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32. Interactions. Steroids–Prostaglandins

REGULATION OF PROSTAGLANDIN FORMATION BY GLUCOCORTICOIDS AND THEIR SECOND MESSENGER, LIPOCORTINS

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Summary—Glucorcorticoids 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. Hence, glucocorticoids reduce the formation of prostaglandins and leukotrienes by inhibiting cellular phospholipases, enzymes that degrade membrane phospholipids to release arachidonic acid, a precursor. The induction by glucocorticoids requires 1 h for the synthesis of mRNA and 5 h for the synthesis of proteins in various tissues and cells. However, glucocorticoids often exert their suppressive effects before the induction of lipocortins. This is now attributed to the nonenzymic formation of the adducts between glucocorticoids and lipocortins. These adducts are easily inserted into the membranes and more resistant to digestion of proteases, thus being more biologically potent with respect to suppression of the release of arachidonic acid, a precursor of prostaglandins and leukotrienes.

Glucocorticoids are clinically used as therapeutics for inflammatory and immunological diseases [1]. Although these steroids can affect a variety of metabolisms at many levels [1, 2], it has been proposed that anti-inflammatory action of glucocorticoids is attributable to reduction in the formation of prostaglandins and leukotrienes, inflammatory compounds derived from arachidonic acid [3]. Glucocorticoids, however, cannot suppress the formation of those inflammatory compounds, when arachidonic acid is given. Furthermore, actinomycin D and cycloheximide, inhibitors of mRNA and protein synthesis, respectively, can block the action of glucocorticoids on the formation of prostaglandins. It has been proposed that glucocorticoids block the release of arachidonic acid by inducing the synthesis of protein(s) that inhibits cellular phospholipases [4]. Such proteins were partially purified and characterized by several laboratories and termed "lipocortins" (for review see Refs [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 protein 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 a 17 amino acid consensus sequence of 4 related proteins, p. 36, pII, calectrin and endonexin [10]. Furthermore, the amino acids 78–236 of lipocortin have a strong homology with the amino acids 22–171 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 lipocortins are more specific for phospholipase A_2 rather than for phosphatidylinositol phospholipase C [12]. Nevertheless, C-MT peptide whose sequence is homologous to the N-terminal amino acids of the consensus amino acid sequence is a strong 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 34,000. Preliminary results obtained with cDNA clone from mRNA of HL60 cells which encodes this protein suggested approximately 40% homology between phospholipase A_2 and phospholipase C inhibitory proteins (Clark and Hirata, unpublished observations). How specificity of these inhibitory proteins for phospholipases is determined awaits further investigation.

INDUCTION OF LIPOCORTIN SYNTHESIS BY GLUCOCORTICOIDS

When rabbit peritonial neutrophils were treated with fluocinolone acetonide, a potent synthetic glucocorticoids, the release of arachidonic acid elicited 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 maximum 10 h after the treat-

Table 1. Induction of mRNA and lipocortins by flucinolone acetonide

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

Rabbit peritoneal neutrophil (6×10^7 cells) were incubated in 6 ml of RPMI 1640 containing 1% bovine serum albumin with 1 μM flucinolone acetonide for varying time. The levels of lipocortins and the release of [^{14}C]arachidonic acid were assayed as described previously [14, 15]. PolyA⁺ mRNA was isolated and translated according to the methods described [26]. The amounts of mRNA for lipocortins are expressed as the radioactivity of [^{35}S]methionine (800 Ci/mmol) incorporated into the immunoprecipitates by affinity purified anti-lipocortin antibody (AFLP1-10GH12).

ment was initiated (Table 1). When cellular levels of lipocortins were measured by radioimmunoassay [15], the contents of lipocortins increased in parallel with the degrees of the suppression of arachidonate release. The level of mRNA for lipocortins was quantitated by incorporation of [^{35}S]methionine into the immunoprecipitates by the anti-lipocortin antibody after polyA⁺-mRNA was translated by the rabbit reticulocyte lysates. The major protein in the immunoprecipitate had an apparent molecular weight of 37,000 on SDS–PAGE. The increase of mRNA for lipocortin proceeded to the increase of lipocortins (Table 1). Furthermore, this increase in mRNA could not be blocked by 5 μg/ml cycloheximide, suggesting that glucocorticoids directly enhance the synthesis of lipocortins through the mechanism of "genomic effects" [2].

POSTTRANSLATIONAL MODIFICATION OF LIPOCORTINS

Lipocortins were first discovered in culture media in which macrophages, neutrophils and kidney cells were cultured together with glucocorticoids [5–7]. As being often the case with other secretory proteins, they were 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 protein for epidermal growth factor (EGF) receptor and retrovirus tyrosine protein kinases [9]. These proteins have been demonstrated by immunological methods to be associated with cytoskeletal elements such as actins. Furthermore, these proteins have no signal peptide sequences. To determine the mechanism for secretion of these cytosolic proteins outside cells, rabbit peritoneal neutrophils were cultured with various radioactive

Table 2. Post-translational modification of lipocortins

	Medium	Cell (cpm/10^7 cells)	
		Membranes	Cytosols
[^{35}S]Methionine	8800 ± 182	1740 ± 300	6530 ± 420
[^{3}H]Palmitic acid	3020 ± 98	2580 ± 180	1020 ± 121
[^{3}H]Myristic acid	2210 ± 68	630 ± 54	3320 ± 162
[^{14}C]Glucosamine	1760 ± 72	630 ± 20	450 ± 32
[^{3}H]Dexamethasone	1760 ± 48	1120 ± 39	280 ± 28

Rabbit neutrophils (2×10^7 cells) were cultured for 16 h at 37°C in 5 ml of RPMI 1640 media containing 1% bovine serum albumin with various radioactive compounds, including 200 μCi/ml [^{35}S]methionine (800 Ci/mmol), 20 μCi/ml [^{3}H]palmitic acid (50 Ci/mmol), 10 μCi/ml [^{14}C]glucosamine (50 mCi/mmol), and 10 μCi/ml [^{3}H]dexamethasone (50 Ci/mmol). After incubation, the cells were washed 3 times with 3 ml of sodium phosphate buffered (pH 7.4) saline, and lysed with 5 ml of 10 mM Tris–Cl buffer, pH 7.4, containing 5 mM EGTA. After centrifugation at 27,000 *g* for 60 min, the precipitates were solubilized with 5 ml of sodium phosphate buffered (pH 7.4) saline containing 0.2% Nonidet P40. The aliquots after each centrifugation were subjected to the immunoprecipitation by affinity purified anti-lipocortin antibody (AFLP1-10GH12).

compounds and then lipocortins in the media were immunoprecipitated (Table 2). As expected, [^{14}C]glucosamine was incorporated into the immunoprecipitates by anti-lipocortin antibody, suggesting that lipocortins are glycosylated. Furthermore, the immunoprecipitable proteins were 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 [16].

Surprisingly, lipocortins bound [^{3}H]dexamethasone and this radioactivity was not released by the extraction with chloroform–methanol (2/1, v/v) or ether. Alkaline and hydroxylamine treatments, which generally cleave the acyl groups proteins, had no effects. Therefore, it is conceivable that lipocortins form the adduct with glucocorticoids. Since such adduct formation between lipocortins and dexamethasone could be detected *in vitro*, this reaction appears to be nonenzymic as reported in the case of lens crystaline [17]. In accordance with this interpretation, the rate of the adduct formation was dependent upon concentrations of dexamethasone or purified lipocortins or both. Preliminary experiments showed that this adduct formation takes place even in intact cells, especially AtT 20 cells, a mouse pituitry cell line (Hirata and Luini; unpublished data). After 30-min incubation, approximately 4% of the total lipocortins formed the adduct in AtT20 cells, whereas approximately 1.4% of the total lipocortin did in rabbit neutrophils after 1-h incubation. Furthermore, this adduct was approximately 50–100-fold biologically more potent more than natural lipocortin with respect to suppression of ACTH release from CRF-stimulated AtT 20 cells. This modification may explain so-called "acute effects" and/or "membrane stabilizing effects" of glucocorticoids.

MECHANISM ON REGULATION OF CELLULAR PHOSPHOLIPID METABOLISM BY LIPOCORTINS

Although the synthesis of lipocortins is induced by glucocorticoids in a variety of cells as described above, relatively high contents of lipocortins can be detected in nontreated cells [5, 6]. Stimulation of many, if not all, cells with hormones, neutrotransmitters and drugs causes release of arachidonic acid from cell membranes [3]. Phosphorylation of cellular proteins has been postulated to be associated with signal transduction through many receptors [18]. Lipocortins can be phosphorylated by various kinases including cyclic AMP dependent kinase, protein kinase C and tyrosine protein kinase [12, 19, 20]. In either case, phosphorylated lipocortins lose their capacity to inhibit phospholipase A_2 *in vitro*. Furthermore, the receptor-mediated phosphorylation of lipocortins in mitogen-induced lymphocytes and fMetLeuPhe-stimulated neutrophils appeared to be well correlated with the release of arachidonic acid from these cells [13, 20]. These observations suggest that phosphorylation of lipocortins by kinases associated with various receptors plays an important role in arachidonic acid release.

Now, many receptors have been shown to couple to N(GTP binding) proteins [21, 22]. These proteins are composed of heterotrimer peptides, alpha, beta and gamma subunits. The alpha subunit of some N protein species is now postulated to be responsible for activation of phosphatidylinositol turnover [23]. Although lipocortins have a strong homology in amino acid sequences with *K-ras*, a GTP-binding

Table 3. Interaction between G proteins and lipocortins

	Radioactivity			
	GTP[gamma ^{32}P] (cpm)		GTP[gamma ^{35}S] (cpm)	
	Control	Anti-lipocortin	Control	Anti-lipocortin
None	512 ± 41	469 ± 32	188 ± 24	134 ± 34
fMetLeuPhe (0.1 μM)	554 ± 62	1852 ± 86	134 ± 26	883 ± 62

Plasma membranes from rabbit neutrophils (100 μg) was incubated in 100 μl of the reaction mixtures containing 0.1 M HEPES buffer, pH 7.4, 5 mM $MgCl_2$, 1 μM $CaCl_2$, 10 μM ATP, 0.1 mg/ml spermine, and 1 μM radioactive GTP (18,274 cpm/100 μl) or its analogue (19,608 cpm). After incubation at 37°C for 2 min, the reaction mixtures were centrifuged by an Ependorph minifuge for 5 min. Affinity purified antilipocortin antibody (100 μl, 100 μg/ml) containing 5 μg/ml aprotinin, 5 μl/ml soyabean trypsin inhibitor, 0.5 mM PMSF and 1 mM sodium deoxycholate was added to the aliquots. After an additional 2-h incubation at 37°C, the immune complexes were precipitated by adding 200 μl of Tachisorb. The precipitates were washed 3 times with 0.5 ml of sodium phosphate buffered (pH 7.4) saline by centrifugation on a minifuge. For immunoprecipitation, affinity purified anti-lipocortin antibody (AFLP1-10GH12) and culture media of NS-1 (control) were used.

protein [11], purified lipocortins failed to bind GTP or its analogues. However, anti-lipocortin antibody can immunoprecipitate GTP-[gamma ^{35}S], a non-hydrolizable analogue of GTP, from fMeLeuPhe-stimulated plasma membranes of rabbit neutrophils (Table 3). Lipocortins have been reported to be contaminated in the highly purified preparation of transducin, another type of GTP-binding protein in the retina. Our preliminary results showed that transducin can reverse the suppressive effects of lipocortins on porcine pancreas phospholipase A_2 in the presence of GTP and its analogues but not by GDP and GMP (Hirata, Fraser and Rodbell; unpublished data). These results suggest that the alpha subunit–GTP complex can strongly interact with lipocortins. The mechanism and nature of this interaction between N proteins and lipocortins require further investigation. However, such interaction may activate phospholipases by dissociating the phospholipase–lipocortin complex.

Recently, *ras-onc* gene products (which can bind GTP) has been reported to activate cellular phospholipase A_2 when they are injected into intact fibroblasts [24]. Since lipocortins are shown to be phosphorylated in proliferating cells [9, 19, 20], these observations suggest that phospholipase A_2 activation either by phosphorylation at a tyrosine site of lipocortins or by interaction between the GTP-*ras-onc* gene product complex and lipocortins may play a role in the events taking place during the transformation and/or proliferation of cells. There is still a possibility that some products of *ras-onc* genes such as *Ha-ras* genes, can phosphorylate lipocortins with GTP as substrate, because this protein has been shown to autophosphorylate at threonine site with GTP [25].

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