Elevation of the intracellular cAMP concentration in agonist-activated human neutrophils (PMN) leads to the concomitant inhibitions of arachidonic acid (AA) release, 5-lipoxygenase (5-LO) translocation, and leukotriene (LT) biosynthesis. We report herein that exogenous AA completely prevents cAMP-dependent inhibition of 5-LO translocation and LT biosynthesis in agonist-activated PMN. Moreover, the group IVA phospholipase A2 inhibitor pyrrophenone and the MEK inhibitor U-0126 inhibited AA release and 5-LO translocation in activated PMN, and these effects were also prevented by exogenous AA, demonstrating a functional link between AA release and 5-LO translocation. Polyunsaturated fatty acids of the C18 and C20 series containing at least three double bonds located from carbon 9 (or closer to the carboxyl group) were equally effective as AA in restoring 5-LO translocation in pyrrophenone-treated agonist-activated PMN. Importantly, experiments with the 5-LO-activating protein inhibitor MK-0591 and the intracellular Ca\(^{2+}\) chelator BAPTA-AM demonstrated that the AA-regulated 5-LO translocation is FLAP- and Ca\(^{2+}\)-dependent. Finally, the redox and competitive 5-LO inhibitors L-685,015, L-739,010, and L-702,539 (but not cyclooxygenase inhibitors) efficiently substituted for AA to reverse the pyrrophenone inhibition of 5-LO translocation, indicating that the site of regulation of 5-LO translocation by AA is at or in the vicinity of the catalytic site. This report demonstrates that AA regulates the translocation of 5-LO in human PMN and unravels a novel mechanism of the cAMP-mediated inhibition of LT biosynthesis.

LT\(^4\) are lipid mediators of inflammation involved in host defense and diseases such as allergy, asthma, rheumatoid arthritis, and ulcerative colitis. The main sources of LT are phagocytes and mast cells. The initial steps of LT biosynthesis following cell activation include Ca\(^{2+}\) mobilization and the subsequent release of AA from membrane phospholipids by the cPLA\(_2\). AA is then transformed by the 5-LO to the unstable allylic epoxide LTA\(_4\). The conjugation of glutathione to LTA\(_4\) is mediated by LTC\(_4\) synthase and results in the formation of LTC\(_4\), whereas the LTA\(_4\) hydrolase generates LTB\(_4\), a potent chemotactant and activator of human PMN.

Although LT biosynthesis has been investigated in many types of leukocytes over the past 25 years, the molecular mechanisms underlying the regulation of 5-LO activity are still incompletely understood. Early studies revealed that 5-LO was unique among the lipoxygenase family for its ability to translocate to nuclear membranes following cell activation (1–9). The mechanisms by which 5-LO translocates to nuclear membranes are, however, not well defined yet. The observation that 5-LO hydrophobicity increases when the enzyme is exposed to Ca\(^{2+}\) (10) led to the finding that 5-LO has a Ca\(^{2+}\) binding domain similar to the C2 domain described for protein kinase C and cPLA\(_2\) (11). This domain, localized at the N terminus of the enzyme, has been demonstrated to be essential and sufficient for the translocation of the enzyme in HEK 293-transfected cells stimulated with A23187 (12), emphasizing the importance of Ca\(^{2+}\) for 5-LO activity and translocation to the perinuclear membranes.

Another important feature of 5-LO regulation in intact cells is its functional association with FLAP, which has been shown to strikingly enhance cellular LT biosynthesis in different cell types (13, 14). The role of FLAP in LT biosynthesis is intriguing and has not been fully characterized yet. The general consensus is that FLAP serves as a helper protein, presenting AA and 5-hydroperoxyeicosatetraenoic acid to the 5-LO, thereby increasing its catalytic potential (14–16). Moreover, two FLAP antagonists, MK-886 and MK-0591, have been shown to inhibit LT biosynthesis as well as the translocation of 5-LO to FLAP-containing nuclear membranes in activated leukocytes (9, 17, 18), suggesting a direct interaction of FLAP and 5-LO.

The phosphorylation of 5-LO by tyrosine kinases, MAPKAPK-2, ERK, and PKA (19–22), as well as interactions of 5-LO with other cellular proteins such as Grb2 (23), transforming growth factor-\(\beta\) receptor-associated protein-1, and coactosin-like protein (24, 25) have also been implicated in the modulation of 5-LO activity and LT biosynthesis in experimental conditions involving whole cells or in vitro enzyme assays.

We recently described the first physiological inhibitory mechanism of 5-LO translocation to the FLAP-containing membranes in activated protein kinase; MAPKAPK, MAPK-activated protein kinase; PAF, platelet-activating factor; fMLP, formylmethionylleucylphenylalanine; 19-OH-PGB\(_2\), 19-hydroxy-prostaglandin B\(_2\); CB, cytochalasin B; MEK,MAPK/ERK kinase; HPLC, high-performance liquid chromatography; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_2\)-tetraacetic acid tetrakis.
Arachidonic Acid Regulates 5-LO Translocation

**FIG. 1.** Reversal of the CGS-21680-induced inhibition of 5-LO translocation by exogenous AA. Pre-warmed human PMNs suspensions (37 °C, 10⁷ cells/ml) were preincubated with the adenosine A₁₅ receptor agonist CGS-21680 (10 μM) then stimulated with either 300 nM thapsigargin, 600 nM PAF or 600 nM fMLP as described under “Experimental Procedures.” Incubations were stopped by the addition of 1 volume of cold (4 °C) incubation buffer, and cell suspensions were immediately centrifuged (600 x g, 90 s, 4°C). Supernatants were collected and processed for the analysis of 5-LO products by reversed phase-HPLC, and cell pellets were used for analysis of (A and B) nuclear 5-LO content following Nonidet P-40 lysis and Western blotting with a 5-LO monoclonal antibody, as described under “Experimental Procedures,” or (C) membrane 5-LO content following sonication, membrane isolation, and Western blotting with LO-32 antiserum, as described previously (9, 18). The sum of LTB₄, 20-COOH-LTB₄, 20-OH-LTB₄, 6-(5S)-LTB₄, 6-(5R)-LTB₄, and 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE) is referred to as 5-LO products and expressed in picomoles/10⁶ PMNs. The data presented are from one experiment representative of three. N.D., not detectable.

PMNs by autacoids such as adenosine and prostaglandin E₂, and pharmacological agents that elevate intracellular cAMP concentration ([cAMP]) (18). Interestingly, we and others previously reported that cAMP-elevating agents also inhibited the release of AA in activated human leukocytes (26–30). In this study, we demonstrate that the translocation of 5-LO to the FLAP-containing membranes requires the release of AA in human PMNs. This represents the first demonstration that, under physiological conditions (PAF or fMLP stimulation), AA itself plays a key role in the cellular localization of 5-LO in human PMNs.

**EXPERIMENTAL PROCEDURES**

**Materials—**Arachidonyl trifluoromethyl ketone (AACOCF₃), methylenearachidonyl fluorophosphonate, stearic acid (C₁₈:0), oleic acid (9-C₁₈:1), linoleic acid (9,12-C₁₈:2), α-linolenic acid (9,12,15-C₁₈:3), eicosaenoic acid (C₂₀:0), 11,14(Z,Z,Z)-eicosadienoic acid (11,14-C₂₀:2), 5,8,11(Z,Z,Z)-eicosatrienoic acid (5,8,11-C₂₀:3), dihomo-γ-linolenic acid (8,11,14-C₂₀:3), 11,14,17(Z,Z,Z)-eicosatrienoic acid (11,14,17-C₂₀:3), 5,8,11,14,17(Z,Z,Z,Z)-eicospentaenoic acid (EPA), 5-oxoETE, 5(S)-, 5(R)-, 8(S)-, 8(R)-, 12(S)-, 12(R)-, 15(S)-, 15(R)-, and 20-HETE were purchased from Cayman Chemical (Ann Arbor, MI). AA (purity > 99%) was obtained either from Nu-Chek Prep Inc. (Elyssan, MN) or Sigma; ethanol solutions were stored under nitrogen at −80 °C and replaced every 4 months. 19-OH-PGB₂, adenosine deaminase, arachidonylethylester, cytochelain B (CB), Me₃SO, fMLP, β-mercaptoethanol, Nonidet P-40, PAF, P₄₂₅, and phenylmethylsulfonyl fluoride were obtained from Sigma. The mouse monoclonal 5-LO antibody was purchased from Research Diagnostics Inc. (Flanders, NJ). The rabbit phospho-ERK polyclonal antibody was purchased from Cell Signaling (Beverly, MA). L-685,015 (CPhU), L-739,010, L-702,539, MK-0591, and anti-5-LO antiserum LO-32 were a gift of Dr. Denis RienDeau from Merck Frosst Laboratories (Pointe Claire, Québec, Canada). Pyrrophenone was a gift of Dr. Kaoru Seno from Shionogi Research Laboratories (Osaka, Japan). The MEK inhibitors PD 98,059 and U-0216 were obtained from Calbiochem. The ECL Detection Kit was purchased from PerkinElmer Life Sciences. Hanks’ balanced salt solution, Hepes, Ficoll-Paque, and Trypan blue were purchased from Wisent Laboratories (St. Bruno, Québec, Canada). Thapsigargin was from Research Biochemicals, Inc. (Natick, MA).

**Isolation of Human PMNs—**Venous blood was obtained from healthy volunteers using heparin as anticoagulant, and PMNs were isolated as previously described (31). Briefly, blood was centrifuged (250 x g, 20 min, at room temperature), the platelet-rich plasma was discarded, and the erythrocytes were removed by dextran sedimentation. Mononuclear cells were then separated from the granulocytes by centrifugation on Ficoll-Paque cushions. Hypotonic lysis was performed on the granulocyte cell pellet to remove the remaining erythrocytes. The resulting suspensions contained mainly PMNs (≥95%) with eosinophils as the major contaminant. PMNs were then re-suspended at 10⁷ cells/ml in Hank’s balanced salt solution containing 1.6 mM CaCl₂.

**PMNs Stimulations—**In experiments where PMNs were stimulated with agonists, pre-warmed PMNs suspensions (10⁷ cells/ml, 37 °C) were treated with 700 pM granulocyte macrophage-colony stimulating factor, 1.5 nM tumor necrosis factor-α, and 10 μM CB during 30 min for priming. Cells were then stimulated with 600 nM of either PAF or fMLP for 5 min. In experiments where PMNs were stimulated with thapsigargin, pre-warmed PMNs suspensions (10⁷ cells/ml, 37 °C) were preincubated for 30 min at 37 °C, then stimulated with 300 nM thapsigargin for 10 min. When indicated, CGS-21680 was added 5 min before stimulation of PMNs. In experiments where endogenous AA release was blocked by the cPLA₂ inhibitor pyrrophenone, pre-warmed PMNs suspensions (10⁷ cells/ml, 37 °C) were incubated with 10 μM CB and 100 nM pyrrophenone for 20 min then stimulated with 600 nM fMLP for 5 min. PD 98,059, U-0126, and CGS-21680 were added 20 min before stimulation of PMNs with the agonists. To eliminate the inhibitory constraint of endogenous adenosine on LT biosynthesis by PMNs (32), 0.3 unit/ml adenosine deaminase was added 20 min before PMNs stimulation in all experimental settings. All incubations were stopped by the addition of one volume of cold (4 °C) incubation buffer, and samples were immediately centrifuged (600 x g, 90 s, 4 °C). Supernatants and cell pellets were processed for the analysis of 5-LO products and nuclear 5-LO, respectively, as described below.

**5-LO Product and AA Analysis—**For the determination of 5-LO products and AA, supernatants were treated with 0.5 volume of a cold
products (20-OH- and 20-COOH-LTB4), LTB4 isomers (6-(E)- and
6-(E)-12-epi-LTB4), and 5(S)-HETE), was compiled and is referred to as
5-LO products. For AA analysis, the AA-containing HPLC fractions
were collected, evaporated to dryness under reduced pressure using a
SpeedVac evaporator, and redissolved in 50 μl of MeCN for analysis by
liquid chromatography-mass spectrometry using electrospray ioniza-
tion in the negative mode as described previously (33).

Analysis of 5-LO and Phospho-ERK—PMNs pellets were resus-
pended with 600 μl of cold (4 °C) lysis buffer (0.1% Nonidet P-40, 10 mM
Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 10 μg/ml aproti-
nin and leupeptin, 1 mM phenylmethylsulfonyl fluoride), vortexed for
15 s, kept on ice for 5 min, then centrifuged (525 × g, 10 min, 4 °C).
Electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 2% (w/v) SDS, 10%
(v/v) glycerol, 5% β-mercaptoethanol, 0.01% (w/v) bromphenol blue, 10
μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride)
was then added to the resulting supernatants and pellets (the nuclear
fraction) as described previously (9), and samples were heated to 100 °C
for 10 min. Samples were analyzed by SDS-PAGE (34) using 10% poly-
acrylamide gels. Proteins were transferred at 0.5 Å for 3 h at 4 °C onto
Immobilon-P polyvinylidene difluoride membranes. Transfer efficiency
and equal loading were visualized by Ponceau red staining. The mem-
branes were soaked for 30 min at 25 °C in Tris-buffered saline (25 mM
Tris-HCl, pH 7.6, 200 mM NaCl, 0.15% Tween 20) containing 5% dried
milk (w/v), blotted with 5-LO or phospho-ERK antibody, and revealed
using a horseradish peroxidase-coupled monoclonal antibody and the
ECL detection kit.

RESULTS

AA Regulates 5-LO Translocation—We and others have shown that
the inhibitory effect of elevated [cAMP], on LT biosynthesis in PMNs
correlates with inhibition of AA release (26–30). Moreover, we found
that another key event of LT biosynthesis, the translocation of 5-LO, is
also strongly and consistently inhibited by all cAMP-elevating agents

FIG. 2. Inhibitory effect of the cPLA2 inhibitor pyrrophenone on 5-LO translocation in activated human PMNs. Pre-warmed human PMNs suspensions (37 °C, 107 cells/ml) were preincubated with 100 nM pyrrophenone for 10 min then stimulated with either 600 nM PAF or 600 nM fMLP as described under “Experimental Procedures.” Incubations were stopped, and the analysis of 5-LO products and nuclear 5-LO content was performed as described in Fig. 1 (A and B) legend. The data presented are from one experiment representative of three. N.D., not detectable.

FIG. 3. Effects of the MEK inhibitors U-0216 and PD 98,059 on AA release, ERK phosphorylation, and 5-LO translocation in activated human PMNs. Pre-warmed human PMNs suspensions (37 °C, 107 cells/ml) were preincubated for 20 min with the MEK inhibitors U-0126 or PD 98,059, then stimulated with either 5 μM AA, 600 nM PAF, 600 nM fMLP, or 300 nM thapsigargin as described under “Experimental Procedures.” Incubations were stopped after 2 min by the addition of 0.5 volume of cold (4 °C) stop solution containing 20 ng of [3H]AA and 12.5 ng of both 19-OH-PGB2 and PGB2 as internal standards; for AA analysis, the AA-containing HPLC fractions were collected, evaporated to dryness under reduced pressure using a SpeedVac evaporator, and redissolved in 50 μl of MeCN for analysis by liquid chromatography-mass spectrometry using electrospray ionization in the negative mode as described previously (33). 

Electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% β-mercaptoethanol, 0.01% (w/v) bromphenol blue, 10 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride) was then added to the resulting supernatants and pellets (the nuclear fraction) as described previously (9), and samples were heated to 100 °C for 10 min. Samples were analyzed by SDS-PAGE (34) using 10% polyacrylamide gels. Proteins were transferred at 0.5 Å for 3 h at 4 °C onto Immobilon-P polyvinylidene difluoride membranes. Transfer efficiency and equal loading were visualized by Ponceau red staining. The membranes were soaked for 30 min at 25 °C in Tris-buffered saline (25 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.15% Tween 20) containing 5% dried milk (w/v), blotted with 5-LO or phospho-ERK antibody, and revealed using a horseradish peroxidase-coupled monoclonal antibody and the ECL detection kit.

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tested (18), suggesting a causal relationship between inhibition of AA release and 5-LO translocation in human PMNs. A first series of experiments was therefore performed to assess the involvement of AA in the cAMP-mediated inhibition of 5-LO translocation. As shown in Fig. 1 (A and B) the adenosine A2A receptor agonist CGS-21680, which increases [cAMP], in PMNs, efficiently inhibited 5-LO translocation and LT biosynthesis in thapsigargin- and ligand-activated PMNs, in agreement with our previous observations (18). Interestingly, increasing concentrations of exogenous AA fully reversed this inhibitory effect of CGS-21680 on LT biosynthesis and 5-LO translocation in all stimulatory conditions tested, thereby providing the first evidence of a functional link between AA release and 5-LO translocation in activated human PMNs. Fig. 1C shows that similar results were obtained in experiments where 5-LO was analyzed in PMN membranes prepared by sonication and centrifugation (as opposed to detergent lysis) and Western blotting with LO-32 polyclonal antibody (as opposed to a monoclonal antibody for Fig. 1, A and B) using previously described methods (9, 18). Finally, it seems important to mention that the effects of AA (and CGS-21680) on unstimulated human PMNs (not shown in this report) have been reported previously (35): addition of AA to unstimulated human PMNs is a peculiar experimental condition where PMN activation (Ca2+ mobilization, 5-LO translocation, and LT biosynthesis) is strictly dependent on an autocrine stimulatory loop by LTβ and, consequently, results in cellular responses (to pharmacological agents) that distinctly differ from those observed in agonist-activated PMNs (herein and Ref. 18).

There is now compelling evidence that the PLA2 involved in AA release and LT biosynthesis in activated PMNs is the cPLA2 (36–38). To confirm the regulatory role of AA in 5-LO translocation, we investigated the effect of the potent and specific cPLA2 inhibitor pyrrophenone (39, 40) on LT biosynthesis and 5-LO translocation. Fig. 2 shows that LT biosynthesis was inhibited by pyrrophenone in FMLP-, PAF-, and thapsigargin-activated human PMNs; moreover, 5-LO translocation was completely blocked in ligand- and thapsigargin-activated PMNs treated with pyrrophenone, and this inhibitory effect of pyrrophenone on 5-LO translocation (and LT biosynthesis) was fully reversed by the addition of AA (Fig. 2B).

It is well recognized that the MAPK pathway is involved in the activation cPLA2. Indeed, several studies have shown that the MEK inhibitors PD 98,059 and U-0126 are potent inhibitors of both cPLA2 phosphorlyation and AA release in many cell types (36, 41–43). It was also shown that these MEK inhibitors block 5-LO translocation in FMLP-activated human PMNs (44). Therefore, we investigated the effect of PD 98,059 and U-0126 on both AA release and 5-LO translocation in activated PMNs. Fig. 3A shows that U-0126 blocked AA release in agonist-activated PMNs in a dose-dependent manner (similar results were obtained with PD 98,059, not shown). This inhibition of AA release correlated with the inhibition of both ERK phosphorylation and 5-LO translocation (Fig. 3, B and C). Interestingly, addition of AA to the incubation media restored the translocation of 5-LO without affecting the phosphorylation of ERK, strongly suggesting that the inhibitory effect of MEK inhibitors on 5-LO translocation is the consequence of AA release inhibition.

Specificity of the Regulatory Effect of AA on 5-LO Translocation—Studies were next undertaken to determine the specificity of the effect of AA on 5-LO translocation. In these experiments, the cPLA2 inhibitor pyrrophenone was used to block endogenous AA release and 5-LO translocation in FMLP-activated PMNs; in these conditions, the restoration of 5-LO translocation by exogenous fatty acids could be assessed without the interference of endogenous AA.

We first investigated the effect of fatty acids of the C20 series (Fig. 4A). Whereas AA and EPA were both strong inducers of 5-LO translocation, C20:0 and 11,14-C20:2 did not significantly inhibit 5-LO translocation in activated PMNs. Interestingly, among the trienoic acids tested, 11,14,17-C20:3 did not elicit 5-LO translocation, whereas the C20:3 isomers with double bonds 5,8,11- or 8,11,14- were equipotent in their stimulatory effect on 5-LO translocation in FMLP-activated PMNs. As expected, the biosynthesis of LTB5, 8(S)-HETE, and LTB3 was observed in presence of 5,8,11-C20:3, 8,11,14-C20:3 and EPA, respectively (data not shown). Additional experiments were performed to assess the impact of products of the 5-, 12-, and 15-lipoxygenases on 5-LO translocation in pyrrophenone/CB-treated, FMLP-activated human PMNs in the analysis of 5-LO products and nuclear 5-LO content were performed as described in Fig. 1 (A and B) legend. The data presented are the mean ± S.E. of at least three separate experiments.
PMNs (Figs. 4B and 5C). 5(S)-, 15(S)-, and 15(R)-HETE strongly induced 5-LO translocation to the nuclear membranes. 5-oxo-ETE, 8(S)- and 12(R)-HETE also induced 5-LO translocation, but to a lower extent, and 8(R)-, 12(S)- and 20-HETE were inactive. Interestingly, 5(R)-HETE was repeatedly found to be much less active than its enantiomer 5(S)-HETE. Similar experiments were performed with fatty acids of the C18 series (Fig. 5A); among the several compounds tested, only 9,12,15-C18:3 elicited significant 5-LO translocation in pyrrophenone/CB-treated, fMLP-activated PMNs. The effect of 9,12,15-C18:3 was similar to that elicited by AA at the same concentration (5 μM). In contrast, C18:0 and 9-C18:1 did not promote 5-LO translocation, while 9,12-C18:2 had a weak effect. Analogs of AA with modified carboxylic end such as AACOCF₃, methylarachidonyl fluorophosphonate, AADCOCF₂, or arachidonylethylester (8); and the indicated LO products or AA for 5 min (C). Incubations were stopped, and the analysis of 5-LO products and nuclear 5-LO content were performed as described in Fig. 1 (A and B) legend. The data presented are from one experiment representative of three.

5-LO Inhibitors Promote 5-LO Translocation—We next addressed the issue of the site of the regulatory effect of AA on 5-LO translocation, and first investigated the putative involvement of the catalytic site of 5-LO. Experiments were undertaken with two competitive 5-LO inhibitors from the naphthalenic lactone class previously demonstrated to bind close to the prosthetic iron atom of the 5-LO. These experiments with the 5-LO inhibitors were carried out under the same experimental conditions as above (with various PUFAs). As shown in Fig. 6, L-702,539 and L-739,010 did not promote (or to a small extent only) 5-LO translocation in resting PMNs. In fMLP-activated PMNs however, the 5-LO inhibitors strikingly promoted 5-LO translocation at nM concentrations, suggesting that the binding of the drug at or near the 5-LO catalytic site triggers translocation of the enzyme to nuclear structures. Similar results were obtained with L-691,816, a competitive 5-LO inhibitor from the thiopyranoindole family (data not shown). Fig. 6 also shows that L-702,539 and L-739,010 are more potent than AA to promote 5-LO translocation by more than 2 orders of magnitude, in agreement with the much higher affinity of these inhibitors (compared with AA) for the catalytic site of 5-LO (45).

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Arachidonic Acid Regulates 5-LO Translocation

redox inhibitor CPHU (L-685,015) for its ability to promote 5-LO translocation in activated PMNs. Fig. 7 shows that similarly to the competitive 5-LO inhibitors, CPHU efficiently restored the pyrophenone-blocked 5-LO translocation in a concentration-dependent manner, both in fMLP- and thapsigargin-activated PMNs. Interestingly, another well known 5-LO redox inhibitor, nordihydroguaiaretic acid (25 μM) also restored 5-LO translocation (not shown). In contrast, cyclooxygenase inhibitors (indomethacin and NS-398), eicosatetraenoic acid, and the LTD4 and PAF antagonists (MK-571 and BN 50,730, respectively) (all tested at 10 μM) had no effect (data not shown).

The importance of intracellular Ca2+ on AA-induced 5-LO translocation was next investigated using the membrane permeable Ca2+ chelator BAPTA-AM. As shown in Fig. 8A, the 5-LO translocation observed in presence of AA or 9,12,15-C18:3 were inhibited by 10 μM BAPTA-AM in pyrophenone-treated fMLP-activated PMNs, whereas the L-739,010-mediated 5-LO translocation was strongly impaired. These results confirm that the 5-LO translocation elicited by the exogenous PUFA and 5-LO inhibitors in this experimental model are Ca2+-dependent. Finally, it seemed important to establish whether the observed PUFA-induced 5-LO translocation was a FLAP-dependent event. As shown in Fig. 8B, AA-, L-739,010-, (S)HETE-, and 8,11,14-C20:3-induced 5-LO translocation in pyrophenone-treated fMLP-activated PMNs was efficiently inhibited by the FLAP antagonist MK-0591. In fact, the 5-LO translocation observed in presence of any of the PUFA tested in this study was consistently inhibited by MK-0591 suggesting that the 5-LO translocation observed was a FLAP-dependent event rather than an unspecific effect of these fatty acids and pharmacological agents on 5-LO localization.

**DISCUSSION**

Although the involvements of Ca2+, ATP, cytoskeleton-associated proteins, and protein kinases in LT biosynthesis have been established, the regulation of a critical event associated with LT biosynthesis, the translocation of 5-LO to the FLAP-containing peri-nuclear membranes remains largely unsolved.

We and others had previously demonstrated that elevated [cAMP], leads to an inhibition of AA release in activated leukocytes (26–30). Moreover, we recently demonstrated that another key event in LT biosynthesis, 5-LO translocation, was also inhibited by elevated [cAMP], in activated PMNs (18). In fact, our consistent observations that all cAMP-elevating agents tested, including various ligands of Gs-coupled receptors, type IVA phosphodiesterase inhibitors, the adenylyl cyclase activator forskolin, and cholaera toxin concomitantly inhibited AA release and 5-LO translocation in activated PMNs, led us to the hypothesis of a causal relationship between these two cellular events, more specifically that AA (or a metabolite of AA) might act as a physiological regulator of 5-LO translocation.

To assess this hypothesis, we carried out studies using three distinct experimental conditions resulting in inhibition of AA release. Firstly, we demonstrated that the addition of 1–10 μM AA restores 5-LO translocation and LT biosynthesis in thapsigargin- or ligand-activated PMNs treated with the A2A receptor agonist CGS-21680, which otherwise efficiently blocks AA release and 5-LO translocation through elevation of [cAMP], (18). Secondly, we showed that the potent and selective cPLA2 inhibitor pyrrophenone, which completely inhibits AA release (data not shown) and LT biosynthesis in activated PMNs, also inhibits 5-LO translocation; these effects of pyrrophenone were fully reversed by exogenous AA, ruling out an unspecific effect of the inhibitor on 5-LO translocation. Thirdly, we have shown that the MEK inhibitors U-0126 and PD 98,091 inhibit AA release and 5-LO translocation in agonist-activated PMNs and that the latter is efficiently restored by exogenous AA. Altogether, these data point to a pivotal role of AA in the regulation of 5-LO translocation in intact PMNs and suggest a functional relationship between the cPLA2, and the 5-LO. These data also provide a complementary mechanism by which cAMP regulates 5-LO product synthesis in PMNs. Indeed, elevation of [cAMP], results in inhibition of AA release, which in addition to limiting substrate availability has a further impact on the translocation of the 5-LO, an essential event in LT biosynthesis. Interestingly, it was recently shown, using 3T3 cells transfected with 5-LO and PKA, that PKA phosphorylates 5-LO on Ser-523,
which results in loss of enzymatic activity without altering 5-LO membrane association nor redistribution from the nucleus to the cytoplasm (22). Because we have clearly shown that cAMP-elevating agents drastically suppress 5-LO translocation in agonist-stimulated human PMNs (18), it would seem that PKA-mediated phosphorylation of 5-LO (Ser523) does not prevail in human PMNs as the mechanism involved in cAMP-mediated inhibition of LT biosynthesis. Finally, in the experimental conditions used in the present study, cAMP-elevating agents such as the A2A and H2 receptor agonists CGS-21680 and histamine do not block Ca2+ release from internal stores (an inositol-1,4,5-triphosphate-mediated process) (28, 29) in PAF-stimulated human PMNs, indicating that phospholipase C is not involved in the inhibitory effects of CGS-21680 described herein.

The structure-activity relationship study of the effects of fatty acids on 5-LO translocation performed in the present study provided intriguing results. First, the data demonstrated that not all fatty acids could induce 5-LO translocation, but only those with three or more double bonds located from C9 or closer to the carboxylic group. More intriguing was the observation that several mono-HETE were as active as AA in inducing 5-LO translocation, whereas others (epimers and positional isomers) showed very little effect. In particular the potent effect of 5(S)-HETE, a natural product of the conversion of AA by the 5-LO, raised the possibility that 5(S)-HETE (or 5(S)-hydroperoxyeicosatetraenoic acid) rather than AA could be the endogenous regulator of 5-LO translocation. However, treatment of PMNs with the redox 5-LO inhibitor CPHU (100 nM), which completely blocks 5-LO product biosynthesis, did not inhibit the subsequent fMLP-induced translocation of 5-LO, demonstrating that AA transformation by 5-LO was not required for 5-LO translocation (data not shown). These data do not, however, rule out that 5(S)-HETE also contributes to promote 5-LO translocation. Altogether these results point to a significant level of specificity in the ability of AA to enable 5-LO translocation, and demonstrate that there is no relationship between the ability of PUFA to promote 5-LO translocation and to serve as 5-LO substrate. This is clearly illustrated by 12(S)-HETE and 15(S)-HETE, two 5-LO substrates with contrasting abilities to promote 5-LO translocation.

In-gel kinase assays showed 5-LO phosphorylation by ERK and/or the downstream target of p38, MAPKAPK-2 (21, 46). It was also recently demonstrated that AA and oleic acid strongly potentiated 5-LO phosphorylation at Ser-271 (47). Because AA itself has been shown to activate several signal transduction pathways (e.g. AA or its metabolites activate the p38 pathway in HL-60 cells and rabbit PMNs, leading to increased cellular responses (48–50)), these data might suggest that the regulatory role of AA on 5-LO translocation described herein implicates an increased activity of MAPKAPK-2 and 5-LO phosphorylation on Ser-271. However, in our experiments, oleic acid (in contrast to AA) did not induce 5-LO translocation in iMLP-activated PMNs. Furthermore, preliminary data obtained with the p38 kinase inhibitors SB 202,190 and SB 203,580 suggest that the effect of AA on 5-LO translocation in activated PMNs described herein is not the consequence of an enhanced activation of the p38 kinase pathway (data not shown).

In this study, in agreement with previous studies, the 5-LO translocation to the nuclear membranes observed in iMLP-, PAF-, and thapsigargin-activated PMNs was prevented by the FLAP inhibitor MK-0591. This observation, together with the previous reports that FLAP is an AA-binding protein suggested that FLAP could have been the target of the regulatory effect of AA on 5-LO translocation. However, important discrepancies in the reported affinity of various fatty acids for FLAP and their ability to elicit 5-LO translocation in this study do not support this hypothesis. For instance, it was shown that, in addition to AA, FLAP binds AAOCCF3 (a compound that did not induce 5-LO translocation in the present study) with the same affinity as AA (51). In the same study, 12(S)-HETE and 15(S)-HETE equally inhibited the binding of L-691,831 (an AA-derived radioactive probe) to FLAP, whereas in our study, 15(S)-HETE strongly induced 5-LO translocation while 12(S)-HETE did not.

Besides FLAP, the 5-LO itself, which possesses at least one binding site for AA (the catalytic site) represented a likely target for the regulatory effect of AA on 5-LO translocation. This was investigated using two classes of non-redox competitive inhibitors of 5-LO, the tiophyronanoides and the naphtalenic lactones, as well as the redox inhibitor CPHU. These compounds have been shown to potently inhibit 5-LO in whole cells and in vitro assays. It was demonstrated that the naphtalenic lactone L-697,198 and the tiophyronanoidole L-691,816 bind an AA binding site on 5-LO with a much higher affinity (>2 orders of magnitudes) than AA itself (52). Because the 5-LO redox inhibitor CPHU was shown to compete with L-708,714 (a tiophyronanoidole photofinity probe), the binding site of these compounds on 5-LO was proposed to be at the catalytic site, close to the non-heme iron atom of 5-LO (45). These data and our observations that AA, L-702,539, L-739,018, and CPHU restored translocation of 5-LO in pyrrophene and fMLP-treated PMNs strongly support that the binding site of AA implicated in 5-LO translocation is the 5-LO catalytic site or lies in its proximity. Interestingly, this observation is reminiscent of the finding that the cPLA2α catalytic site is important for the localization of cPLA2α on the peri-nuclear membranes (53).

It was previously described that 5-LO binds Ca2+ at its N terminus leading to an increased hydrophobicity of the enzyme (10, 11). The N-terminal β-barrel of 5-LO contains a Ca2+-binding domain similar to those found in protein kinase C and cPLA2α, a domain previously demonstrated essential and sufficient to induce 5-LO translocation in transfected HEK-293 cells (54). The present observation of the complete inhibition by BAPTA-AM of 5-LO translocation confirms the essential role played by Ca2+ in the AA-regulated translocation of 5-LO in fMLP-activated PMNs.

In summary, this study unravels an important mechanism of regulation by AA of 5-LO translocation to the peri-nuclear membranes in activated PMNs. These data also demonstrate a novel biological activity for AA, and a functional relationship between the cPLA2α and the 5-LO, two key enzymes of the 5-LO pathway in leukocytes. Finally, these studies pointed to a novel mechanism by which cAMP-elevating agents down-regulate LT biosynthesis in human PMNs.

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