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IDENTIFICATION OF SEVERAL SPECIES OF PHOSPHOLIPASE INHIBITORY PROTEIN(S) BY RADIOIMMUNOASSAY FOR LIPOMODULIN

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SUMMARY: Radioimmunoassay of lipomodulin has been developed using a monoclonal anti-lipomodulin antibody and ^{125}I-labelled lipomodulin. Lipomodulin activity was measured in peritoneal lavage fluids obtained from rats injected with dexamethasone by radioimmunoassay and by enzymatic assay with phospholipase A_2. Three species of immunoreactive substances with Mr= 40,000, 30,000 and 16,000 were found. While two species of Mr= 40,000 and 30,000 had phospholipase inhibitory activities, the species of Mr= 16,000 could inhibit phospholipase A_2 only after dephosphorylation by alkaline phosphatase treatment.

Glucocorticoids exert a variety of biological activities on various tissues and organs of mammals (1). Anti-inflammatory activity, a major action of glucocorticoids has been proposed to be associated with the induction of the phospholipase inhibitory proteins (2,3). These phospholipase inhibitory proteins have been isolated from glucocorticoid-treated rabbit neutrophils (lipomodulin with Mr = 40,000) and rat macrophages (macrocortin with Mr = 15,000) (2,3). Partially purified preparations of these proteins have similar activities with respect to inhibition of arachidonate release from many cells and tissues, and exert anti-inflammatory activity on carageenan induced paw edema and pleurisy (4,5). However, no evidence is available showing that macrocortin and lipomodulin are closely related proteins. Recently, we have prepared a monoclonal antibody against lipomodulin and

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have found autoantibodies against lipomodulin in sera from patients with rheumatic diseases (such as systemic lupus erythematosus). To measure the level of phospholipase inhibitory protein(s) in animals, we developed the radioimmunoassay of lipomodulin. Peritoneal lavage fluids obtained from glucocoricoid treated rats (samples which have been reported to be rich in macrocortin (3,5)) were analyzed by inhibition of phospholipase A_2 activity and by the newly developed radioimmunoassay. Here we demonstrate that three species of proteins (with Mr= 40,000, 30,000 and 16,000 in the preparation) are immunologically reactive with anti-lipomodulin antibody. Some properties of these three species are described with respect to their inhibitory activities on phospholipase A_2 from porcine pancreas.

MATERIALS AND METHODS

Monoclonal antibody - Anti-lipomodulin antibody was obtained from ascites fluids of mice bearing hybridoma 4-4C3 cells (6). This clone was obtained by fusion of P3 x 63 Ag8 mouse myeloma cells with splenocytes of Balb/c mice immunized with rat cerebral synaptosomes (7). Antigen(s) of this antibody was identified as lipomodulin (6). This clonal hybridoma cell line was kindly provided by Dr. M. Nirenberg, of the National Institute of Heart and Lung and Blood.

Purification of lipomodulin - Lipomodulin was purified by a slight modification of the method described previously (8). The concentrated conditioned media (50 ml) of rabbit peritoneal neutrophils treated with 0.1 μM fluocinolon acetonide were passed through DEAE-cellulose column. The eluates not absorbed to the column were concentrated to 10 ml using diaflow membrane P10. The concentrate was passed through 25 ml of phospholipase A_2-coupled agarose and washed with 10 mM phosphate buffer pH 7.4 containing 150 mM NaCl and 0.25% sodium deoxycholate. Phospholipase A_2 from porcine pancreas (50 mg) was coupled with 25 ml of Affi-Gel 15 at pH 5.0. The fractions containing phospholipase inhibitory activity were eluted with 0.01 N acetic acid and were neutralized to pH 7.0 with 0.1N NaOH. The concentrate was applied on a Sephadex G-75 column. The fractions corresponding with Mr= 40,000 were collected.

Preparation of macrocortin - Rats (Wistar, male, 200-250 g) were injected subcutaneously with dexamethasone (1 mg/kg) or saline and killed 1 h later by exposure to CO_2. The peritoneal cavity was washed with 10 ml heparinized, buffered saline containing 50 μM phenylmethylsulfonylflouride (PMSF) to prevent proteolysis. Alternatively, phospholipase inhibitory protein(s) was obtained from culture media of peritoneal cells treated with dexamethasone. Male Wistar rats (weighing 150 to 200 g) were killed by exposure to ether and bled. The peritoneal cavity was washed with 20 ml of RPMI medium enriched with bovine serum albumin (100 μg/ml). The final suspension contained 3 to 4 x 10^6 cells per ml (80% mononuclears and 20% polymorphonuclears). Samples (2 ml) of the cell suspension were incubated for 120 min in a metabolic shaker at 37°C. Dexamethasone (sodium phosphate salt) was added to some samples at a final concentration of 1 μM. After the cells were removed by centrifugation, the conditioned medium or peritoneal extracts were dialysed against 2 x 100 vols of 0.1% ammonium bicarbonate buffer (pH 8.0) and lyophilized.

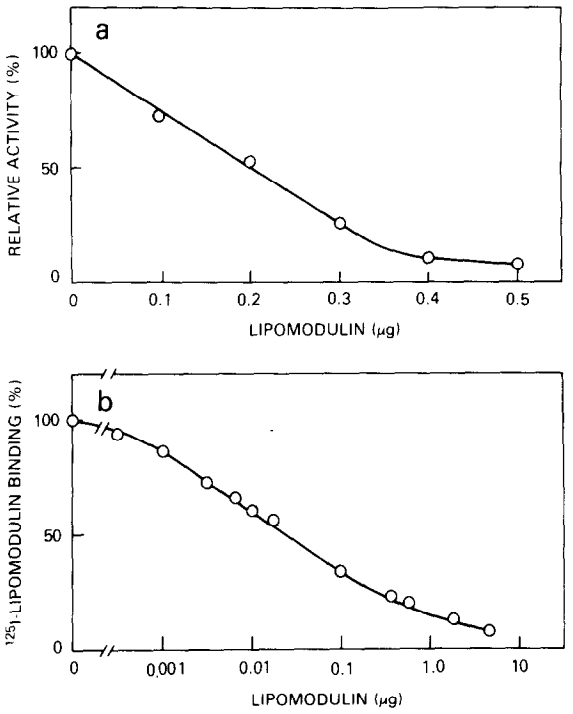

Fig 1. Enzymatic assay (a) and radioimmunoassay (b) of lipomodulin Purified lipomodulin (0 to 5 μg) was assayed as described in the text.

Radioimmunoassay - The reaction mixture contained 10 mM sodium phosphate buffer pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2% Nonidet P40, 0.1 mM PMSF, 0.1 μg pepstatin, 1 μg aprotinin, 10 μg soybean trypsin inhibitor in a total volume of 100 μl. Lipomodulin in Mr= 40,000 fraction, obtained as described above, was labelled with ^{125}I-Bolton Hunter's reagent as described (9). ^{125}I-lipomodulin (20,000 cpm/0.05 μg) and 0.5 μg of IgG fraction of the ascites fluid were incubated for 20 hrs at 4°. Five μl of the anti-mouse IgG (light and heavy chains) rabbit serum were added to each tube and incubated for another 14 hrs at 4°. The immune complex was precipitated by adding 1 ml of 10% polyethyleneglycol (PEG 4,000) and centrifuged at 6,000 x g for 20 min. The precipitates were dissolved in 0.5 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and then 0.5 ml of 20% PEG 4,000 were added. After sitting on ice for 30 min, tubes were centrifuged (6,000 x g for 20 min) and the supernatants were aspirated. The precipitates were measured for radioactivity.

Enzymatic assay of lipomodulin: Lipomodulin was assayed by its ability to inhibit phospholipase A_2 from porcine pancreas (Sigma, approximately 600 unit/mg) (8). The reaction mixture contained 25 mM Tris-glycine buffer, pH 8.8, 0.2 mM α-palmitoyl-β-[1-^{14}C]arachidonyl phosphatidylcholine (New England Nuclear, 50 μCi/μmol) and 0.125 μg porcine pancreas phospholipase A_2 in a total volume of 25 μl. The mixture was incubated at 37° C for 5 min and [1-^{14}C]arachidonate released was measured as described (8). One unit of lipomodulin was defined as ability to inhibit 50% of 1 μg of phospholipase A_2 by 50%. Specific activity of purified lipomodulin was approximately 500 U/mg.

Table 1. Phospholipase inhibitory activities and anti-lipomodulin antibody immunoprecipitable proteins in sources of macrocortin

Materials	Glucocorticoid treatment	Radioimmunoassay	Enzymatic assay
		(μg equivalent/ml)	
Peritoneal lavage (17.2 mg protein)	-	42	25
	+	106	47
Rat macrophage culture (0.57 mg protein)	-	1.44	0.72
	+	2.72	1.44

Peritoneal lavage fluids and culture media of rat macrophages were prepared as described in the text and lyophilyzed materials were dissolved into 1 ml of distilled water. Lipomodulin levels were measured by their inhibitory activities on phospholipase A_2 as shown in Fig. 1a and by radioimmunoassay with Fig 1b as a standard curve.

RESULTS

Enzymatic assay and radioimmunoassay of lipomodulin: Lipomodulin inhibits phospholipae A_2 by changing Vmax but not Km for phospholipids (8). Lipomodulin purified from rabbit neutrophils could be assayed by its ability to inhibit phospholipase A_2 from porcine pancreas (2,4,8). The degree of inhibition of phospholipase A_2 by lipomodulin was dose dependent (Fig. 1a). Maximal inhibition of phospholipase A_2 required a stoicheometric amount of lipomodulin on a molar basis. When ^{125}I-labelled lipomodulin was precipitated by anti-lipomodulin antibody, the radioactivity in the precipitates was inversely proportional to the amounts of nonlabelled lipomodulin present in the assay (Fig. 1b). The presence of bovine serum albumin (1 mg), detergents (1%) or Ca^{2+} (10 mM) in the assay medium, which disturb the enzymatic assay of lipomodulin, did not affect the radioactivity of immunoprecipitates. In this study, lipomodulin could be measured in the range from 1 ng to 100 ng of protein, while the sensitivity of the enzymatic assay was 50 ng to 300 ng.

Enzymatic and immunological assay: To apply the radioimmunoassay and enzymatic assay of lipomodulin to biological materials, we analyzed peritoneal lavage fluids from glucocorticoid-treated and nontreated rats in

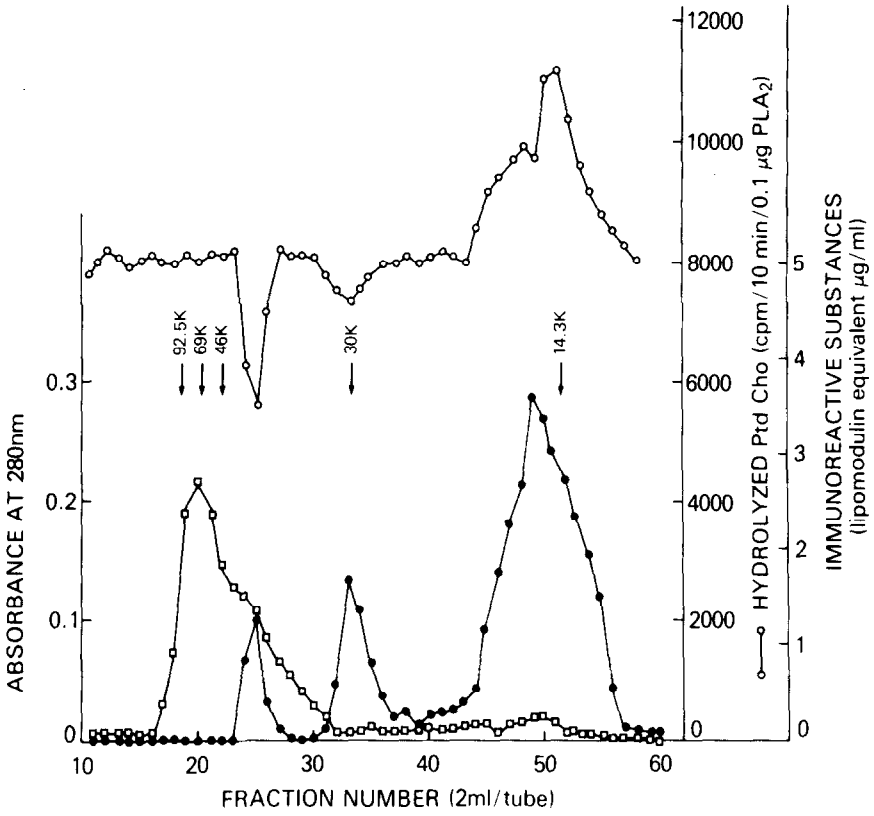

Fig 2. Fractionation of peritoneal lavage fluid from rats treated with dexamethasone by Sephadex G 200 column chromatography.

Peritoneal lavage fluid from rats treated with dexamethasone (1 mg/kg) was obtained as described in the text. Lyophylized material was dissolved into 1 ml of distilled water and 0.5 ml of aliquot was applied on Sephadex G 200 column (1 x 90 cm). The column was eluted with 10 mM sodium phosphate buffer containing 150 mM NaCl and each fraction contained 2 ml. Lipomodulin was assayed by either enzymatic method (O) using phospholipase A_2 (PLA_2) and phosphatidylcholine (PtdCho) or radioimmunoassay (●) as described in the text. Proteins was measured by absorbance at 280 nm (□).

addition to culture media of rat macrophages incubated with and without dexamethasone. Both preparations have been reported to be rich in macrocortin (3,5). When these preparations were measured by the enzymatic and immunological assays, the radioimmunoassay always gave two-fold higher values than those obtained by the enzymatic method (Table 1). Dexamethasone doubled the apparent level of lipomodulin regardless of the methods. Furthermore, these results suggest that lipomodulin and macrocortin might be mutually related proteins, although the sources for their isolation are different.

Table 2. Phospholipase inhibitory activities of Mr = 40,000, 30,000 and 15,000 fractions before and after alkaline phosphatase treatment

	Alkaline phosphatase treatment (U/mg equivalent)	
	before	after
Mr= 40,000 fraction	150	480
Mr= 30,000 fraction	118	260
Mr= 15,000 fraction	0	160

The fractions shown in Fig 2 were collected and treated with 100 μl of alkaline-phosphate-coupled agarose (10 U/ml, Sigma). As a control, the fractions were treated with 100 ul of lipomodulin-coupled agarose (10 μg of lipomodulin was coupled to 1 ml of Affi-Gel 10 at pH 8.0). After the incubation at room temperature for 30 min, the mixtures were centrifuged at 15,000 x g for 20 min and the aliquot was used for the assay. The specific activity was calculated by measuring the inhibitory activity on phospholipase A_2 and the amount of apparent lipomodulin by radioimmunoassay.

Sephadex G 200 column chromatography of crude macrocortin: To obtain additional evidence that the lavage fluids and culture media contain the phospholipase inhibitory protein(s), the peritoneal lavage fluids from rats injected with dexamethasone were separated by Sephadex G 200 column chromatography (Fig. 2). When the column was eluted with 10 mM sodium phosphate buffer containing 150 mM NaCl, most of the protein as measured by absorbance at 280 nm was eluted in the void volume. The activity to inhibit phospholipase A_2 was found in the fractions corresponding with Mr= 40,000 and 30,000. On the other hand, the immunoreactive substances were eluted in the Mr= 40,000, 30,000 and 16,000 fractions. The fraction with Mr= 16,000 did not exhibit inhibitory activity on phospholipase A_2. On the contrary, this fraction stimulated phospholipase A_2 activity, probably due to contamination of phospholipase(s) activators such as Ca^{2+} (5). Since we have previously reported that phosphorylated lipomodulin has no inhibitory activity on phospholipase A_2 (8), all three fractions were dephosphorylated by treatment with fetal calf intestinal alkaline phosphatase-coupled agarose after concentrated using diaflow PM 10 membrane. Such treatment of all the fractions increased their inhibitory activities on phospholipase A_2 (Table 2). Marked enhancement of phospholipase inhibition after phosphatase treatment was

observed in the fraction with Mr= 16,000. Since treatment of this fraction with lipomodulin-coupled agarose had no effect, these results suggest that the dephosphorylation caused the recovery of inhibitory activity. The specific activity of this fraction was calculated to be approximately one-third that of the Mr= 40,000 species.

DISCUSSION

The radioimmunoasssay of lipomodulin has been newly developed in addition to its enzymatic assay. Using this method, we demonstrated several species of phospholipase inhibitory protein(s) in materials from glucocorticoid-treated rats. These species had Mr= 40,000, 30,000 and 16,000. Among them, the species of Mr= 40,000 has the highest inhibitory activity on phospholipase A_2. In addition, an inhibitory activity was also detected in the Mr= 30,000 fraction. Although most of the immunoreactive material was eluted in fractions with Mr= 16,000 (macrocortin fraction), no inhibitory activity on phospholipase A_2 could be detected until after dephosphorylation by alkaline phosphatase treatment. Since many cells contain alkaline phosphatase on cell surfaces (10), it is likely that the Mr= 16,000 peptide also acts to inhibit arachidonate release in vivo. The previous reports on macrocortin demonstrated that a majority of the inhibitory activity with respect to prostaglandin formation in perfused lung is present in the Mr= 15,000 fraction, although the major inhibitory activity on phospholipase A_2 could be detected in the Mr= 40,000 fraction (2,3,5). In the IgE synthesis system of rat lymphocytes, the species of Mr= 15,000 has the ability to induce the formation of IgE suppressor factors by inhibiting phospholipase(s) (11,12). Since macrophages and neutrophils, major sources for purification of lipomodulin or macrocortin have various proteases secreted by stimulation, it is quite likely that the Mr= 40,000 species is cleaved to the smaller species by these proteases. In keeping with this interpretation, omission of protease inhibitors such as PMSF, aprotinin and soya bean trypsin inhibitor from buffers used for purification results in poor recovery of the Mr= 40,000 species. These results, taken together, suggest but do not necessarily

prove that macrocortin is a phosphorylated fragment of lipomodulin with Mr= 16,000, and that monoclonal anti-lipomodulin antibody cross reacts with macrocortin (Mr= 16,000). Thus, the biological materials obtained from dexamethasone-treated and nontreated animals contain three species of phospholipase inhibitory protein(s) with different molecular weights. Since each species has a different specific activity to inhibit phospholipase A_2, the enzymatic assay of lipomodulin is not adequate to measure the level of phospholipase inhibitory protein(s). Furthermore, the majority of these proteins appeared to be phosphorylated and inactive unless they were treated with alkaline phosphatase. Thus, the radioimmunoassay of lipomodulin has several advantages to measure phospholipase inhibitory protein(s) in biological materials, compared with the enzymatic assay.

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