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Cyclic AMP-Mediated Inhibition of 5-Lipoxygenase Translocation and Leukotriene Biosynthesis in Human Neutrophils

NICOLAS FLAMAND, MARC E. SURETTE, SERGE PICARD, SYLVAIN BOURGOIN, AND PIERRE BORGEAT

Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du CHUQ, and Faculté de Médecine, Université Laval, Québec, Canada

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ABSTRACT

5-Lipoxygenase (5-LO) catalyzes the transformation of arachidonic acid to leukotrienes (LT). In stimulated human PMN, activation of 5-LO involves calcium, p38 MAP kinase (p38) phosphorylation, and translocation of 5-LO from the cytosol to nuclear membranes containing the 5-LO activating protein (FLAP). In this study, cAMP-elevating agents such as isoproterenol, prostaglandin E_2, CGS-21680 (an adenosine A_{2a} receptor agonist), the type IV phosphodiesterase inhibitor RO 20-1724, the adenylate cyclase activator forskolin, and the Gs-protein activator cholera toxin all inhibited LT biosynthesis and 5-LO translocation to the nucleus in cytokine-primed human PMN stimulated with platelet-activating factor and in human PMN stimulated with the endomembrane Ca^{2+}-ATPase blocker thapsigargin. Furthermore, monophosphorothioate analogs of cAMP, which activate protein kinase A (PKA), also inhibited LT biosynthesis and 5-LO translocation in stimulated cells. Treatment of PMN with CGS-21680 also prevented the phosphorylation of p38 by thapsigargin. Treatment of PMN with the PKA inhibitors H-89 and KT-5720 prevented the inhibitory effect of cAMP-elevating agents on LT biosynthesis, 5-LO translocation, and p38 phosphorylation, whereas the p38 inhibitor SB 203,580 dose-dependently inhibited arachidonic acid–induced LT biosynthesis. The 5-LO translocation was also inhibitable by the FLAP antagonist MK-0591 and correlated with LT biosynthesis in all experimental conditions tested. These results indicate that cAMP-mediated PKA activation in PMN results in the concomitant inhibition of 5-LO translocation and LT biosynthesis and support a role of p38 in the signaling pathway involved. This represents the first physiological down-regulation mechanism of 5-LO translocation in human PMN.

Leukotrienes (LT) are lipid mediators of inflammation that have been implicated in a number of pathological conditions including allergy, asthma, and other inflammatory diseases. The biosynthesis of LT involves the sequential release of arachidonic acid (AA) from cellular glycerolipids and its initial transformation by 5-lipoxygenase (5-LO), which catalyzes both the hydroperoxydation of AA at carbon 5 and a dehydrase reaction resulting in the formation of LTA_4. In human PMN, LTA_4 is further metabolized to the potent PMN activator and chemoattractant LTB_4 by the LTA_4 hydrolase.

Pharmacological agents such as Ca^{2+} ionophores or the Ca^{2+}-ATPase blocker thapsigargin are routinely used as tools to study the regulation of AA metabolism in human PMN because they are potent inducers of LT biosynthesis. Physiological ligands such as platelet-activating factor (PAF) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) also stimulate LT biosynthesis in human PMN, and this biosynthesis is strongly potentiated when cells are pre-exposed to priming agents such as TNF-α, GM-CSF, or lipopolysaccharides (Roubin et al., 1987; DiPersio et al., 1988; McColl et al.,

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N.F. and M.E.S. contributed equally to this work.

ABBREVIATIONS: LT, leukotriene; AA, arachidonic acid; 5-LO, 5-lipoxygenase; PMN, polymorphonuclear neutrophils; PAF, platelet-activating factor; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulating factor; $cPLA_2$, type IV cytosolic phospholipase A_2; PDE, phosphodiesterase; PG, prostaglandin; RO 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; PMSF, phenylmethylsulfonyl fluoride; ADA, adenosine deaminase; CGS-21680, 2-[*p*-(2 -carboxyethyl)]phenylethyl-amino-5-*N*′-ethylcarbox-amidoadenosine; 8CPT, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate; cAMPS, adenosine-3′,5′-cyclic monophosphorothioate; p38, p38 MAP kinase; MK-0591, 3-[1-(4-chlorobenzyl)-3-(t-butylthio)-5-(quinolin-2-yl-methoxy)-indol-2-yl]-2,2-dimethyl propanoic acid; SB 203,580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; HBSS, Hanks' balanced salt solution; RP, reversed-phase; HPLC, high-performance liquid chromatography; 5-HETE, 5-hydroxyeicosatetraenoic acid; NP-40, Nonidet P-40; DMSO, dimethyl sulfoxide; PKA, protein kinase A; KT-5720, [9*R*,10*S*,12*S*]-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-*fg*:3,2,1-*kl*]pyrrolo[3,4-*l*][1,6]benzodiazocine-10-carboxylic acid hexyl ester; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MK2, MAPKAP kinase 2; FLAP, 5-lipoxygenase activating protein.

1989; Poubelle et al., 1989; Surette et al., 1993). Upon PMN stimulation, an increase in intracellular Ca^{2+} concentrations triggers the translocation of type IV cytosolic phospholipase A_2 (cPLA$_2$) and 5-LO to nuclear membranes, where LT biosynthesis probably occurs (Woods et al., 1993; Pouliot et al., 1996). Although much effort has been invested into understanding the up-regulation of the biosynthesis of LT, little is known about the inhibition or suppression of their biosynthesis. However, a small number of studies have shown that agents which elevate cellular cAMP levels ([cAMP]$_i$) can inhibit the stimulated biosynthesis of LT. Ham and colleagues (1983) first showed that prostaglandin E_2 (PGE$_2$) inhibited LT biosynthesis in cytochalasin B-treated/fMLP-stimulated PMN. Other agents, such as cell-permeable phosphodiesterase (PDE)-resistant analogs of cAMP or agents that cause elevation in [cAMP]$_i$, such as the type IV PDE inhibitor RO 20-1724, and agents that act through the G protein-linked receptors, such as isoproterenol and adenosine A_{2a} receptor agonists, have also been shown to inhibit the biosynthesis of LT in human PMN and other leukocyte populations (Peachell et al., 1989; Schudt et al., 1991; Fonteh et al., 1993; Tenor et al., 1996; Krump et al., 1997; Dennis and Riendeau, 1999; Surette et al., 1999; Flamand et al., 2000). It is noteworthy that elevation of [cAMP]$_i$ by adenosine has been shown previously to inhibit several other functional responses in PMN (Cronstein, 1994).

The mechanism by which agents that elevate [cAMP]$_i$ inhibit LT biosynthesis has not been conclusively elucidated. We recently demonstrated that this inhibition of LT biosynthesis was associated with an inhibition of AA release in ligand-activated PMN (Flamand et al., 2000). In the present study, we provide evidence that cAMP-elevating agent-mediated inhibition of LT biosynthesis also involves an inhibition of the translocation of 5-LO to the nucleus in agonist- or thapsigargin-stimulated human PMN, a process demonstrated previously to be implicated in the activation of LT biosynthesis in intact PMN. This is the first description of a mechanism by which PMN down-regulates the translocation of 5-LO.

Materials and Methods

Materials. PAF, cytochalasin B, cholera toxin, forskolin, isoproterenol, PGB$_2$, PGE$_2$, 19-OH-PGB$_2$, PGF$_{2\alpha}$, leupeptin, aprotinin, PMSF, adenosine deaminase (ADA), and horseradish peroxidase-linked donkey anti-rabbit antibodies were purchased from the Sigma Chemical Co. (St. Louis, MO). H-89 and KT-5720 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Thapsigargin, RO 20-1724, and CGS-21680 HCl were purchased from Sigma/RBI (Natick, MA). The *R*p- and *S*p-isomers of 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate (8CPT) as well as the *S*p-isomer adenosine-3′,5′-cyclic monophosphorothioate (cAMPS) were obtained from Biolog Life Science Institute (Hayward, CA). MK-0591 was a gift from Dr. Robert Young (Merck Frosst, Kirkland, PQ, Canada). Rabbit polyclonal anti-5-LO (LO-32) was kindly supplied by Dr. Jillian F. Evans (Merck Frosst). Mouse mAb against 5-LO was purchased from RDI (Flanders, NJ), and the enhanced chemiluminescence detection kit was purchased from Amersham Biosciences (Oakville, ON, Canada). Immobilon-P polyvinylidene difluoride blotting membrane was from Millipore Corporation (Mississauga, ON, Canada). Ficoll medium was obtained from Pharmacia (Montréal, PQ, Canada). SB 203,580 was obtained from Calbiochem (La Jolla, CA). The rabbit p38 antiserum and the mouse phospho-p38 monoclonal antibody were purchased from New England Biolabs (Beverly, MA).

Isolated Cell Preparations. Venous blood was obtained from healthy donors and collected in 10-ml tubes containing 143 USP units of heparin. Human PMN were isolated from peripheral blood after dextran sedimentation and centrifugation on Ficoll cushions as described previously (Boyum, 1968). Final preparations contained ≥95% PMN, and viability was greater than 95% as assessed by trypan blue exclusion.

Cell Stimulations. In experiments in which PMN were stimulated with PAF, cells were preincubated at 37°C in HBSS plus 1.6 mM $CaCl_2$ (10^7 cells/ml) containing 1.2 nM TNF-α and 700 pM GM-CSF for 10 min. Cytochalasin B (10 μM) was added for an additional 20 min of preincubation, and cells were then stimulated with 600 nM PAF for 5 min. In experiments in which PMN were stimulated with thapsigargin, cells were preincubated at 37°C in HBSS plus 1.6 mM $CaCl_2$ (10^7 cells/ml) for 20 min before stimulation with 300 nM thapsigargin for 10 min. In all experimental settings, 0.1 U/ml ADA was added 5 min before stimulation to eliminate the inhibitory constraint exerted by extracellular adenosine (Krump et al., 1997). All incubations were stopped by the addition of 1 volume of cold (4°C) HBSS plus 1.6 mM $CaCl_2$ and immediately centrifuged at 500*g* (1 min; 4°C). Cell pellets were used for 5-LO immunoblot analysis, and supernatants were used for the determination of 5-LO products. In some experiments, cAMP-elevating agents or enzyme inhibitors were included in the incubation media for the indicated periods of time before stimulation (see figure legends).

For the determination of 5-LO products, cell supernatants were denatured by the addition of 0.5 volumes of ice-cold MeOH/MeCN (1:1, v/v) containing 12.5 ng each of PGB$_2$ and 19-OH-PGB$_2$ as internal standards, and the samples were processed and analyzed by reversed-phase (RP)-HPLC using an on-line extraction procedure as described previously (Borgeat et al., 1990). The sum of LTB$_4$, 20-COOH-LTB$_4$, 20-OH-LTB$_4$, 6(*E*)-LTB$_4$, 6(*E*)-12-epi-LTB$_4$, and 5-hydoxyeicosatetraenoic acid (5-HETE) was compiled and is referred to as 5-LO products.

Cell Fractionation and Protein Analysis. For the preparation of nuclei, PMN incubated under the described conditions were pelleted and resuspended in 600 μl of ice-cold NP-40 lysis buffer (0.1% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). The cells were vortexed for 15 s, kept on ice for 5 min, and centrifuged at 525*g* (10 min, 4°C). The resulting supernatants (i.e., the non-nuclear fractions) and pellets (the nuclei-containing fractions) were then immediately solubilized in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% bromphenol blue, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and boiled for 10 min as reported previously (Pouliot et al., 1996).

In some experiments, cellular membranes were prepared by sonication. Briefly, PMN (2×10^7) were pelleted and resuspended in 600 μl of cold (4°C) sonication buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Cell disruption was performed at 4°C using a Branson sonifier 450 (25 s at a power setting of 1.5 and 100% duty cycle). Sonicates were centrifuged at 12,000*g* for 10 min, and the resulting supernatants were centrifuged at 180,000*g* for 45 min. The resulting supernatants (referred to as the cytosolic fractions) and pellets (referred to as cellular membranes) were immediately solubilized in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% bromphenol blue, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and boiled for 10 min as described above.

Non-nuclear and nuclear proteins (equivalent to 2×10^6 cells for nuclei-containing fractions obtained from NP-40–treated cells and 4×10^6 cells for cellular membranes obtained by sonication) were separated by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) on 9% acrylamide gels. Proteins were then transferred at 0.5 A for 3 h at 4°C onto an Immobilon-P polyvinylidene difluoride blotting membrane. Transfer efficiency as well as loading was visualized with the use of Ponceau Red staining. For the deter-

mination of phospho-p38, p38, and 5-LO, the membranes were soaked for 30 min at 25°C in Tris-buffered saline (25 mM Tris-HCl, pH 7.6, 0.2 M NaCl, and 0.15% Tween 20) containing 5% nonfat dried milk (w/v), were blotted with the primary antibody, and were revealed using a horseradish peroxidase-coupled monoclonal antibody and the enhanced chemiluminescence detection kit.

Results

The translocation of cytosolic 5-LO to the nuclear structures after the stimulation of human PMN is recognized as an important regulatory step in 5-LO activation and LT biosynthesis. To determine whether the inhibition of LT biosynthesis by agents that elevate $[cAMP]_i$ involves an effect at the level of 5-LO activation, TNF-α/GM-CSF–primed human PMN were stimulated with PAF in the presence or absence of agents that are known to elevate $[cAMP]_i$, and 5-LO translocation was assessed by immunoblot analysis of 5-LO content in subcellular fractions containing nuclei. As shown in Fig. 1, A and B, unstimulated (DMSO)-primed human PMN do not synthesize measurable quantities of LT and show little nuclei-associated 5-LO. This result is consistent with previous reports that the translocation of 5-LO to the nucleus requires an increase in the intracellular Ca^{2+} concentration because it occurs after agonist stimulation. When primed PMN were stimulated with PAF, the biosynthesis of LT and the translocation of 5-LO to cell nuclei were observed clearly.

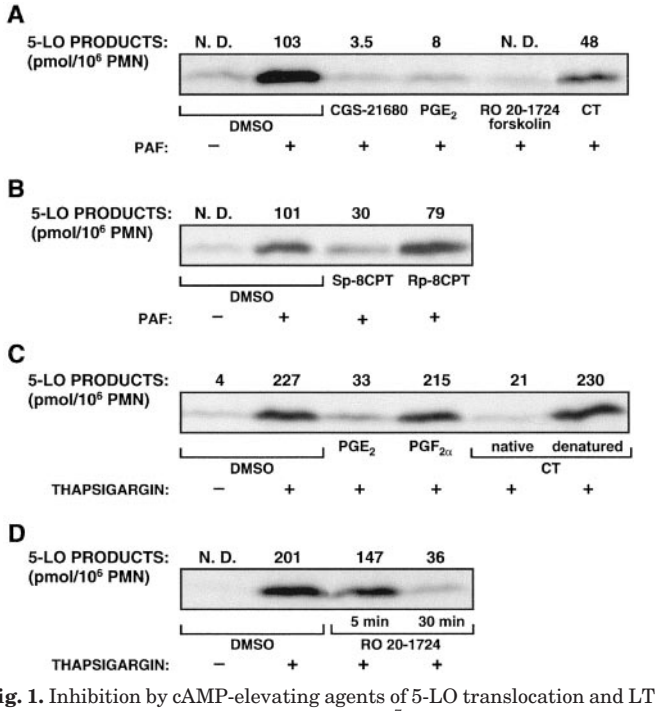

Fig. 1. Inhibition by cAMP-elevating agents of 5-LO translocation and LT biosynthesis in PMN. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. Additions of the cAMP-elevating agents in the incubation media before stimulation with either 600 nM PAF or 300 nM thapsigargin were performed as follows: PGE_2 (1 μM), $PGF_{2\alpha}$ (1 μM), or CGS-21680 (1 μM) were added 5 min before stimulation; cholera toxin (1 μg/ml), *S*p-cAMPS (50 μM), *S*p- and *R*p-8CPT-cAMPS (75 μM), as well as the combination of RO 20-1724 (10 μM) and forskolin (50 μM) were added 15 min before stimulation. Inactivation of cholera toxin was achieved by heating the toxin at 100°C for 5 min. DMSO did not exceed 0.1% (v/v) in incubation media. Supernatants were analyzed by RP-HPLC to determine LT biosynthesis, and cell pellets were disrupted, processed, and analyzed as described under *Materials and Methods*. Data shown are from one experiment and are representative of at least three separate experiments.

The presence of cytochalasin B was required in these experiments (PAF stimulations) to achieve experimental conditions in which 5-LO translocation can be properly evaluated. In the absence of cytochalasin B, identical translocation patterns are obtained but levels of 5-LO translocation (and LT biosynthesis) are lower, and the differences between treatments are more difficult to assess (data not shown). In these experiments, the addition of the adenosine A_{2a} receptor agonist CGS-21680, PGE_2, or the adenylate cyclase activator forskolin and the type IV PDE inhibitor RO 20-1724 to incubation media resulted in the strong inhibition of PAF-induced LT biosynthesis and 5-LO translocation (Fig. 1A). In the experiment shown, cholera toxin [which elevates $[cAMP]_i$ by catalyzing the ADP-ribosylation of the α subunit of Gs proteins, leading to a persistent activation of the adenylate cyclase (van Heyningen, 1982)] had an intermediate effect on both LT biosynthesis and 5-LO translocation, inhibiting both by approximately 50%.

The effect of cAMP on 5-LO translocation and LT biosynthesis was further confirmed by experiments in which cells were incubated with the PDE-resistant cell permeable phosphorothioate analog of cAMP, *S*p-8CPT, which also inhibited PAF-induced LT biosynthesis and 5-LO translocation (Fig. 1B). In contrast, the inactive enantiomer *R*p-8CPT had no effect on PAF-stimulated LT biosynthesis or 5-LO translocation, indicating that the inhibitory effect was specific to the ability of *S*p-8CPT to activate the PKA.

Similar results were obtained when PMN were activated with the endomembrane Ca^{2+}-ATPase blocker thapsigargin. In PMN, inhibition of the Ca^{2+}-ATPase with thapsigargin results in an emptying of intracellular Ca^{2+} stores, which is followed by an influx of extracellular Ca^{2+} (Foder et al., 1989; Demaurex et al., 1994); such Ca^{2+} fluxes result in 5-LO translocation and LT biosynthesis (Fig. 1, C and D). As observed with PAF-stimulated cells, the exposure of PMN to PGE_2 was also effective in inhibiting the thapsigargin-mediated biosynthesis of LT and translocation of 5-LO. This inhibitory effect was not observed when cells were incubated with $PGF_{2\alpha}$, which does not cause the activation of adenylate cyclase or the increase in $[cAMP]_i$ in human PMN. Additionally, native cholera toxin also caused an inhibition of thapsigargin-induced 5-LO translocation and LT biosynthesis, whereas the heat-inactivated toxin, which does not activate the cyclase, was without effect (Fig. 1C). Furthermore, treatment of PMN with the type IV PDE inhibitor RO 20-1724 was also inhibitory to thapsigargin-induced LT biosynthesis and 5-LO translocation (Fig. 1D); in these experiments, the inhibitory effect of RO 20-1724 occurred in a time-dependent manner, which is consistent with its time-dependent effect on increasing $[cAMP]_i$ (data not shown).

The above experiments provide strong evidence that treatment of cells with agents known to elevate $[cAMP]_i$ in PMN inhibit LT biosynthesis and 5-LO translocation to nuclear structures. To confirm that this inhibitory activity is due to the sequential elevation of $[cAMP]_i$ and the activation of PKA, we assessed the ability of PKA inhibitors to reverse the inhibitory actions of cAMP-elevating agents in thapsigargin-stimulated human PMN. Figure 2A shows that the PKA inhibitor H-89 reverses the inhibitory effects of isoproterenol and forskolin/RO 20-1724 on thapsigargin-induced LT biosynthesis and 5-LO translocation in human PMN. H-89 alone had no stimulatory effect on 5-LO translocation or LT bio-

synthesis in unstimulated PMN or in thapsigargin-stimulated cells. Essentially identical results were obtained with the structurally distinct PKA inhibitor KT-5720 (Fig. 2B). Together, these data indicate that cAMP-mediated activation of PKA exerts a suppressive effect on 5-LO translocation and subsequent LT synthesis in human PMN. The data also strongly support the concept that 5-LO translocation is an essential process for LT biosynthesis in intact human PMN, because in all experimental conditions investigated, 5-LO translocation correlated with increased formation of LT.

In a previous study (Pouliot et al., 1996), immunoblotting with an antibody raised against a PMN cell surface marker, 13F6, was performed on both fractions from NP-40 lysis of PMN and showed that the 13F6 marker was specifically found into the non-nuclear fraction, whereas immunoblotting with an antiserum raised against FLAP showed that FLAP was only present in the nuclear fraction in all experimental conditions tested, indicating that 5-LO translocation events observed in the present study by using the NP-40 fractionation method were specific (i.e., indicated translocation of 5-LO to FLAP containing nuclear membranes, as opposed to cytoplasmic membranes). Accordingly, as shown in Fig. 3A, translocation of 5-LO to the nuclear fraction in thapsigargin-stimulated PMN correlated with a loss of 5-LO from the non-nuclear fraction. Moreover, preincubation of PMN with the PDE-resistant cAMP analog *S*p-cAMPS or with PGE_2 inhibited the translocation of 5-LO to the nuclear fraction, and this inhibitory effect was prevented by incubating cells with the PKA inhibitor KT-5720.

To demonstrate that similar 5-LO translocation data could be obtained regardless of the method used for analysis of membrane-bound 5-LO, additional experiments were conducted in which cellular membranes (including nuclear membranes) were prepared by sonication. As shown in Fig. 3B, identical results were obtained when thapsigargin-induced 5-LO translocation, and its inhibition by various agents was assessed using either isolated nuclei obtained by NP-40 lysis or membranes prepared by sonication. Finally, to confirm that the observed translocation of 5-LO induced by PAF and thapsigargin reflected an interaction of 5-LO with nuclear structures, cells were stimulated in the presence and absence of the FLAP antagonist and LT biosynthesis inhibitor, MK-0591. As shown in Fig. 3C, the preincubation of cells with MK-0591 effectively inhibited the translocation of 5-LO induced by both PAF and thapsigargin. This result further confirms the specificity of the translocation events observed in our experimental conditions because FLAP is predominantly associated with nuclear structures in human PMN (Woods et al., 1993). Figure 4 shows representative RP-HPLC chromatograms of 5-LO products from the supernatants of PMN stimulated with thapsigargin in the presence or absence of the adenosine A_{2a} receptor agonist CGS-21680. The evident inhibition of LT biosynthesis caused by CGS-21680 was clearly prevented when the PKA inhibitor KT-5720 was included in the incubation medium.

Werz et al. (2000, 2001) previously characterized the effect

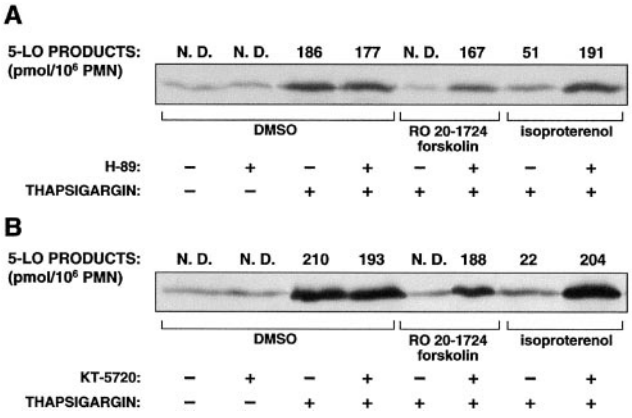

Fig. 2. Reversal of cAMP-mediated inhibition of 5-LO translocation by PKA inhibitors. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. Addition of the PKA inhibitors H-89 (10 μM) or KT-5720 (3 μM) was done 20 min before stimulation of the cells with 300 nM thapsigargin, whereas isoproterenol (1 μM) or the combination of RO 20-1724 (10 μM) and forskolin (50 μM) were added 5 and 15 min, respectively, before the addition of thapsigargin. DMSO did not exceed 0.1% (v/v) in incubation media. Supernatants were analyzed by RP-HPLC to determine LT biosynthesis, and cell pellets were disrupted, processed, and analyzed for 5-LO content as described under *Materials and Methods*. Data shown are from one experiment and are representative of at least three separate experiments.

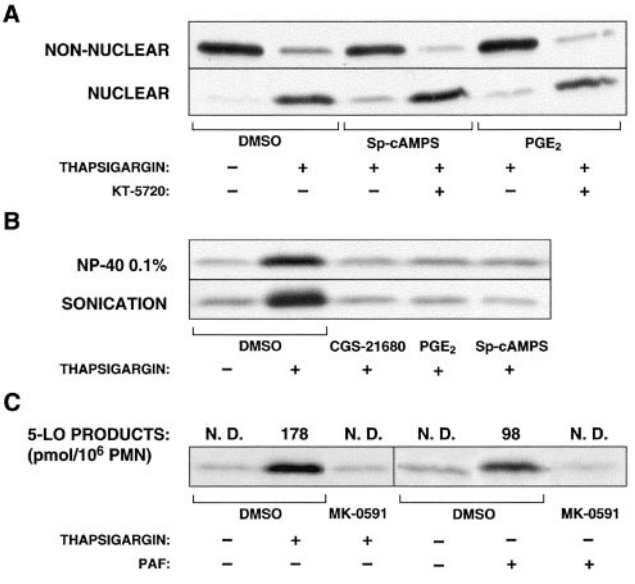

Fig. 3. Characterization of 5-LO translocation in various experimental conditions. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. A, analysis of 5-LO in non-nuclear and nuclear fractions of NP-40 treated PMN. Addition of 3 μM KT-5720 was done 20 min before stimulation of the cells, whereas 1 μM PGE_2 and 50 μM *S*p-cAMPS were added 5 and 15 min, respectively, before the stimulation of the cells with 300 nM thapsigargin. Incubations were stopped, and cell pellets were disrupted with 0.1% NP-40 as described under *Materials and Methods*. Both fractions obtained (non-nuclear and nuclear) were then analyzed using SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-5-LO. B, comparative analysis of 5-LO in the nuclear and membrane fractions obtained by NP-40 and sonication, respectively, of PMN. After stimulation of PMN with thapsigargin, the incubations were stopped by the addition of 1 volume of cold (4°C) HBSS plus $CaCl_2$. PMN were then disrupted by NP-40 lysis or sonication (Pouliot et al., 1996). Cellular membranes obtained by sonication, and nuclear fractions obtained by NP-40 lysis were analyzed for 5-LO content as described under *Materials and Methods*. C, reversal of 5-LO translocation by the FLAP antagonist MK-0591. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. Addition of 100 nM MK-0591 was done 5 min before addition of either 600 nM PAF or 300 nM thapsigargin. Incubations were stopped by the addition of one volume of cold (4°C) HBSS plus 1.6 mM $CaCl_2$. Supernatants were analyzed using RP-HPLC to determine LT biosynthesis, and cell pellets were disrupted with 0.1% NP-40, processed, and analyzed for 5-LO content as described under *Materials and Methods*. Data shown are from one experiment and are representative of at least three separate experiments.

of the serine/threonine kinases p38 and MAPKAP kinase 2 (MK2) on 5-LO activity in PMN and in Mono Mac 6 cells. They showed that the p38-dependent activation of MK2 correlated with an increase in LT biosynthesis and 5-LO translocation to the nuclear structures. To assess the putative inhibitory effect of cAMP-elevating agents in PMN on the regulation of p38, experiments were undertaken to evaluate the status of p38 phosphorylation in activated PMN. As shown in Fig. 5A, activation of PMN with thapsigargin enhanced the levels of phosphorylated p38, and 100 nM CGS-21680 abrogated this effect of thapsigargin on both p38 phosphorylation and 5-LO translocation. Furthermore, the PKA inhibitor KT-5720 restored both the phosphorylation of p38 and the translocation of 5-LO to the nuclear structures. In all experimental conditions tested, p38 was found in the non-nuclear fraction, and the phosphorylation pattern of p38 observed in whole cell lysates was identical to that observed in the non-nuclear fraction (0.1% NP-40 lysis) (data not shown). Finally, Fig. 5B shows that the p38 inhibitor SB 203,580 dose-dependently inhibited 5-LO products biosynthesis in PMN stimulated with AA, in agreement with the proposed role of p38 in 5-LO activation (Werz et al., 2002).

Although Figs. 1 through 5 show representative experiments, Fig. 6 illustrate the results (p38 phosphorylation, 5-LO translocation, and 5-LO products biosynthesis) obtained in four separate experiments in which PMN were exposed to DMSO (control) or thapsigargin in the presence or absence of the A_{2a} receptor agonist CGS-21680 and the PKA inhibitor KT-5720. The data show that the differences observed on the three parameters investigated were significant (two-tailed t test, $p < 0.005$).

Discussion

Initial observations in the late 1980s indicated that an important regulatory step in the biosynthesis of LT in human PMN involves the translocation of 5-LO from the cytosol to a membrane compartment (Rouzer et al., 1985; Rouzer and Samuelsson, 1987). Ca^{2+} mobilization is required for 5-LO translocation. However, Ca^{2+} mobilization alone is not sufficient because little 5-LO translocation occurs when unprimed cells are stimulated with agonists such as fMLP or PAF, which induce the mobilization of Ca^{2+} from intracellular stores and subsequent Ca^{2+} influx. Since those initial reports, it has been established that 5-LO activation in stimulated human PMN involves the Ca^{2+}-dependent movement of 5-LO from the cytosol to the nuclear envelope in a mechanism not yet completely understood but implicating the membrane protein FLAP (Dixon et al., 1990; Reid et al., 1990). Indeed, it has been clearly established by using FLAP antagonists that FLAP plays an essential role in the translocation of 5-LO in intact PMN and that this process is a key step in 5-LO activation. Chen and Funk (2001) recently

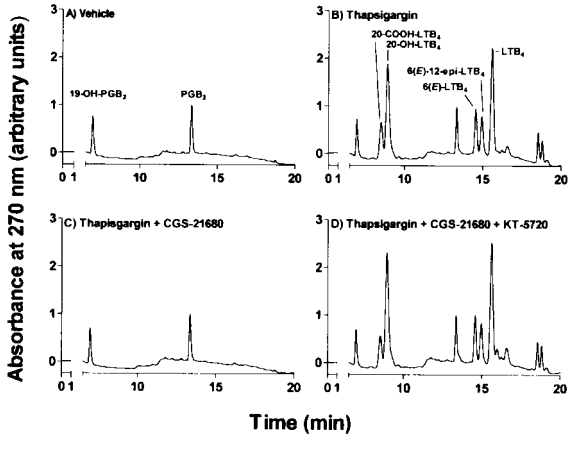

Fig. 4. RP-HPLC analysis of 5-LO products in thapsigargin-stimulated PMN. Supernatants obtained from PMN incubations were analyzed for the biosynthesis of 5-LO products by RP-HPLC using an online extraction procedure as described under *Materials and Methods*. Cells were incubated at 37°C with (A) 0.1% DMSO (diluent), (B) 300 nM thapsigargin for 10 min, (C) 100 nM CGS-21680 for 5 min followed by 300 nM thapsigargin for 10 min, or (D) 3 μM KT-5720 for 20 min and CGS-21680 for 5 min followed by 300 nM thapsigargin for 10 min. Data shown are representative of at least three separate experiments.

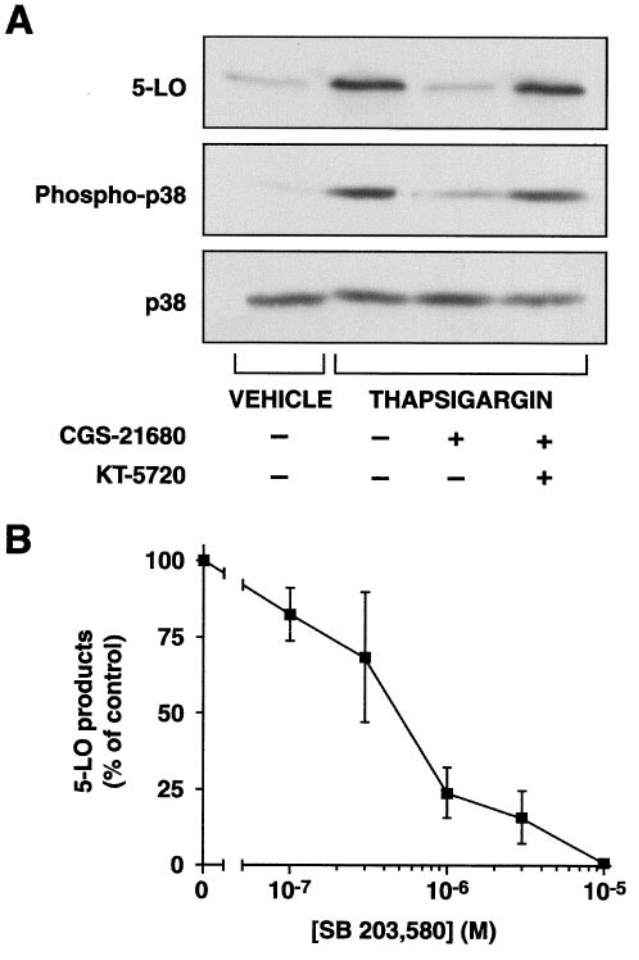

Fig. 5. A, inhibitory effect of CGS-21680 on p38 phosphorylation and 5-LO translocation in thapsigargin-activated PMN. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. Addition of 3 μM KT-5720 and 100 nM CGS-21680 to the incubation media was performed 20 and 5 min, respectively, before stimulation with 300 nM thapsigargin. Cell pellets were disrupted with 0.1% NP-40, processed, and analyzed for 5-LO, p38, and phospho-p38 contents as described under *Materials and Methods*. An immunoblot representative of four separate experiments is shown for each protein studied. Analysis of nuclear 5-LO was performed on the nuclear fractions obtained by NP-40 lysis, and analysis of phospho-p38 and p38 were performed on the non-nuclear fraction obtained by NP-40 lysis. B, 5-LO products biosynthesis inhibition by SB 203,580 in AA-activated PMN. Human PMN (5×10^6) were preincubated (10 min, 37°C) with various concentrations of SB 203,580 (as indicated) and then stimulated for an additional 5 min with 5 μM AA. 5-LO products were analyzed as described under *Materials and Methods*. The data shown are the mean (± S.D.) of three separate experiments, each performed in triplicate.

showed that the N-terminal β-barrel region of the protein is sufficient and necessary for translocation to membranes. Moreover, recent studies suggested that interactions with the cytoskeleton (Lepley and Fitzpatrick, 1994; Provost et al., 1999; Provost et al., 2001), modification of the enzyme by kinases (Lepley et al., 1996; Boden et al., 2000; Werz et al., 2000), or interactions with signaling molecules may be involved in directing 5-LO specifically to the nuclear membrane after cell activation.

Although much effort has been focused on understanding the mechanisms by which 5-LO is activated in stimulated cells, little is known about suppressive mechanisms that may inhibit or prevent the translocation of 5-LO to the nucleus. We and others have reported previously that the treatment of human PMN with agents that cause an elevation of $[cAMP]_i$ results in an inhibition of the capacity for the biosynthesis of LTB_4 after cell stimulation (Ham et al., 1983; Peachell et al., 1989; Schudt et al., 1991; Fonteh et al., 1993; Tenor et al., 1996; Krump et al., 1997; Dennis and Riendeau, 1999; Surette et al., 1999; Flamand et al., 2000). The present studies were undertaken to investigate whether cAMP-mediated inhibition of LT biosynthesis could involve an inhibitory effect at the level of 5-LO translocation. The results presented herein represent the first description of a mechanism by which cells negatively regulate the translocation of 5-LO from the cytoplasm to the nuclear structures of activated PMN.

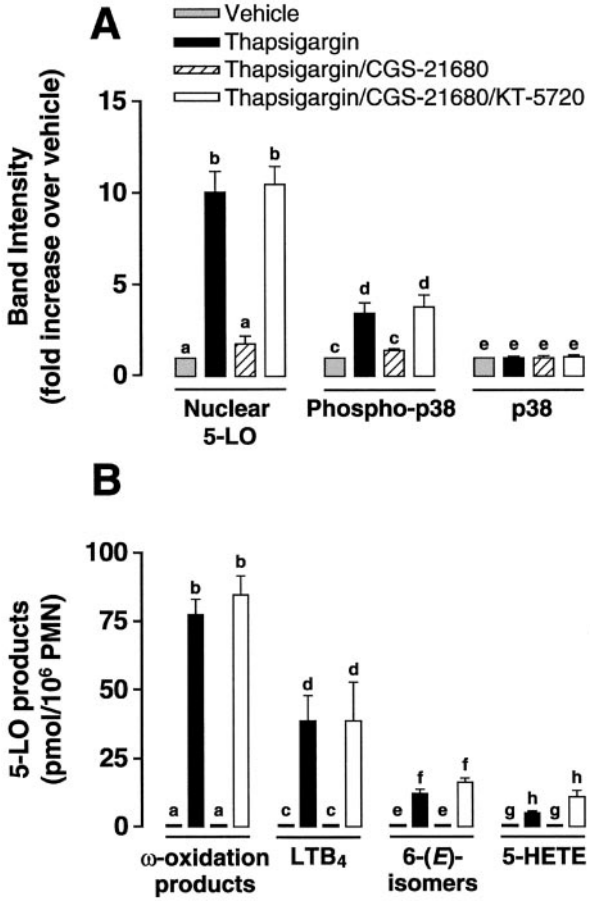

Fig. 6. Computed immunoblot analysis of proteins (A) and RP-HPLC analysis of 5-LO products (B) from thapsigargin-activated PMN. Human PMN (2×10^7) were incubated and stimulated as described under *Materials and Methods*. Addition of 3 μM KT-5720 and 100 nM CGS-21680 to the incubation media was performed 20 and 5 min, respectively, before stimulation with 300 nM thapsigargin for 10 min. Supernatants obtained from PMN incubations were analyzed for the biosynthesis of 5-LO products by RP-HPLC using an online extraction procedure, and cell pellets were disrupted with 0.1% NP-40, processed, and analyzed for nuclear 5-LO, non-nuclear p38, and phospho-p38 contents as described under *Materials and Methods*. Data shown are the mean (± S.D.) of four densitometric determinations on each protein analyzed and four RP-HPLC analysis. Bars identified by different letters are significantly different (two-tailed *t* test, $p < 0.005$).

In the present study, the inhibition of 5-LO translocation resulting from the elevation of $[cAMP]_i$ was clearly demonstrated using several approaches. Indeed, agents which elevate $[cAMP]_i$ after cell surface–receptor engagement, type IV PDE inhibitors, Gs protein activator (cholera toxin), adenylate cyclase activator (forskolin), and cell-permeable cAMP analogs all caused an inhibition of 5-LO translocation in activated PMN. In further support of a role of cAMP and PKA in the observed inhibitory effects of the above-mentioned pharmacological agents, the two structurally distinct PKA inhibitors used in this study effectively reversed the inhibitory effects of cAMP-elevating agents on 5-LO translocation. It must be pointed out, however, that the cAMP-mediated regulation of 5-LO translocation has not yet been investigated in other cell types (such as eosinophils and mononuclear phagocytes). The important differences in the regulation of LT biosynthesis between PMN and these other cell types already reported dictate caution in extrapolating the present findings to other cells containing the 5-LO.

The mechanism by which 5-LO translocation is inhibited by cAMP is not clear. Two putative PKA phosphorylation consensus sequences can be found on 5-LO between amino acids 247–250 (RRCT) and 521–524 (RKSS). Thus, PKA might directly phosphorylate 5-LO, inducing a conformational change that could result in a decreased ability of the enzyme to associate with nuclear membranes and/or FLAP. A recent study suggests that a PKA-dependent phosphorylation of 5-LO at the putative phosphorylation sites mentioned above is unlikely. Indeed, an S271A mutant of 5-LO was not phosphorylated by PKA in an in vitro kinase assay (Werz et al., 2002). Elevation of cAMP and PKA activation could also result in the inhibition of a kinase upstream of MAPKAP kinases, which have been shown to phosphorylate 5-LO in vitro (Werz et al., 2000). Phosphorylation of 5-LO by MK2 from stimulated PMN and Mono Mac 6 cells has been demonstrated in in-gel kinase assays. This phosphorylation, as well as 5-LO activation, was inhibited by the p38 inhibitor SB 203,580, suggesting that p38 activation upstream of MK2 is required for 5-LO phosphorylation and activation. Moreover, it was recently shown that MAPK kinase (MEK) inhibition strikingly decreased 5-LO translocation in fMLP-activated PMN, suggesting that MEK plays an important role in 5-LO activation and translocation (Boden et al., 2000). Consistent with a MEK-dependent 5-LO activation and translocation and a p38- and MK2-dependent phosphorylation of 5-LO in activated cells, Lepley et al. (1996) showed that tyrosine kinase inhibitors also inhibited 5-LO activation and translocation; because many of the kinases upstream of MEK and p38 are tyrosine kinases, their inhibition could affect MEK, p38, and MK2 activation and ultimately 5-LO phosphorylation, activation, and translocation. Our observation that cAMP-mediated PKA activation profoundly affects 5-LO translocation and that this inhibition correlated with a marked decrease of p38 phosphorylation in thapsigargin-stimulated PMN (Figs. 5 and 6) suggests that PKA activation

negatively affect the putative 5-LO activating cascade described above. We believe that this is the first report of an inhibitory effect of cAMP-elevating agents on p38 activation in stimulated human PMN.

Another putative mechanism for the cAMP-mediated inhibition of 5-LO translocation could implicate AA itself in the regulation of the localization of the enzyme on nuclear structures. We recently showed that the cAMP-dependent inhibition of LT biosynthesis by adenosine was at least in part caused by the inhibition of $cPLA_2$ activation and AA release from phospholipids (Flamand et al., 2000); we showed in that study that the cAMP-elevating agent CGS-21680 tested in the present study and shown to block 5-LO translocation also dramatically inhibits the release of AA in PAF-stimulated PMN. This raises the interesting possibility of a causal relationship between the two processes. In this regard, it is noteworthy that we have already observed that exogenous AA causes significant 5-LO translocation in agonist- and thapsigargin-activated PMN exposed to cAMP-elevating agents[1]; Werz et al. (2002) have shown that AA directly promotes MK2-mediated phosphorylation of 5-LO, indicating a mechanism by which AA may directly affect 5-LO activation in intact PMN. The hypothesis that the $cPLA_2$-mediated release of AA and the translocation of 5-LO may be causally related is currently under investigation in our laboratory.

Finally, recent preliminary data from our laboratory suggest that the cAMP-mediated abrogation of 5-LO translocation is partially reversed by the addition of exogenous 12-HETE and 15-HETE (data not shown), suggesting that PMN exposed to cAMP-elevating agents can still generate 5-LO–derived lipid mediators at inflammatory sites from exogenous substrates through transcellular mechanisms. The putative formation of an anti-inflammatory lipid mediator such as lipoxin A_4 would strengthen the anti-inflammatory signals triggered by autacoids such as adenosine (Cronstein, 1994; Ohta and Sitkovsky, 2001) and contribute to the down-regulation and/or resolution of inflammation (Levy et al., 2001).

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Address correspondence to: Dr. Pierre Borgeat, Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du CHUQ, Pavillon CHUL, Office T1–49, 2705 Laurier, Ste.-Foy, Québec, Canada, G1V 4G2. E-mail: pierre.borgeat@crchul.ulaval.ca