ± 31	36 ± 3	832 ± 57
24	1030 ± 20	38 ± 3	531 ± 61

cytosolic proteins are secreted outside cells, rabbit peritoneal neutrophils were cultured with various radioactive compounds, and then, lipocortin in the media was immunoprecipitated (Table 3). As expected, ^{14}C-glucosamine was incorporated into the immunoprecipitates by anti-lipocortin antibody, suggesting that lipocortin is glycosylated. Furthermore, the immunoprecipitable protein(s) was found to be acylated with palmitic and myristic acids. These fatty acids are reported to modify the different sites of proteins; proteins acylated with palmitic acid are often observed to be integrated into membranes [17].

Surprisingly, lipocortins bound [^{3}H]dexamethasone and this radioactivity was not released by the extraction with chloroform-methanol (2/1, v/v) or ethylether. Alkaline and hydroxylamine treatments, which generally cleave the acyl groups of proteins, had no effects. Therefore, it is likely that lipocortin forms the adduct with glucocorticoids. Since such adduct formation between lipocortin and dexamethasone could be detected *in vitro*, this reaction appears to be nonenzymic as reported in the case of lens crystalins [18]. In accordance with this interpretation, the rate of the adduct formation was dependent upon concentrations of dexamethasone or purified lipocortin or both. Preliminary experiments showed that this adduct formation takes place even in intact cells, especially AtT 20 cells, a mouse pituitary cell line (Hirata, F. and Lupini, A., unpublished data). Lipocortin in the adduct form was approximately 50 to 100 fold more biologically potent than lipocortin in the nonadduct form with respect to suppression of the CRF-induced ACTH release form AtT 20 cells. This post-translational modification of lipocortin may explain so called "acute effect" and/or "membrane stabilizing effect" of glucocorticoids.

Mechanism of inhibition of phospholipases by lipocortins

The activity of lipocortin is generally assayed as its inhibitory activity against phospholipases *in vitro* [15]. The maximal inhibition of porcine pancreatic phospholipase A_2 is observed, when the stoichiometric amount (on the molar basis) of lipocortin is given. Lipocortin noncompetitively inhibits porcine pancreas phospholipase A_2 with respect to Ca^{2+}, an activator, and phosphatidylcholine, a substrate [12]. The Km of phospholipase A_2 for phosphatidylcholine is not changed, whereas the Ka of phospholipase A_2 for Ca^{2+} increases in the presence of lipocortin. Essentially similar results are obtained with the C-MT peptide, although the affinity of this peptide for phospholipase A_2 is much lower, compared with that of lipocortin [13]. Two moles of Ca^{2+} are proposed to bind to phospholipase A_2; one is at a high affinity site (active site of phospholipase) and the other at a low affinity site [19]. The low affinity site is proposed to play a role in bridging phospholipid substrates with the active site of phospholipase. The C-MT peptide interferes the action of Ca^{2+} at the low affinity site. The C-MT peptide is homologous to the consensus sequence of the calcium binding proteins [13], and lipocortin can bind Ca^{2+} even in the absence of acidic phospholipids [20]. From these observations, we concluded that lipocortin inhibits phospholipase A_2 by binding to the Ca^{2+} binding sites of phospholipase A_2 and making a one-to-one stoichiometric complex with phospholipase A_2.

Table 3. - *Post-translational modification of lipocortins* [31]

		Cell	
	Medium	Membranes	Cytosols
		(cpm/10^7 cells)	
^{35}S-methionine	8,800 ± 182	1,740 ± 300	6,530 ± 420
^{3}H-palmitic acid	3,020 ± 98	2,580 ± 180	1,020 ± 121
^{3}H-myristic acid	2,210 ± 68	630 ± 54	3,320 ± 162
^{14}C-glucosamine	1,760 ± 72	630 ± 20	450 ± 32
^{3}H-dexamethasone	1,760 ± 48	1,120 ± 39	280 ± 28

However, Davidson *et al.* reported that lipocortin and its related proteins inhibit phospholipase A_2 by binding phospholipid substrate rather than by making a complex with phospholipase A_2 [21]. Only in the presence of Ca^{2+}, these proteins bind to phosphatidylserine and other acidic phospholipids but not to phosphatidylcholine and phosphatidylethanolamine, better substrates for phospholipase A_2. A concentration of Ca^{2+} for half maximal binding of lipocortin to acidic phospholipids is around 5 to 10 μM, concentrations which are much beyond a physiological concentration. For the assay, they used bacterial phospholipids which contained phosphatidylserine in addition to phosphatidylethanolamine, a major phospholipid substrate. Therefore, it is likely that in the reaction mixture containing high concentrations of Ca^{2+}, lipocortin bound to phospholipid micelles before it interacted with phospholipase A_2. Alternatively, it is still possible that p35 and p36, substrate proteins for tyrosine kinases, are not identical to lipocortin that we originally reported. This possibility is more favored, since cDNA clones which we recently isolated from libraries of human placenta and dexamethasone treated U937 cells have distinct sequences from those previously reported (Hirata, F. *et al.*, unpublished data).

Mechanism on regulation of cellular phospholipid metabolism by lipocortins

Stimulation of many, if not all, cells with hormones, neurotransmitters and drugs causes release of arachidonic acid cell membranes [3]. In the resting state, phospholipases appear to be inactive, although these lipases are surrounded by phospholipid substrates. We hypothesized that phospholipases make the complexes with inhi-

bitory proteins in intact resting cells. To prove it, we investigated the mechanism as to how lipocortin is dissociated from phospholipases after stimulation of receptors, and how phospholipases are allowed to be maximal active. Phosphorylation of cellular proteins has been postulated to be associated with signal transduction through many receptors [22]. Lipocortins can be phosphorylated *in vitro* by various kinases including cyclic AMP dependent kinase, protein kinase C and tyrosine protein kinase [12, 20, 23]. In either case, phosphorylated lipocortin loses its capacity to inhibit phospholipase A_2 *in vitro*. Tyr^{20} or Tyr^{23} and $threonine^{23}$ ($serine^{25}$) are now proposed to be the phosphorylation sites. Furthermore, the amounts of phosphorylated lipocortin in the mitogen-induced lymphocytes and the fMetLeuPhe-stimulated neutrophils appeared to correlate well with the rates of release of arachidonic acid from these cells [13, 23]. These observations suggest that the phosphorylation of lipocortin by kinases associated with various receptors plays an important role in arachidonic acid release.

Now, many receptors have also been shown to couple to N(GTP binding) proteins [24, 25]. These proteins are composed of heterotrimer peptides, alpha, beta and gamma subunits. The alpha subunit of some species of N proteins are now postulated to be responsible for activation of phosphatidynositol turnover [26]. Although lipocortin shares the homologous amino acid sequence with K-ras, a GTP binding protein [11], purified lipocortin failed to bind GTP or its analogues. However, anti-lipocortin antibody can immunoprecipitate GTP-[$gamma^{35}S$], a nonhydrolyzable analogue of GTP, from the fMetLeuPhe-stimulated plasma membranes of rabbit neutrophils (Table 4). Lipocortin has been reported to be copurified in highly purified preparations of transducin, another type of GTP binding proteins in the retina [9]. Our preliminary results showed that transducin can overcome the suppressive effect of lipocortin on porcine pancreatic phospholipase A_2 when GTP and its analogues are present (Hirata, F., Fraser, C. and Rodbell, M., unpublished data). GDP and GMP had almost no effect on the reversal action of transducin to anti-phospholipase action of lipocortin. These results suggest that the alpha subunit-GTP complex may strongly interact with lipocortin. The mechanism and nature of this interaction between N proteins and lipocortin require further investigation. However, it is quite likely that such interaction may activate phospholipases by dissociating the phospholipase-lipocortin complex. Recently, ras-onc gene products (which can bind GTP) have been reported to activate cellular phospholipase A_2, when they are injected into intact fibroblasts [27]. Since lipocortins are shown to be phosphorylated in proliferating cells [9, 20, 23], these observations suggest that activation of cellular phospholipase A_2 either by phosphorylation at tyrosine and/or serine or threonine sites of lipocortin or by interaction between the ras-onc gene product-GTP complex and lipocortin may play a role in the events taking place during transformation and/or proliferation of cells. It is not yet ruled out that products of ras-onc genes such as Ha-ras gene, may phosphorylate lipocortin with GTP as substrate, because this protein has been shown to autophosphorylate threonine site with GTP but not ATP [28].

Table 4. - *Interaction between G proteins and lipocortins* [31]

	Radioactivity			
	GTP[gamma ^{32}P]		GTP[gamma ^{35}S]	
	Control	Anti-lipocortin	Control	Anti-lipocortin
	(cpm)		(cpm)	
None	512 ± 41	469 ± 32	188 ± 24	134 ± 34
fMetLeuPhe (0.1 μM)	554 ± 62	1,852 ± 86	134 ± 26	883 ± 62

Review submitted on invitation by the Editorial Board of the *Annali*. Accepted for publication: 5 May 1987.

REFERENCES

1. SAYERS, G. & TRAVIS, R.H. 1978. Adrenocorticotrophic hormone; adrenocortical steroids and their synthetic analogs. In: *The pharmacological basis of therapeutics*. L.S. Goodman & A. Gilman (Eds). McMillan, New York. pp. 1604-1642.
2. THOMPSON, E.B. & LIPPMAN, M.E. 1974. Progress in endocrinology and metabolism. Mechanism of action of glucocorticoids. *Metabolism* **23**: 159-202.
3. KUEHL, F.A. Jr & EGAN, R.W. 1980. Prostaglandins, arachidonic acid, and inflammation. *Science* **210**: 978-984.
4. DANON, A. & ASSOULINE, G. 1978. Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature* **273**: 552-554.
5. HIRATA, F. 1984. Role of lipomodulin: a phospholipase inhibitory protein in immunoregulation. *Adv. Inflammation Res.* 7: 71-78.
6. FLOWER, R.J., WOOD, J.N. & PARENTE, L. 1984. Macrocortin and mechanism of action of glucocorticoids. *Adv. Inflammation Res.* 7: 61-70.

7. DI ROSA, M., FLOWER, R. J., HIRATA, F., PARENTE, L. & RUSSO-MARIE, F. 1984. Letter to the editor: anti-phospholipase proteins, nomenclature announcement. *Prostaglandins* **28**: 441-442.

8. WALLNER, B.P., MATTALIANO, R.J., HESSION, C., CATE, R.L., TIZARD, R., SINCLAIR, L.K., FOELLER, C., CHOW, E.P., BROWNING, J.L., RAMACHANDRAN, K.L., & PEPINSKY, R.B. 1986. Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. *Nature* **320**: 77-80

9. HUANG, K.S., WALLNER, B.P., MATTALIANO, R.J., TIZARD, R., BURNE, C., FREY, A., HESSION, C., MCGRAY, P., SINCLAIR, L. K., CHOW, E.P., BROWNING, J. L., RAMACHANDRAN, K.L., TANG, J., SMART, J.E. & PEPINSKY, R.B. 1986. Two human 35 Kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase.*Cell* **46**: 191-199.

10. KRETSINGER, R.H. & CREUTZ, C.E. 1986. Consensus in exocytosis. *Nature* **320**: 573.

11. MUNN. T.Z. & MUES, G.I. 1986. Human lipocortin similar to ras gene products. *Nature* **322**: 314-315.

12. HIRATA, F. 1981. The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. *J. Biol. Chem.***256**: 7730-7733.

13. NOTSU, Y., NAMIUCHI, S., HATTORI, T., MATSUDA, K. & HIRATA, F. 1985. Inhibition of phspholipase by Met-Leu-Phe-Ile-Leu-Ile-Lye-Arg-Ser-Arg-His-Phe, C terminus of middle-sized tumor antigen. *Arch. Biochem. Biophys.* **236**: 195-204.

14. HIRATA, F., SCHIFFMANN, E., VENKATASUBRAMANIAN, K., SOLOMON, D. & AXELROD, J. 1980. A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl Acad. Sci. USA* **77**: 2533-2536.

15 HIRATA, F., NOTSU, Y., IWATA, M., PARENTE, L., DI ROSA, M. & FLOWER, R. J. 1982. Identification of several species of phospholipase inhibitory protein(s) by radioimmunoassay for lipomodulin. *Biochem. Biophys. Res. Commun.* **109**: 223-230.

16. LOEB, L. & GROSS, R.W. 1986. Identification and purification of sheep platelet phospholipase A_2 isoforms. Activation by physiologic concentrations of calcium ions. *J. Biol. Chem.* **261** (23):10467-10470.

17. OLSON, E.N., TOWLER, D.A. & GLASER, L. 1985. Specificity of fatty acid acylation of cellular proteins. *J. Biol. Chem.* **260**: 3784-3790.

18. BUCALA, R., MANABE, S., URBAN, R.C. & CERAMI, A. 1985. Non enzymatic modification of lens crystallins by prednisolone induces sulphydryl oxidation and aggregate formation: *in vitro* and *in vivo* studies. *Exp. Eye Res.* **41**: 353-363.

19. VAN DEN BOSCH, H. 1980. Intracellular phospholipases A. *Biochim. Biophys. Acta* **604**: 191-246.

20. HIRATA, F. 1983. Lipomodulin: a possible mediator of the action of glucocorticoids. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **11**: 73-78.

21. DAVIDSON, F.F., DENNIS, E.A., POWELL, M. & GLENNEY, J.R. Jr. 1987. Inhibition of phospholipase A_2 by "lipocortins" and calpactins. *J. Biol. Chem.* **262** (4): 1698-1705.

22. COHEN, P. 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296**: 613-620.

23. HIRATA, F., MATSUDA, K., NOTSU, Y., HATTORI, T. & DEL CARMINE, R. 1984. Phosphorylation at a tyrosine residue of lipomodulin in mitogen-stimulated murine thymocytes. *Proc. Natl Acad. Sci. USA* **81**: 4717-4721.

24. RODBELL, M. 1985. Programmable messengers: a new theory of hormone action. *TIBS* **10**: 461-464.

25. GILLMAN, A.G. 1984. G proteins and dual control of adenylate cyclase. *Cell* **36**: 577-579.

26. LITOSCH, I. & FAIN, J.N. 1986. *Minireview*: Regulation of phosphoinositide breakdown by guanine nucleotides. *Life Sci.* **39** (3): 187-194.

27. BAR-SAGI, D. & FERAMISCO, J.R. 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. *Science* **233**: 1061-1068.

28. SHIH, T.Y., PAPAGEORGE, A.G., STOKES, P.E., WEEKS, M.O. & SCOLNICK, E.M. 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the $p21^{src}$ protein of Harvey murine sarcoma virus. *Nature* **287**: 686-691.

29. DAVIS, L.G., DIBNER, M.D. & BATTERY, J.F. 1986. In: *Basic methods in molecular biology*. Elsevier, New York.

30. HIRATA, F. 1987. Role of lipocortins in cellular function as a second messenger of glucocorticoids. In: *Anti-inflammatory steroid action; basic and clinical aspects*. L.M. Lichtenstein, H. Claman, A. Oronsky & R. Schleimer (Eds). Academic Press, New York (in press).

31. HIRATA, F., STRACKE, M.L. & SCHIFFMANN, E. 1987. Regulation of prostaglandin formation by glucocorticoid and their second messenger, lipocortins. In: *Proceedings of the 7th International Congress on Hormonal Steroids*. Madrid, Spain. (in press).