REDISTRIBUTION OF 5-LIPOXYGENASE AND CYTOSOLIC PHOSPHOLIPASE A2 TO THE NUCLEAR FRACTION UPON MACROPHAGE ACTIVATION[1]

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SUMMARY   Both the cytosolic phospholipase A2 and 5-lipoxygenase enzymes redistribute from the high-speed supernatant to a particulate fraction upon cell activation with associated leukotriene synthesis, but the subcellular site to which these enzymes translocate is not known. In this study, we disrupted resting and ionophore A23187-stimulated rat peritoneal macrophages by N2 cavitation and separated lysates into nuclear, cytosolic, and crude membrane fractions; these were then examined by immunoblot analysis for their contents of immunoreactive cytosolic phospholipase A2, 5-lipoxygenase, and 5-lipoxygenase activating protein. 5-Lipoxygenase activating protein was localized predominantly in the nuclear fraction of both resting and activated cells, while both cytosolic phospholipase A2 and 5-lipoxygenase redistributed from the cytosol fraction in resting cells to the nuclear fraction in activated cells. These data demonstrate for the first time coordinate subcellular localization of the key proteins involved in leukotriene synthesis from endogenous arachidonate.   © 1993 Academic Press, Inc.

Arachidonic acid (AA), the precursor for an array of bioactive oxidative metabolites known as eicosanoids, is liberated from membrane phospholipids by phospholipase enzymes, in particular phospholipase A2. Metabolism of AA via the 5-lipoxygenase (5-LO) pathway results in the synthesis of leukotrienes C4 and B4 (LTB4) as well as 5-hydroxyeicosatetraenoic acid. These eicosanoids are potent mediators of inflammation which serve important roles in normal host defense as well as in the pathogenesis of a variety of inflammatory diseases, including asthma, allergic rhinitis, inflammatory bowel disease, psoriasis, and rheumatoid arthritis (1).

The first two committed steps in this metabolic pathway are catalyzed by the 5-LO enzyme itself (2). Because of the biologic significance of these reactions, the molecular mechanisms of 5-LO activation have been the focus of intense investigation. Such studies, performed largely in granulocytes, have demonstrated that i) upon cell activation, 5-LO translocates from the soluble

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Abbreviations: AA, arachidonic acid; 5-LO, 5-lipoxygenase; LTB4, leukotriene B4; FLAP, 5-LO activating protein; cPLA2, cytosolic phospholipase A2; PM, peritoneal macrophages.

fraction to the particulate fraction (3, 4), and ii) leukotriene generation requires an as yet uncharacterized interaction of the translocated 5-LO with an integral membrane protein designated "5-LO activating protein" (FLAP) (5, 6).

To date, subcellular fractionation studies designed to identify the membrane site to which 5-LO translocates have not been reported. In the present study, we addressed this question by using immunoblot analysis on fractions of rat peritoneal macrophages (PM) activated with the potent model agonist, ionophore A23187. In agreement with recent immunoelectron microscopic localization studies performed in neutrophils (7), our data demonstrated that both FLAP as well as translocated 5-LO were localized to the nuclear fraction in stimulated PM. We used the same techniques to examine the localization of the recently-described arachidonoyl-selective high-molecular mass cytosolic phospholipase A2 (cPLA2), which is also known to associate with a particulate fraction upon cell activation (8, 9), and found that cPLA2 also redistributed to the nuclear fraction following A23187 stimulation. To our knowledge, this is the first demonstration of any kind that the nucleus is the site to which cPLA2 translocates, and the first study to demonstrate coordinate translocation of both cPLA2 and 5-LO to the same intracellular locale.

Materials and Methods

Cell isolation and experimental incubations. Resident PM were obtained by lavage of the peritoneal cavity of 125-150 g female Wistar rats as described (10). After hypotonic lysis, cells were resuspended in Hank's balanced salt solution (GIBCO, Grand Island, NY) and enumerated in a hemacytometer. They were then pelleted by centrifuging at 500 x g for 5 min, resuspended in warmed medium 199 containing Earle's salts (GIBCO) at ~10-15 x 10^6 cells/ml, and 2 ml of cell suspension aliquotted to a 15 ml conical polypropylene tube for each experimental condition. Ionophore A23187 (Calbiochem, La Jolla, CA) (final concentration 5 μM) in DMSO (final concentration, 0.25%) or DMSO alone was added to the tube and the cell suspension was lightly vortexed and incubated at 37 °C for 15 min . Following agonist incubation, EDTA was added to each tube to a final concentration of 4 mM and tubes were vortexed and placed on ice. Cells were pelleted at 4 °C for 5 min at 500 x g, and washed by resuspending in 2 ml of iced cavitation buffer (50 mM potassium phosphate, 100 mM NaCl, 1 mM dithiothreitol, 2 mM EGTA, 2 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 60 μg/ml soybean trypsin inhibitor, and 1 μg/ml leupeptin, pH 7.1). They were pelleted once again and resuspended in 1 ml of iced cavitation buffer. At this point cell suspensions were routinely stored at -70 °C for up to 1 wk prior to disruption.

Cell disruption and fractionation. Disruption was accomplished by thawing cell suspensions and cavitating in a Parr (Moline, IL) N2 cavitation bomb at 800 psi for 20 min on ice; preliminary experiments determined that this level of pressure yielded optimal cell breakage. The cavitate was collected and centrifuged at 600 x g and 4 °C for 40 min. This supernatant was recentrifuged at 600 x g for an additional 20 min. The pellets from the two 600 x g spins were pooled to yield crude nuclei. Microscopic examination of Diff-Quik (Difco, Detroit, MI)-stained cytospin preparations of this fraction revealed homogeneous spherical bodies typical of nuclei (not shown). The nuclei were washed by resuspending in 1 ml of cavitation buffer and centrifuging at 1000 x g. They were then resuspended in 250 μl of cavitation buffer and sonicated using a Branson (Danbury, CT) sonifier at 20% duty cycle, setting 1, for 90 sec to yield the nuclear fraction. Preliminary experiments had determined that even two 600 x g spins were not adequate to remove all nuclei; thus, an additional 10 min centrifugation at 1000 x g at 4 °C was performed prior to ultracentrifugation. This 1000 x g pellet was discarded and the nuclei-free low-speed supernatant was then subjected to ultracentrifugation for 1 h at 100,000 x g and 4 °C. The high-speed supernatant was designated the cytosol fraction. The high-speed pellet was washed by resuspending in 100 μl of iced cavitation buffer and dispersed with two brief bursts of sonication at 100% duty cycle. It was recentrifuged at 100,000 x g, resuspended once again in 100 μl of cavitation buffer, sonicated briefly, and designated the membrane fraction.

Immunoblot analysis of cell fractions. The total protein content of nuclear, cytosol, and membrane fractions was determined using a microtiter plate modification of the Coomassie dye-binding assay (Pierce, Rockford, IL) with bovine serum albumin as standard. The distribution of protein among the three fractions was identical in control and A23187-stimulated cells: nuclear, 21 ± 3% and 22 ± 5%; cytosol, 69 ± 3% and 68 ± 5%; and membrane, 10 ± 1% and 10 ± 0%, respectively (p>0.05). Aliquots from the three fractions containing equal amounts of protein (~10-30 μg) were subjected to SDS-PAGE under reducing conditions using 11% acrylamide gels to separate FLAP and 5-LO and 7.5% acrylamide gels to separate $cPLA_2$. Proteins of interest were detected by immunoblot analysis as described (11) using rabbit polyclonal antisera raised against either i) purified human leukocyte 5-LO (1:3000 dilution) (provided by Dr. J. Evans, Merck Frosst, Pointe Claire-Dorval, Quebec), ii) amino acid residues 41-52 of the human FLAP sequence (1:5000 dilution) (provided by Dr. J. Evans, Merck Frosst), or iii) purified human recombinant $cPLA_2$ (1:1000 dilution) (provided by Dr. J. Clark, Genetics Institute, Cambridge, MA). Blots were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL) and developed using the ECL chemiluminescent detection system (Amersham) according to the manufacturer's instructions. The densities of bands corresponding to 5-LO, FLAP, and $cPLA_2$ were quantitated by video densitometry of autoradiographs using image analysis software from Scion (Frederick, MD). In each experiment, arbitrary density units per μg protein loaded were multiplied by the total protein content of each fraction to give the relative amounts of each of the three proteins of interest for each of the three fractions in both unstimulated and A23187-stimulated PM.

Enzyme immunoassay for LTB_4. Immunoreactive LTB_4 was quantitated in cell-free supernatants of cell suspensions by an acetylcholinesterase-linked immunoassay kit from Cayman (Ann Arbor, MI), according to the manufacturer's instructions.

Data analysis. All data are presented as the mean ± SEM for n=5 independent experiments. Subcellular fractions from control and A23187-treated cells were compared using a paired Student's *t* test, and significance was assumed when p<0.05.

Results and Discussion

Although the organellar membrane to which 5-LO translocates upon cell activation was not defined in the original reports describing such redistribution, this site had long been assumed to be the plasma membrane (12), given that leukotrienes were known to be secreted extracellularly. It was therefore quite surprising that immunogold electron microscopic studies recently localized both FLAP as well as translocated 5-LO in ionophore-activated neutrophils to the nuclear envelope (7). In retrospect, it is clear that the original descriptions of this phenomenon were incapable of recognizing the role of the nucleus, since i) cells were disrupted by sonication, a relatively harsh method that tends to fragment intracellular membranes as well, and ii) low-speed particulate fractions likely to include much of the nuclear material were discarded. Certainly, cell disruption and fractionation methods appropriate for studying the nuclear translocation of 5-LO will be essential if the mechanisms and biologic significance of such localization are to be characterized.

Towards that end, we utilized N_2 cavitation as a gentler disruption method more likely to spare intracellular organellar membranes, and employed for subcellular fractionation a differential centrifugation technique whose primary goal was to separate nuclei from other components. Macrophages comprise an appropriate cellular model for study because they are the major leukotriene-synthesizing cells normally resident within most organs. Activation of AA hydrolysis and 5-LO metabolism in response to agonist stimulation was documented by determining levels of immunoreactive LTB_4, the major macrophage 5-LO metabolite, in the supernatant of PM suspensions (control, 25 pg/ml; A23187, 5859 pg/ml).

Figure 1A depicts the distribution of immunoreactive FLAP in nuclear, cytosol, and membrane fractions from a representative immunoblot analysis experiment. FLAP was recognized as an 18 kDa protein localized exclusively to the nuclear and membrane fractions. No differences in distribution were apparent between control and A23187-stimulated cells. The distribution of total FLAP among all three fractions was estimated in each individual experiment by multiplying the density of the FLAP band per μg of protein loaded by the total protein content of each fraction. Figure 1B shows the mean distribution of immunoreactive FLAP from all 5 experiments. The nuclear fraction contained ~75% and the membrane fraction, ~20% of the total cellular FLAP, and the distribution was not affected by stimulation. These results are consistent with the electron microscopic immunolocalization data reported in neutrophils (7), in which FLAP was localized predominantly in the nuclear envelope and to a lesser extent in the endoplasmic reticulum of both resting and stimulated cells. Figure 2A and 2B present analogous representative and mean data for immunoreactive 5-LO protein. 5-LO was recognized as a 78 kDa band predominantly localized to the cytosol fraction of control cells. Stimulation resulted in significant (p<0.02) decreases in the 5-LO content of the cytosol fraction and significant (p<0.01) increases in the 5-LO content of the nuclear fraction, with no significant change in the membrane fraction. These results, too, are consistent with the data indicating immunolocalization of 5-LO to the nuclear envelope of ionophore-stimulated, but not resting, neutrophils (7).
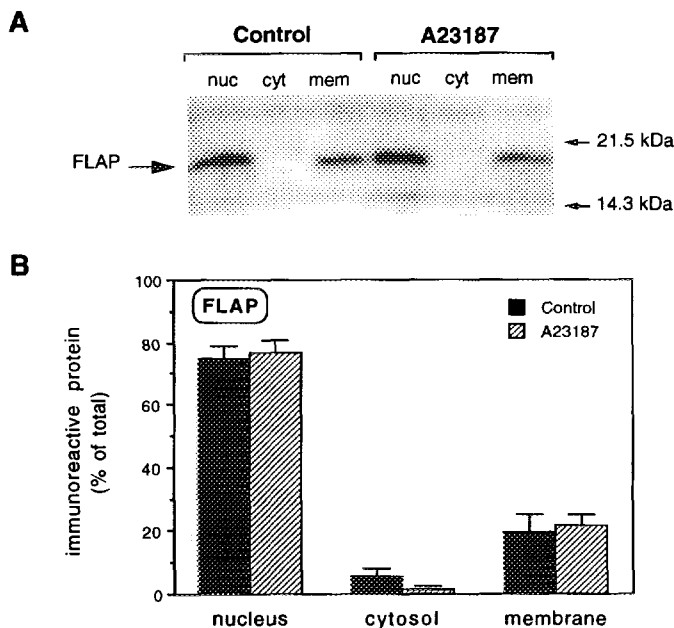

Figure 1. Subcellular distribution of immunoreactive FLAP protein in control and A23187-stimulated PM. Aliquots of PM fractions containing equal amounts of total protein were subjected to immunoblot analysis as described in Materials and Methods. A) Autoradiograph of a blot from a representative experiment. Migration positions of molecular weight markers are indicated on the right; (nuc, nucleus; cyt, cytosol; mem, membrane). B) Distribution of total FLAP protein among the three fractions. Total FLAP protein content of each fraction was derived from the product of band density/μg protein loaded x μg protein per fraction. Each bar represents the mean ± SEM from n=5 independent experiments.
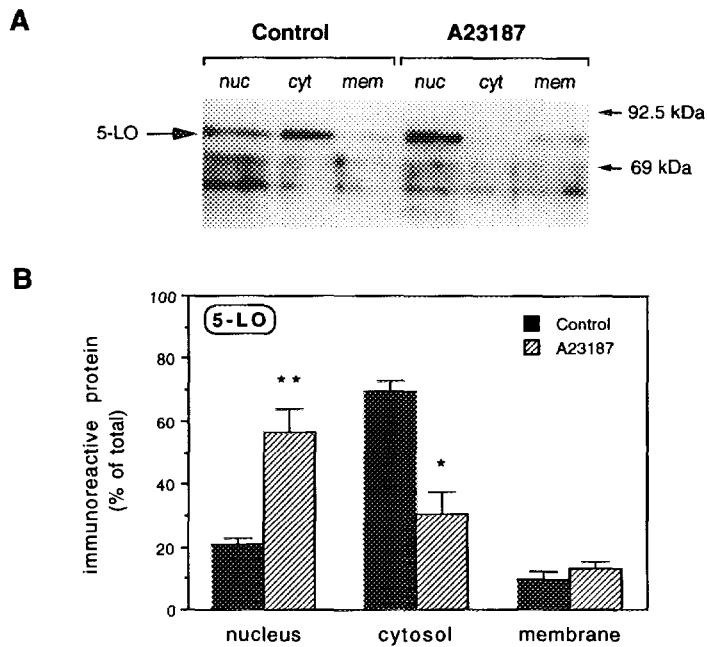
Figure 2. Subcellular distribution of immunoreactive 5-LO protein in control and A23187-stimulated PM. Methods and data expression are as described in the legend to Figure 1. A) Autoradiograph of a blot from a representative experiment. B) Distribution of total 5-LO protein among the three fractions. *p<0.02; **p<0.01.

cPLA2 is a recently-described hormonally-regulated (9, 13) form of PLA2 that exhibits biochemical characteristics (arachidonoyl selectivity, submicromolar calcium requirement, and resistance to reducing agents such as glutathione) (14, 15) consistent with its functioning as a key intracellular enzyme responsible for hydrolyzing AA destined for eicosanoid synthesis. Prior studies examining the translocation of cPLA2, which indicated redistribution from the cytosol to a high-speed particulate fraction, either utilized sonication (8), or discarded the low-speed pellet (8, (9). To our knowledge, no studies employing immunolocalization techniques have been undertaken with cPLA2. Since the disruption and fractionation methods which we employed demonstrated subcellular distributions of FLAP and 5-LO which were in agreement with the ultrastructural immunolocalization data mentioned above, we also utilized them to examine the redistribution of cPLA2. Figure 3A presents a representative immunoblot, and Figure 3B, the mean distribution of immunoreactive cPLA2 from all of the experiments. cPLA2 protein was recognized as a 97 kDa band which, in resting cells, was predominantly localized to the cytosol fraction. Upon A23187 activation, cPLA2 content of the cytosol fraction decreased significantly (p<0.05), while that of the nuclear fraction increased significantly (p<0.05); the cPLA2 content of the membrane fraction did not change (p>0.05).

These data indicate that both cPLA2 and 5-LO translocate to the nuclear fraction upon activation of rat PM. Of note, this is also the constitutive site of localization for FLAP. Although not directly examined in the present study, it is likely on the basis of ultrastructural immunolocalization data in neutrophils (7) that the precise site at which these proteins are localized within the nucleus of
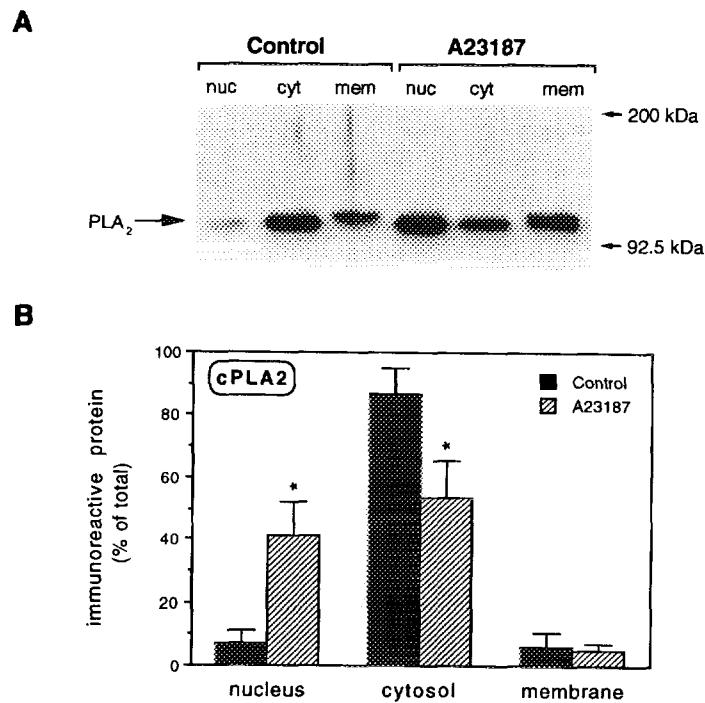
Figure 3. Subcellular distribution of immunoreactive cPLA2 protein in control and A23187-stimulated PM. Methods and data expression are as described in the legend to Figure 1. **A)** Autoradiograph of a blot from a representative experiment. **B)** Distribution of total cPLA2 protein among the three fractions. *p<0.05.

activated cells is the nuclear envelope. Since FLAP has the capacity to bind AA (16), it may serve to present to the 5-LO enzyme its fatty acid substrate which has been liberated from nuclear membrane phospholipids by cPLA2. This scenario is consistent with published data which have linked AA hydrolysis from nuclear membrane phospholipids to eicosanoid synthesis (17). Interestingly, CTP:phosphocholine cytidyltransferase, a key enzyme in phosphatidylcholine synthesis, has also been recently demonstrated to translocate to the nuclear membrane upon activation (18). Finally, the two isoforms of cyclooxygenase (prostaglandin endoperoxide synthase), which catalyzes the initial steps in the conversion of AA to prostaglandins and thromboxane, have also been localized to the nuclear membrane and perinuclear endoplasmic reticulum (19). Taken together, these findings provide compelling evidence that the nucleus, in particular the nuclear membrane, is an important intracellular site for both phospholipid remodeling as well as the oxidative metabolism of AA to bioactive eicosanoids. The approach described in this report should prove useful in efforts to further elucidate the role of the nucleus in these processes.

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