Rapid Import of Cytosolic 5-Lipoxygenase into the Nucleus of Neutrophils after in Vivo Recruitment and in Vitro Adherence*

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5-Lipoxygenase catalyzes the synthesis of leukotrienes from arachidonic acid. The subcellular distribution of 5-lipoxygenase is known to be cell type-dependent and is cytosolic in blood neutrophils. In this study, we asked whether neutrophil recruitment into sites of inflammation can alter the subcellular compartmentation of 5-lipoxygenase. In peripheral blood neutrophils from rats, 5-lipoxygenase was exclusively cytosolic, as expected. However, in glycogen-elicited peritoneal neutrophils, abundant soluble 5-lipoxygenase was in the nucleus. Upon activation with calcium ionophore A23187, intranuclear 5-lipoxygenase translocated to the nuclear envelope. Elicited neutrophils required a greater concentration of A23187 for activation than did blood neutrophils (half-maximal response, 160 versus 52 nM, respectively) but generated greater amounts of leukotrienes from arachidonic acid (AA). They have important roles in regulating inflammatory processes implicated in disease states, such as both normal cell functions, such as proliferation (1), and in inflammation can alter the subcellular compartmentation of 5-lipoxygenase. In peripheral blood neutrophils from rats, 5-lipoxygenase was exclusively cytosolic, as expected. However, in glycogen-elicited peritoneal neutrophils, abundant soluble 5-lipoxygenase was in the nucleus. Upon activation with calcium ionophore A23187, intranuclear 5-lipoxygenase translocated to the nuclear envelope. Elicited neutrophils required a greater concentration of A23187 for activation than did blood neutrophils (half-maximal response, 160 versus 52 nM, respectively) but generated greater amounts of leukotrienes from arachidonic acid (AA). They have important roles in regulating inflammatory processes implicated in disease states, such as both normal cell functions, such as proliferation (1), and in inflammation can alter the subcellular compartmentation of 5-lipoxygenase. In peripheral blood neutrophils from rats, 5-lipoxygenase was exclusively cytosolic, as expected. However, in glycogen-elicited...
processed for indirect immunofluorescent microscopy (see below). Alternatively, lung sections (from different animals) were processed for immunohistochemistry (see below). Human alveolar leukocytes were obtained from patients with pneumonia undergoing diagnostic bronchoalveolar lavage, as described (25). The experimental protocol was approved by the University of Michigan Medical Center Institutional Review Board for Approval of Research Involving Human Subjects.

**Indirect Immunofluorescence Microscopy (IFM)**—Cells were prepared for IFM as described previously (4), using methanol (−20 °C, 30 min) followed by acetone (−20 °C, 3 min) to fix and permeabilize. They were then probed with a rabbit polyclonal antibody raised against purified human leukocyte 5-LO (a generous gift of Dr. J. Evans, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dore, Quebec, Canada; titer of 1:150) (8) followed by rhodamine-conjugated goat anti-rabbit antibody (1:200, Sigma). In some cases, cells were also stained for DNA using acridine orange (in the presence of RNase). Preparations were examined with a Nikon Labophot 2 microscope equipped for epifluorescence or imaged by confocal microscopy using a Bio-Rad MRC-600 laser confocal microscope. The rhodamine signal was imaged using a 560-nm-long pass filter followed by a 585-nm bandpass filter. Acridine orange fluorescence was isolated using a 514-nm bandpass filter combined with a 526-nm-long pass dichroic reflector.

**Cell Fractionation/Immunoblotting**—As described previously (9), cells were disrupted by nitrogen cavitation at 400 p.s.i. for 5 min at 4 °C. Cavitate was centrifuged at 1000 × g to pellet nuclei; the postnuclear supernatant was centrifuged at 100,000 × g for 30 min at 4 °C to generate soluble (“cytosolic”) and insoluble (“membrane”) fractions. Nuclei were sonicated, and soluble and insoluble fractions were separated by ultracentrifugation. Equivalent amounts of protein from each fraction were evaluated by immunoblotting using 5-LO antibody (1:5000) and then peroxidase-conjugated goat anti-rabbit secondary (1:50000) with chemiluminescent detection (Amersham) or by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI).

**Immunohistochemistry**—Immunohistochemistry, as described (26), utilized lung tissue specimens fixed in neutral-buffered formalin, embedded in paraffin, sliced, mounted, deparaffinized (AmeriCel), and rehydrated in descending concentrations of ethanol. Tissue was blocked (Power Block; Biogenex, San Ramon, CA), probed with 5-LO antibody (1:5000, 4 °C, 24 h), washed, and then incubated with biotinylated goat anti-rabbit secondary (1:5000) followed by the avidin-biotin-peroxidase complex (Amersham). After incubation, slides were washed and then incubated with True-Blue peroxidase substrate (KPL Laboratories, Gaithersburg, MD), and then counterstained with Contrast Red (KPL Laboratories).

**Cell Activation and Enzyme Immunoassay**—PMNs in M199 at 10⁶ cells/ml were activated with calcium ionophore A23187. After 15 min at 37 °C, cells were pelleted, and the LTβ concentration in the conditioned media was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Average values of duplicate determinations were obtained in each experiment.

**Statistical Analysis**—Statistical significance was evaluated by a paired Student’s t test, using p < 0.05 as indicative of statistical significance.

**RESULTS**

**5-LO Distribution in Peripheral Blood and Glycogen-elicited Peritoneal PMNs**—The distribution of 5-LO was evaluated by two complementary techniques: 1) IFM, and 2) cell fractionation combined with immunoblotting. By IFM, 5-LO was predominantly cytosolic in PMNs purified from peripheral blood (Fig. 1A), as has been described for human blood PMNs (4). This pattern was observed in essentially all blood PMNs. In sharp contrast, most PMNs elicited into the peritoneum after glycogen instillation showed the opposite pattern: the nucleus stained strongly positive for 5-LO, whereas the cytosol had negligible staining (Fig. 1B). This pattern was evident in approximately 80% of all PMNs obtained by peritoneal lavage after elicitation. Other PMNs showed either predominantly cytosolic 5-LO or a mix of both cytosolic and nuclear staining (arrow, Fig. 1B). Peritoneal macrophages, a minor contaminant, had predominantly cytosolic 5-LO (data not shown), as reported previously (5).

When peripheral blood PMNs were analyzed as a population by fractionation and immunoblotting, 5-LO was found to be entirely soluble and exclusively in the cytosol (Fig. 1C), consistent with conclusions based on IFM. By the same method, 5-LO in lavaged peritoneal cells was divided between the cytosolic and nuclear soluble fractions. Thus, two independent methods, one that evaluated individual cells and another that analyzed cell populations, indicated that 5-LO was exclusively in the cytosol of peripheral blood PMNs, whereas glycogen-elicited PMNs contained abundant nuclear 5-LO. The apparent differences in the amount of cytosolic 5-LO in elicited PMNs as judged by the two methods may be attributed to, on the one hand, an underestimation due to loss during permeabilization for IFM and, on the other hand, an overestimation due to unavoidable disruption of some nuclei combined with fractionation of a heterogeneous population (i.e., PMNs with nuclear 5-LO mixed with some peritoneal macrophages, as well as PMNs, with cytosolic 5-LO).

The localization of 5-LO in elicited PMNs was further evaluated by optical sectioning of stained cells, using confocal microscopy (Fig. 2). An optical section through the nucleus of elicited PMNs stained for both DNA (using acridine orange) and 5-LO revealed colocalization of DNA and 5-LO in most cells, although some cells retained cytosolic 5-LO (arrow, Fig. 2A). Serial sections through cells stained only for 5-LO demonstrated that 5-LO was dispersed homogenously throughout the intranuclear volume rather than distributed peripherally near the nuclear envelope (Fig. 2B).

**5-LO Distribution in Other Types of Elicited PMNs**—PMNs can be recruited into a variety of tissue sites in response to a number of different signals. We were interested in determining if the nuclear import of 5-LO was unique to glycogen-elicited peritoneal PMNs. 5-LO was also intranuclear in PMNs recovered from the rat peritoneum 4 h after instillation of heat-killed C. neoformans (Fig. 3A), indicating that the result was not glycogen-specific. 5-LO was also found to be intranuclear in PMNs recovered from rat lung 2 days after intratracheal instillation of bleomycin as determined by either IFM of cells recovered from lung by lavage (Fig. 3B) or immunohistochemistry of lung sections (Fig. 3C). The demonstration of intranuclear 5-LO in elicited PMNs in situ (by immunohistochemistry) indicated that the redistribution did not result from the lavage process or the subsequent handling of PMNs. As an example of elicited PMNs from humans, PMNs recovered by bronchoalveolar lavage of patients with pneumonia also exhibited intranuclear 5-LO (Fig. 3D). These results showed that 5-LO movement from the cytosol into the nucleus could occur in PMNs recruited into different anatomic sites, in response to different
agents, and in humans as well as in rats.

The Effect of Subcellular Distribution on Function—How does 5-LO redistribution affect 5-LO function? One hallmark of 5-LO action is its translocation to the nuclear envelope upon cell stimulation, a process that colocalizes 5-LO with the AA-binding protein, 5-lipoxygenase-activating protein. As reported for human blood PMNs (8, 9), after stimulation, 5-LO in rat blood PMNs was found predominantly at the periphery of the multilobed nuclei, apparently at the nuclear envelope (Fig. 4A). The subcellular distribution of 5-LO in elicited PMNs after activation was also consistent with translocation to the nuclear envelope (Fig. 4B). However, the pattern of distribution was subtly different from that seen in blood PMNs: in blood PMNs, the nuclear lobes and connecting strands were clearly outlined with fluorescence, whereas in elicited PMNs, only portions of the nuclear lobes were labeled. These patterns would be consistent with translocation to different membrane faces of the nuclear envelope, with cytoplasmic 5-LO decorating the outer membrane, and intranuclear 5-LO binding to the inner membrane. Finally, the distribution of 5-LO in activated elicited PMNs was distinctly different from that in resting elicited PMNs (Fig. 1B), again indicating that 5-LO in resting elicited PMNs was not simply associated with the nuclear envelope.

Elicited PMNs, like blood PMNs, generated little LTB₄ without exogenous stimulation (7.3 ± 2.3 versus 12.9 ± 2.4 pg/10⁶ cells, respectively; p = 0.21; n = 3), indicating that elicitation itself did not activate the 5-LO pathway. We hypothesized that a consequence of the shift in 5-LO localization seen with elicitation might be a change in the dose of ionophore required for LT synthesis. Specifically, we reasoned that more ionophore would be needed to cause an increase in nuclear calcium and thus activate nuclear 5-LO than would be needed to have the same result within the cytosol. Indeed, significantly more ionophore was needed to stimulate LTB₄ synthesis in elicited PMNs than in peripheral blood PMNs (half-maximal stimulation at 160 ± 16 versus 52 ± 4.6 nM A23187, respectively; p = 0.0027; n = 4). However, maximal LT synthetic capacity for elicited PMNs (26.6 ± 7.4 ng/10⁶ cells at 407 ± 210 nM A23187) exceeded that for blood PMNs (7.68 ± 0.84 ng/10⁶ cells at 165 ± 78 nM A23187) significantly (p = 0.044; n = 4). Dose-response curves from a representative experiment are shown in Fig. 5. Thus, a greater level of stimulation was needed to initiate LT synthesis in elicited PMNs, but the maximal LT synthetic capacity of elicited PMNs significantly surpassed that of blood PMNs.

Possible Role for Adherence in Redistribution of 5-LO—PMN recruitment is a multistep process, but one prerequisite for recruitment to all anatomic sites is the adherence of PMNs to the endothelium. If adherence triggers the redistribution of 5-LO from the cytosol to the nucleus of PMNs, then PMNs examined at earlier time points after recruitment should also show intranuclear 5-LO. Indeed, PMNs recovered by peritoneal lavage at either 1 or 2 h after glycoegen instillation (in separate animals) exhibited intranuclear 5-LO (Fig. 6). This indicated that the nuclear import of 5-LO occurred relatively early in recruitment, resulting from an early event, such as adherence, and was not necessarily a function of prolonged residence in the inflammatory site. Furthermore, because elicited PMNs from both human and rat sources demonstrated intranuclear 5-LO (Fig. 3), it seemed likely that a common process, like adherence, could cause the nuclear import of 5-LO in PMNs from either source.

As an initial evaluation of the role of adherence in 5-LO redistribution in human PMNs, peripheral blood PMNs were
purified and examined before and after adherence to glass. By IFM, 5-LO was cytosolic in freshly purified human blood PMNs maintained in suspension in Teflon cups (Fig. 7A), as expected. After adherence to glass for 1 h, 5-LO was predominantly intranuclear (Fig. 7C). A shift of 5-LO into the nucleus was evident as early as 15 min after adherence (Fig. 7B). Intranuclear localization of 5-LO was also prominent in PMNs adhered for 1 h to albumin-coated glass or plastic (data not shown). When adherent PMNs were detached by trypsinization, fractionated, and analyzed by immunoblotting, abundant 5-LO was associated with the nuclear soluble fraction, whereas 5-LO was solely cytosolic in PMNs in suspension (Fig. 7C). Thus, adherence alone is sufficient to induce the redistribution of 5-LO from the cytosol into the nucleus.

**DISCUSSION**

It is well established that upon activation, 5-LO undergoes rapid translocation to the nuclear envelope. However, the subcellular distribution of 5-LO in resting cells has previously been viewed as static, although it was known to vary among cell types (27). The principle finding of this study is that the distribution of 5-LO can be dynamic, even in the resting cell: unstimulated PMNs in the peripheral blood have none of the enzyme associated with the nucleus, whereas elicited or adherent PMNs have abundant 5-LO within the nucleus. It is important to distinguish between the import into the nucleoplasm seen here and the translocation to the nuclear envelope associated with activation. The former, unlike the latter, was not associated with LT synthesis.

This study also demonstrated several important aspects of the shift in 5-LO distribution: 1) this redistribution can be relatively rapid. 2) The redistribution of 5-LO into the nucleus is a general feature of PMN recruitment to sites of inflammation: it can occur during elicitation into different sites, in response to different agents, and in both rats and humans. 3) 5-LO within the nucleus, like 5-LO in the cytosol, will translocate to the nuclear envelope upon cell stimulation. 4) Elicited PMNs, with predominantly intranuclear 5-LO, can not only synthesize LTs but can generate significantly greater amounts of LTs than blood PMNs, which have exclusively cytosolic 5-LO. 5) Nuclear sequestration seems to buffer the 5-LO pathway from activation. 6) Adherence alone is sufficient to induce redistribution of 5-LO.

The actual trigger for import is not known. Although adherence seems to rapidly promote nuclear import, different types of cell surfaces, adhesion molecules, and extracellular matrices may have different effects on the import of 5-LO. These have not been examined here. Also, the mechanism of 5-LO import into the nucleus is not known. Large molecules require either a nuclear localization sequence or a chaperone with a nuclear localization sequence to traverse nuclear pores (28). The 5-LO protein has a region that is rich in basic amino acids (amino acids 650–657) that might serve as a nuclear localization sequence to traverse nuclear pores (28). The 5-LO protein itself or a change in an unidentified accessory molecule, such as an import-chaperone protein or a cytosolic tethering protein. These possibilities await clarification.

The functional importance of the 5-LO redistribution that we have described is unclear, but it could have ramifications for enzyme activation. 5-LO activation is calcium-dependent (30). In some cell types, changes in intranuclear calcium levels seem to be independent of changes in cytosolic calcium (31), whereas in other cell types, the two apparently are linked (32). Theoretically, an agonist that increased calcium exclusively in the cytosol would activate 5-LO (and initiate LT synthesis) in blood PMNs but not in elicited PMNs. In such a scenario, the sequestration of 5-LO within the nucleus would thereby serve to decrease the susceptibility of the enzyme to activation. Consistent with this possibility, our data using ionophore demonstrated that more agonist was needed to drive LT synthesis in elicited PMNs than in blood PMNs. A similar pattern exists among macrophages: the threshold for initiation of LT synthesis is higher in alveolar macrophages, which have intranuclear 5-LO (6), than in peritoneal macrophages (24), which have cytosolic 5-LO (5). However, once triggered, the level of LT synthesis produced by nuclear 5-LO in elicited PMNs greatly exceeds that produced by cytosolic 5-LO in blood PMNs. It is interesting that just as alveolar macrophages have a higher activation threshold than peritoneal macrophages, they also have a greater maximal LT synthesis.
patterns of 5-LO distribution and action in macrophages and PMNs suggest a causal relationship between changes in 5-LO localization and altered AA metabolism. Although the basis for the greater maximal LT synthetic capacity in elicited PMNs and alveolar macrophages is not known, these results underscore the importance of intranuclear 5-LO as an active pool in LT generation.

Within the activated cell, cytosolic 5-LO will move to the outer membrane of the nuclear envelope, whereas intranuclear 5-LO will move to the inner membrane. These sites of 5-LO action are spatially distinct, being separated by a lumen. The function of activated 5-LO may therefore be influenced by its topographic proximity to substrate and cofactors, as well as other enzymes that act either upstream or downstream from it. In this manner, the disparate distributions of 5-LO in resting and elicited PMNs would also be expected to have further ramifications on molecular aspects of enzyme action. Furthermore, the subcellular localization of these other elements may also be dynamic rather than static. For example, the “cytosolic” phospholipase A2 is indeed cytosolic in quiescent endothelial cells but becomes intranuclear when the same cells are growing rapidly (33). Thus, the element of dynamic redistribution adds a new level of complexity to the process of AA liberation and 5-LO action.

The changeable compartmentation of 5-LO suggests novel roles for 5-LO products and also for 5-LO itself. The rapid redistribution of 5-LO may serve to regulate the site of 5-LO enzymatic action. This would be particularly interesting if 5-LO products acted at the immediate site of synthesis. For example, if the products of cytosolic 5-LO affected cytoskeletal processes (e.g., cytoskeletal function), and the products of nuclear 5-LO affected nuclear processes (e.g., transcription), then 5-LO compartmentation could be a way to regulate cell function. Such localized effects could be achieved by much lower concentrations of LTs than are needed for extracellular release and paracrine action. These effects might be mediated by soluble intracellular receptors like the peroxisome proliferator-activated receptor α, which can bind LTB4 in the nucleus (34). Alternatively, the 5-LO protein itself has sites for interacting with other proteins, including a Src homology 3-binding domain (35). Such domains mediate the interaction of 5-LO with other proteins (35), suggesting the possibility of function(s) for 5-LO independent of its enzymatic activity. The rapid redistribution of 5-LO, then, could serve as a mechanism for regulating the accessibility of 5-LO to other proteins and thus controlling such (hypothetical) effects.

The potential for rapid movement of 5-LO between the cytosol and the nucleus in a single cell type, independent of activation, indicates a level of complexity in 5-LO action that has heretofore been unappreciated. Furthermore, this study correlates the import of 5-LO into the nucleus with an increase in cellular LT synthetic capacity. The molecular mechanisms of both 5-LO redistribution and altered LT synthetic capacity remain to be determined.
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