Leukotriene B_4 mediates p47phox phosphorylation and membrane translocation in polyunsaturated fatty acid-stimulated neutrophils

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Abstract: Polyunsaturated fatty acids (PUFAs) and leukotriene B_4 (LTB_4) are involved in many inflammatory and physiological conditions. The role of arachidonic acid (AA) and linoleic acid (LA) in promoting the assembly of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits is well known, but the involvement of LTB_4 and other 5-lipoxygenase (5-LO) pathway metabolites of AA in hydrogen peroxide (H_2O_2) production by PUFA-stimulated polymorphonuclear leukocytes (PMNs) has not been investigated. We examined this question by determining H_2O_2 production as well as phosphorylation and membrane translocation of the p47phox subunit of NADPH oxidase. Elicited peritoneal PMNs from rats and from 5-LO-deficient or wild-type mice were pretreated with or without inhibitors of LT biosynthesis and antagonists of the receptors for LTB_4 and cysteinyl LTs for 20 min before stimulation with AA (at 5 and 20 μM) or LA (at 20 μM). PUFAs elicited H_2O_2 production in a dose-dependent manner, and pharmacologic or genetic inhibition of LT synthesis decreased H_2O_2 production by ~40% when compared with untreated controls. LTB_4 was the moiety responsible for H_2O_2 production, as revealed by studies using receptor antagonists and its exogenous addition. LTB_4 itself also promoted p47phox phosphorylation and translocation. These results identify a heretofore unrecognized role for activation of 5-LO and subsequent production of LTB_4 in stimulation of PMN NADPH oxidase activation by PUFAs. *J. Leukoc. Biol.* 78: 976–984; 2005.

***Key Words:** PMN · PUFAs · lipid mediators · NADPH oxidase · BLT1*

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are involved in many physiological and pathological situations, including alteration of plasma lipid levels, cardiovascular function, insulin action, neuronal development, and activation and regulation of the immune system [1]. These lipids are classified according to the position of the double bond nearest their methyl (ω) end into those of the n-3 (ω-3) and those of the n-6 (ω-6) series. PUFAs are essential constituents of mammalian diets, required for maintenance of normal cell structure and function. Examples of n-6 PUFAs are linoleic acid (LA; 18:2n-6) and arachidonic acid (AA; 20:4n-6). AA and LA are liberated from membrane phospholipids by the action of phospholipase A_2 (PLA_2) enzymes. AA serves the well-characterized role of substrate for the generation of bioactive eicosanoids, including prostaglandins and leukotrienes (LTs). Besides its role as a precursor for eicosanoids, endogenous AA may function as an intracellular signaling molecule [2, 3] or as a cell activator when released into the extracellular space.

Numerous signaling components have been reported to be activated by exogenous AA. These include guanosine 5′-triphosphate-binding proteins [4], plasma membrane Ca^{++} channels [5], extracellular signal-regulated kinase 1/2 (ERK 1/2) [6], Raf-1/mitogen-activated protein kinase kinase (MEK) [7], c-Jun N-terminal kinase [8], phosphatidylinositol 3-kinase [9], PLC [9], nuclear factor-κ B [10], and protein kinase C (PKC) [11]. In neutrophils [polymorphonuclear leukocytes (PMNs)], it has been suggested that activation of ERK 1/2 and Raf-1/MEK by AA could be mediated by formation of its lipoxygenase (LO) metabolic products [6, 7]. One of the most well-studied actions of AA is the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in cell-free systems as well as intact PMNs [4, 5, 12–19]. The NADPH oxidase complex is formed by the cytosolic proteins p47phox, p40phox, p67phox, and Rac-1 or -2 and the membrane-associated components gp91phox, p22phox, and Rap-1 [20]. Upon cell activation, the cytosolic components are phosphorylated by specific serine kinases. This leads to structural modification of the phox proteins, allowing their translocation to the cell membrane [20]. Most activators of NADPH oxidase, such as cytokines, lipids, and bacteria, act via receptor-mediated sig-

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naling pathways; however, lipids, such as AA, LA, diacylglycerol, or phosphatidic acid, may also interact directly with p47phox or p67phox proteins by unmasking their membrane-binding domains [19, 21] or may interact with other relevant regulatory components [22–27].

AA is converted to LTs by 5-LO, and the major products formed in PMNs are LTB_4 and 5-hydroxy-eicosatetraynoic acid (5-HETE) [28]. It is important that LTB_4 has been shown to activate NADPH oxidase [29, 30], although the mechanism of this activation remains unknown. However, we recently demonstrated that in rat alveolar macrophages, LTB_4 activates NADPH oxidase through phosphorylation and translocation of p47phox to the membrane, a process that was itself dependent on PKC-δ activity [31]. It is unclear whether the ability of AA to activate NADPH oxidase depends, in part, on its metabolism to LTB_4. Conversely, as LTB_4 activates cytosolic PLA_2 ($cPLA_2$) [32], it is possible that NADPH oxidase activation depends on LTB_4-stimulated AA. As AA and LTB_4 can stimulate Ca^{++} release [33, 34] as well as PLC and PKC activities [9, 11, 30, 35], these actions may affect NADPH oxidase indirectly as well. Thus, the goals of this work were to evaluate the role of 5-LO metabolites in PUFA-induced NADPH oxidase activation in PMNs and to determine the molecular mechanisms involved in these actions. Herein, we report that LTB_4 is a significant mediator of the ability of AA to activate NADPH oxidase in PMNs. LTB_4 participates in NADPH oxidase activation by modulating p47phox phosphorylation and translocation to the membrane. To our knowledge, this is the first report showing that LTB_4 is able to directly affect the assembly of NADPH oxidase components in PMNs.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) without phenol red was purchased from Gibco-Invitrogen (Carlsbad, CA). Type IV horseradish peroxidase (HRP) was purchased from Sigma Chemical Co. (St. Louis, MO). AA, LA, LTB_4, LTC_4, LTD_4, phorbol myristate acetate (PMA), MK886 [5-LO-activating protein (FLAP) inhibitor], AA-861 (5-LO inhibitor), MK571 [cysteinyl LT receptor 1 (cysLT1) antagonist], and diphenyleneiodonium (DPI; NADPH oxidase-like flavoprotein inhibitor) were purchased from Biomol (Palo Alto, CA). CP105,696 [LTB_4 receptor 1 (BLT1) antagonist] was a generous gift of Dr. Henry Showell (Pfizer, Groton, CT). Abbott Laboratories (Abbott Park, IL) provided zileuton. Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased from Molecular Probes (Eugene, OR). Compounds requiring reconstitution were dissolved in ethanol or dimethyl sulfoxide. Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

Animals

5-LO knockout (KO; 129-$Alox5^{tm1Fun}$) [36] and strain-matched wild-type (WT) sv129 mice were bred in the University of Michigan Unit for Laboratory Animal Medicine (Ann Arbor) from breeders obtained from Jackson Laboratories (Bar Harbor, ME), and female Wistar rats were obtained from Charles River Laboratories (Portage, MI). The University Committee on Use and Care of Animals approved animal protocols.

Cell harvest

Glycogen-elicited rat or murine PMNs were obtained following injection of 20 mL 4% glycogen (Sigma Chemical Co.) into the peritoneal cavity. After 5–6 h, the peritoneal exudates were harvested by lavage with phosphate-buffered saline (PBS). Contaminating red blood cells were lysed with H_2O, and the cells were washed 2× with PBS. The percentage of PMNs was determined microscopically using a modified Wright-Giemsa stain, and a typical experiment yielded ~90% PMNs.

Cell viability

No experimental compounds or vehicle showed any adverse effects on PMN viability as determined by a cell-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (data not shown).

Hydrogen peroxide (H_2O_2) detection

Rat PMNs (5×10^5/well) were plated in 96-well dishes as described above. H_2O_2 production was assessed in a HRP-coupled reaction using Amplex Red as a probe, according to the manufacturer (Molecular Probes). To assess the role of 5-LO metabolites in H_2O_2 production stimulated by PUFAs, the cells were first pretreated with zileuton, AA-861, MK886, MK571, or CP105,696 for 20 min followed by the addition of the Amplex Red solution containing AA or LA. The solution containing 50 μM Amplex Red reagents and 10 U/mL HRP was prepared in PBS, 0.1 mL was added to PMNs at 37°C for 30 min, and the H_2O_2 levels were determined colorimetrically (absorbance at 560 nm).

LTB_4 measurement

Rat PMNs (5×10^5 cells/well) were cultured in 96-well plates in DMEM. Cultures were then incubated for 5, 15, and 30 min with 20 μM AA to stimulate LT production. Culture supernatants were collected, and LTB_4 levels were quantified by enzyme immunoassay, according to the manufacturer (Assay Designs, Ann Arbor, MI).

Fractionation, immunoprecipitation, and Western blotting

PMNs (1×10^7) were plated in six-well tissue-culture dishes and pretreated with AA-861 or CP105,696 for 20 min, followed by stimulation for 5 min with 20 μM AA or vehicle control. After this, PMNs were lysed by sonication in ice-cold lysis buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanodate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 μg/mL leupeptin, followed by ultracentrifugation at 100,000 *g* for 20 min at 4°C. The cytosolic (soluble) fraction was harvested, and the membrane (insoluble) fraction was washed and subjected to another ultracentrifugation step as described above. The resulting pellet was resuspended in lysis buffer and sonicated. Protein concentrations were determined by a modified Coomassie dye-binding assay (Pierce Chemical Co., Rockford, IL). The cytosolic fraction was used for immunoprecipitation as described previously [37] with some modifications. The fraction was incubated overnight at 4°C with anti-p47phox antibody (1:80; Upstate Biotechnology, Lake Placid, NY). Protein A-Sepharose was added to each sample and incubated for 3 h with rotation at 4°C. The beads were washed briefly 3× with lysis buffer without Triton X-100, and samples containing 20 μg protein were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to nitrocellulose membranes. Following blocking with 5% nonfat milk, membranes were probed with antibodies directed against p47phox (1:500 dilution; Upstate Biotechnology) or antiphosphoserine (1:900; KI-191, clone 1C8; Biomol) for 90 min, followed by peroxidase-conjugated goat anti-rabbit (Amersham, Piscataway, NJ) or anti-mouse secondary (1:5000; Zymed, South San Francisco, CA) and developed using enhanced chemiluminescence detection (Amersham).

Statistical analysis

Data are represented as mean ± SE and were analyzed with the Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Comparisons between two experimental groups were performed with Student's *t*-test. Comparisons among more than or equal to three experimental groups were performed with ANOVA followed by the Bonferroni analysis. Differences were considered significant if $P \leq 0.05$. All experiments were performed on more than or equal to three separate occasions unless otherwise specified.

RESULTS

PUFAs induce H_2O_2 release in glycogen-elicited PMNs via NADPH oxidase

To determine if PUFAs activate NADPH oxidase (as determined by H_2O_2 production), we performed dose-response experiments using AA or LA. As shown in **Figure 1, A** and **B**, AA and LA induced H_2O_2 generation in a dose-dependent manner. At the plateau dose (20 μM) of either PUFA, we observed the production of approximately five times more H_2O_2 than by untreated cells. As concentrations of AA above 20 μM can induce cell necrosis [38], we used 20 μM of both PUFAs for subsequent experiments.

Reactive oxygen intermediates (ROI) may be generated by a variety of sources, including enzymes other than NADPH oxidase [39, 40]. To determine if ROI produced during PUFA activation is from NADPH oxidase activation, we pretreated PMNs with DPI, a flavoprotein inhibitor that has relative specificity for NADPH oxidase at a concentration of 10 μM, followed by addition of 20 μM AA or LA. Our data show that the major source of ROI during AA or LA stimulation is through activation of NADPH oxidase, as H_2O_2 production was abolished completely in DPI-treated as compared with untreated cells (**Fig. 2**).

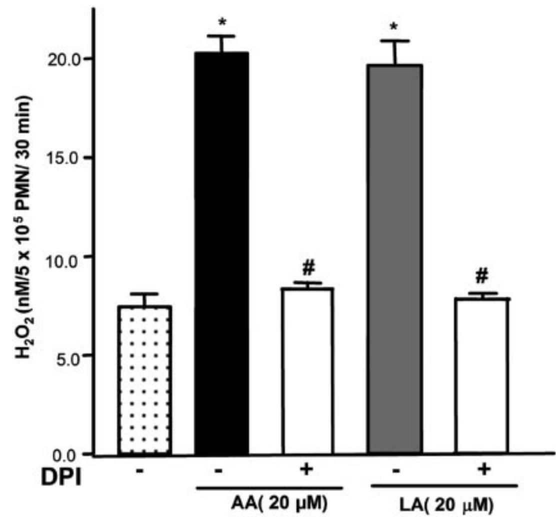

Fig. 2. PMN H_2O_2 production in response to PUFAs is dependent on NADPH oxidase activity. PMNs were treated with the NADPH oxidase inhibitor DPI (10 μM) for 20 min and then stimulated for 30 min with 20 μM AA or 20 μM LA. H_2O_2 production was determined using Amplex Red as a probe, as described in Materials and Methods. Data represent mean ± SE from quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$, versus control and #, $P < 0.05$ versus PUFA alone by ANOVA.

NADPH oxidase activation by PUFAs is partially dependent on 5-LO activity

The role of $cPLA_2$ activation and AA release in NADPH oxidase activation is well-established [18, 41]. As AA is the substrate for 5-LO, and 5-LO metabolites are themselves able to induce NADPH oxidase activation [29, 30, 42], we sought to determine if such products are important in mediating the effects of PMN stimulation by PUFAs.

Our first approach was to determine whether endogenous 5-LO metabolites play a role in PUFA-stimulated H_2O_2 production. Rat PMNs were pretreated with either of two inhibitors of LT synthesis, namely, the 5-LO inhibitor zileuton (10 μM) or the FLAP inhibitor MK886 (1 μM), for 20 min before activation with AA or LA (20 μM). As observed in **Figure 3, A** and **C**, zileuton and MK886 treatment decreased by ~40% the AA- or LA-induced H_2O_2 production as compared with untreated cells. Similar experiments using cells from 5-LO-deficient mice confirmed these results (Fig. 3, B and D). It is interesting that when the PMNs were stimulated with a lower dose of AA (5 μM; a concentration of AA that is perhaps more relevant to its possible function as an endogenous second messenger), the role of endogenous 5-LO metabolites in inducing H_2O_2 generation was even more pronounced. For example, stimulation of H_2O_2 generation with 20 μM AA was inhibited by ~40% by

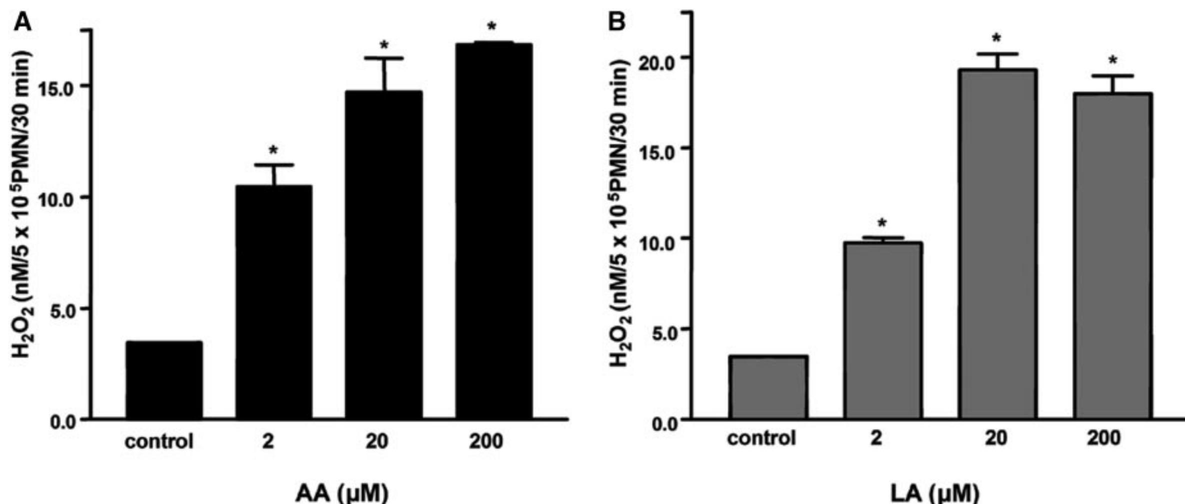

Fig. 1. PUFAs stimulate PMN H_2O_2 release in a dose-dependent manner. H_2O_2 from glycogen-elicited rat PMNs was measured 30 min after stimulation with AA (A) or LA (B). H_2O_2 concentrations were determined using Amplex Red as a probe as described in Materials and Methods. Data represent mean ± SE from quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$, versus control by ANOVA.

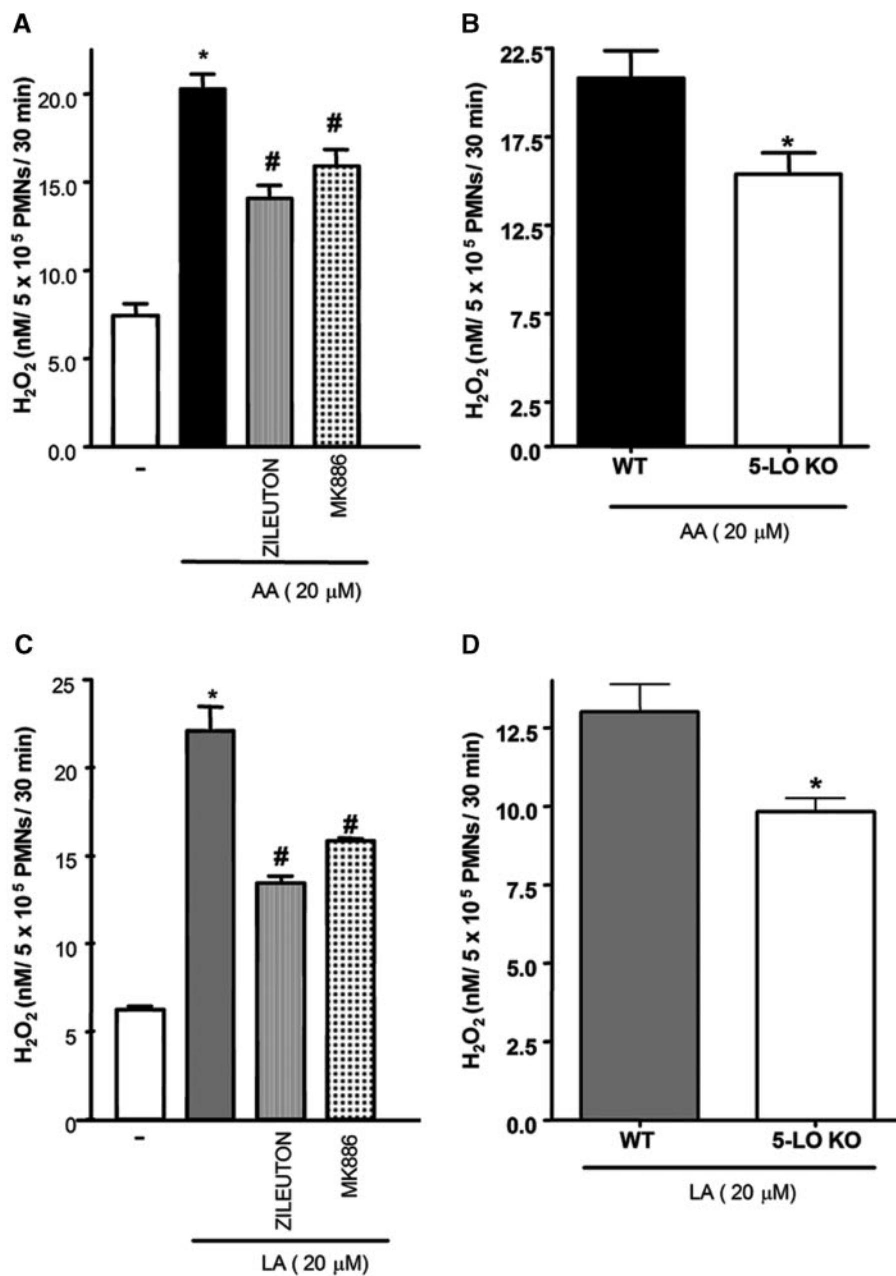

Fig. 3. H_2O_2 production in response to PUFAs in LT-deficient PMNs. Glycogen-elicited rat PMNs were pretreated with the 5-LO inhibitor zileuton (10 μM) or the FLAP inhibitor MK886 (1 μM) for 20 min and then stimulated for 30 min with 20 μM AA (A) or 20 μM LA (C). Glycogen-elicited PMNs from WT or 5-LO KO mice were stimulated with AA (B) or LA (D) for 30 min. H_2O_2 concentrations were determined using Amplex Red as a probe as described in Materials and Methods. Data represent the mean ± SE of quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$ versus control and #, $P < 0.05$ versus PUFA alone by ANOVA.

the 5-LO inhibitor AA-861, but with 5 μM AA, the inhibition was ~75% when compared with untreated control (control, 2.93±0.129; AA, 11.23±0.134; AA+AA-861, 5.080±0.240 nM $H_2O_2/2\times10^5$ PMN/30 min; $P<0.05$ for AA+AA-861 vs. AA alone). Taken together, these data indicate that H_2O_2 production stimulated by PUFAs in glycogen-elicited PMNs is dependent, in part, on endogenous 5-LO products.

NADPH oxidase activation by endogenous 5-LO products involves the BLT1 receptor

The major products of the 5-LO pathway include LTB_4 as well as the cysteinyl LTs C_4, D_4, and E_4. Both act in leukocytes primarily through BLT1 or cysLT1 receptors. To evaluate which of these LTs are involved in the activation of NADPH oxidase by PUFAs, we pretreated cells with the BLT1 antagonist CP105,696 (10 μM), or the cysLT1 antagonist MK571 (10 μM) for 20 min before PUFA stimulation. Antagonism of BLT1 abolished H_2O_2 secretion in PMNs stimulated with AA or LA. In contrast, PMNs pretreated with the cysLT1 antagonist MK571 did not demonstrate altered H_2O_2 production when compared with the untreated group (**Fig. 4, A** and **B**). To exclude the possibility that CP105,696 was merely acting as a H_2O_2 scavenger in this circumstance, we assayed a range of concentrations of added reagent H_2O_2 in the presence or absence of 10 μM CP105,696 and detected no influence of the antagonist (data not shown). Furthermore, we examined if the BLT1 antagonist influenced NADPH oxidase activation in response to the strong stimulus PMA, and we observed no effect of the antagonist (data not shown). To investigate the role of BLT1 in PMNs stimulated with 5 μM AA, we pretreated PMNs with the BLT1 antagonists CP105,696 (10 μM) or U75302 (1 μM) followed by AA stimulation. As observed above, the effects of both antagonists were again more evident at this lower AA concentration (control, 2.93±0.129; AA,

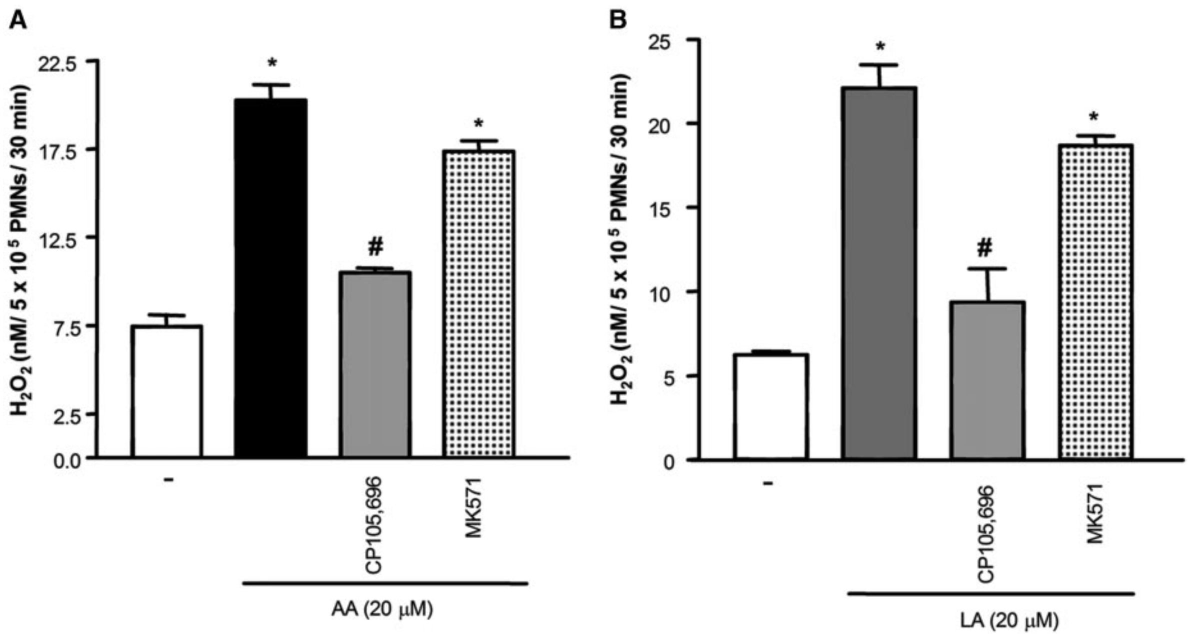

Fig. 4. Role of LT receptors in PMN H_2O_2 production by PUFAs. Glycogen-elicited rat PMNs were treated with the BLT1 antagonist CP105,696 (10 μM) or the cysLT1 antagonist MK571 (10 μM) and then stimulated for 30 min with 20 μM AA (A) or 20 μM LA (B). H_2O_2 production was determined using Amplex Red as a probe as described in Materials and Methods. Data represent mean ± SE of quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$ versus control and # $P < 0.05$, versus PUFA alone by ANOVA.

11.23±0.134; AA+CP105,696, 2.700±0.385; AA+U75302, 6.725±0.125 nM $H_2O_2/2\times10^5$ PMN/30 min. $P<0.05$ for AA+CP105,696 or AA+U75302 vs. AA alone).

To confirm that LTB_4 itself can increase NADPH oxidase activity in rat PMNs, we incubated cells with increasing concentrations of LTB_4 or cysteinyl LTs (LTC_4 and LTD_4). The stimulation of PMNs with 0.01–10 nM LTB_4 induced H_2O_2 secretion in a dose-dependent manner (**Fig. 5A**). However, we observed no H_2O_2 production in PMNs stimulated by cysLTs at any concentration tested (10–1000 nM LTC_4 or LTD_4; data not shown). Thus, our results show that only exogenous LTB_4 can induce NADPH oxidase activation in PMNs.

To determine the kinetic relationship between LTB_4 production and H_2O_2 generation in response to exogenous AA, we performed a time-course experiment in PMNs stimulated with 20 μM AA. LTB_4 production was detected within 5 min of AA addition, appeared to plateau at 15 min, and increased once again by 30 min. By contrast, H_2O_2 demonstrated an initial lag period, until an increase could be observed between 5 and 15 min, which increased further by 30 min (Fig. 5B). Thus, our

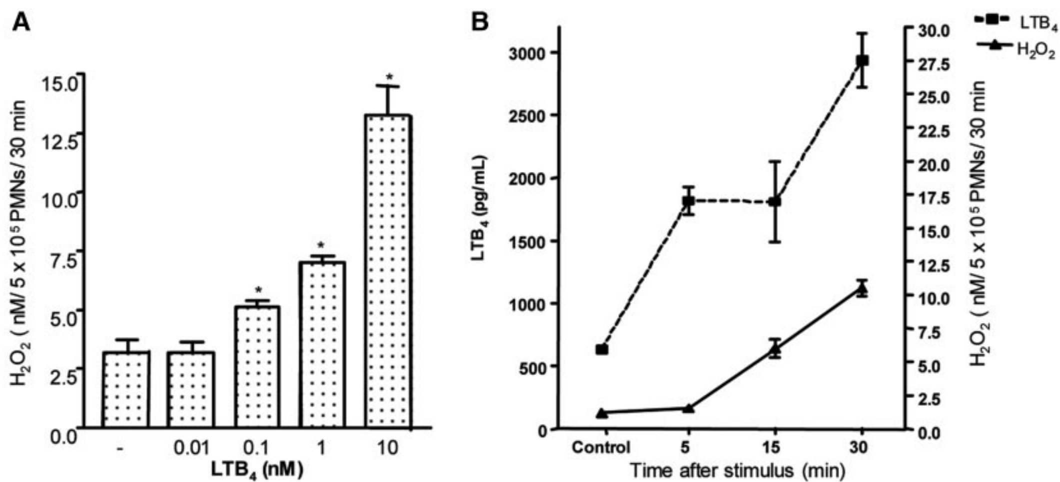

Fig. 5. AA stimulates LTB_4 production followed by H_2O_2 secretion in PMN from rats. (A) H_2O_2 levels were measured as described in Materials and Methods after stimulation with different LTB_4 concentrations. (B) LTB_4 and H_2O_2 levels were measured as described in Materials and Methods in PMN supernatants at different time-points following addition of 20 μM AA. Data represent mean ± SE of quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$ versus control by ANOVA.

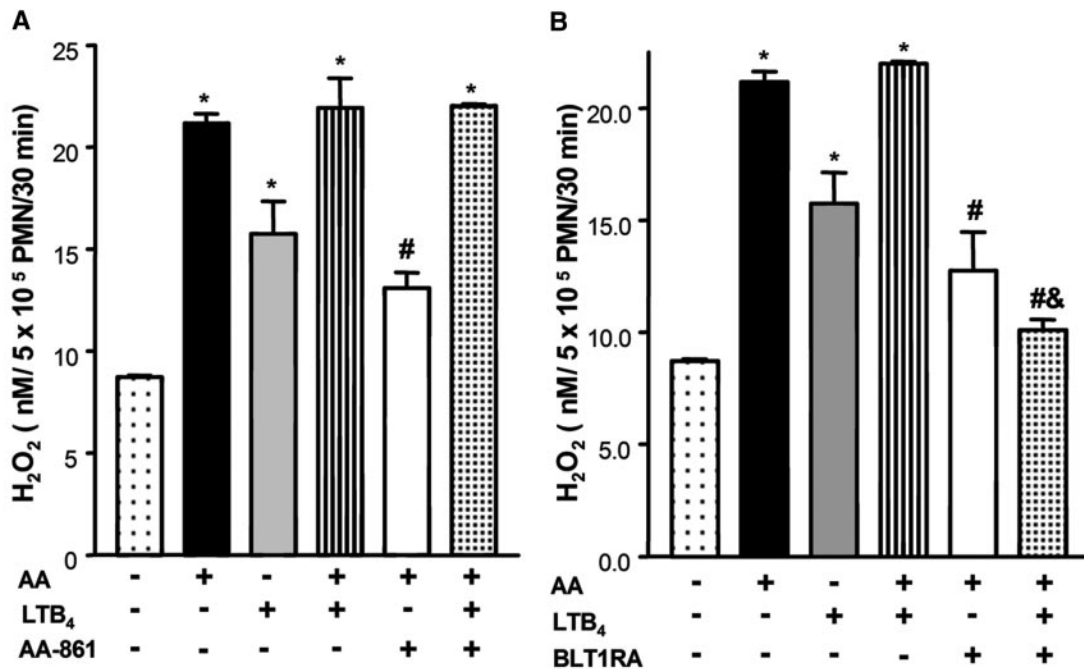

Fig. 6. Role of BLT1 receptor in mediating H_2O_2 stimulation. Glycogen-elicited rat PMNs were treated with the 5-LO inhibitor AA-861 (10 μM; A) or the BLT1 antagonist CP105,696 (10 μM; B) and then stimulated for 30 min with 20 μM AA and/or 10 nM LTB_4. H_2O_2 production was determined using Amplex Red as a probe as described in Materials and Methods. Data represent mean ± SE of quadruplicate values from one experiment representative of a total of four. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AA; and &, $P < 0.05$ versus LTB_4 by ANOVA.

results indicate that LTB_4 production precedes NADPH oxidase activation in AA-stimulated PMNs, a temporal relationship consistent with the possibility that the former response contributes to the latter.

Next, we wished to investigate whether it was possible to restore H_2O_2 secretion with exogenous LTB_4 in PMNs treated with inhibitors of endogenous LT synthesis and if the LTB_4 effect was a result of interaction with the BLT1 receptor. We therefore pretreated PMNs for 20 min with the 5-LO inhibitor AA-861 (10 μM; **Fig. 6A**) or the BLT1 antagonist CP105,696 (10 μM; Fig. 6B), followed by stimulation with AA and/or LTB_4. Our results show that exogenous LTB_4 restored the inhibition of H_2O_2 generation in PMNs treated with the 5-LO inhibitor. In contrast, the addition of LTB_4 to AA-treated PMNs incubated with the BLT1 antagonist did not overcome this inhibition. These results indicate that endogenous LTB_4 is important in NADPH oxidase activation by AA, and this effect is mediated by interaction with the BLT1 receptor.

LTB_4 induces p47phox phosphorylation and translocation in AA-stimulated PMNs

As our data identified a role for LTB_4 in AA activation of NADPH oxidase, we sought to determine the relevant molecular mechanism. We first evaluated if LTB_4 contributed to p47phox phosphorylation in PMNs stimulated by AA. As can be observed in **Figure 7A**, AA elicited phosphorylation of p47phox, and pretreatment of PMNs with a 5-LO inhibitor as well as a BLT1 antagonist decreased AA-induced p47phox phosphorylation. In addition, LTB_4 itself induced p47phox phosphorylation. As p47phox translocation is dependent on phosphorylation in some cell types [43], we evaluated the effects of a 5-LO inhibitor and a BLT1 antagonist on this phenomenon in AA-stimulated PMNs. Our results show that 5-LO inhibition as well as BLT1 antagonism inhibited p47phox translocation to the membrane fraction, indicating that endogenous LTB_4 contributes to p47phox translocation in PMNs stimulated by AA (Fig. 7C). In addition, we observed the same profile in LA-stimulated cells (data not shown). To directly test

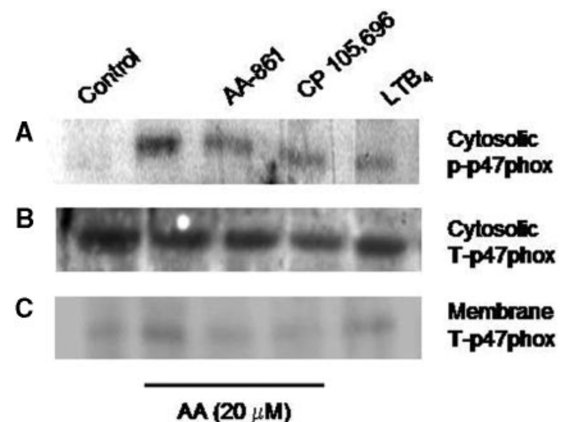

Fig. 7. AA induces phosphorylation and translocation of p47phox in glycogen-elicited rat PMNs in a 5-LO- and BLT1-dependent manner. PMNs (4×10^6/well) were pretreated or not with CP105,696 or AA-861, stimulated by 20 μM AA or 10 nM LTB_4 for 5 min, and then harvested and fractionated as described in Materials and Methods. The cytosolic fraction was immunoprecipitated, and immunoprecipitated protein was immunoblotted using antiphosphoserine (1:900), and phosphorylated protein is designated p-p47phox (A). The total cytosolic fraction (B) and membrane fraction (C) were probed for total (T) p47phox (1:500). Experiment is representative of a total of two.

the capacity of LTB_4 to promote NADPH oxidase activation, we stimulated PMNs with 10 nM LTB_4 and observed phosphorylation and membrane translocation of p47phox. This finding therefore extends to PMNs what we have recently demonstrated with LTB_4 in alveolar macrophages [31]. Together, these results suggest that LTB_4 is partially responsible for the NADPH oxidase activation induced by AA, through effects on phosphorylation as well as membrane translocation of p47phox.

DISCUSSION

This study establishes a role for LTB_4 in NADPH oxidase activation by exogenous PUFAs in elicited rodent PMNs. Specifically, our results show that AA and LA are able to induce secretion of H_2O_2 in elicited PMNs; NADPH oxidase is the major source of H_2O_2 in PUFA-stimulated PMNs; PUFA-induced H_2O_2 generation is dependent on 5-LO activity and LTB_4 biosynthesis, and effects of LTB_4 are mediated by the BLT1 receptor; and LTB_4 contributes to p47phox phosphorylation and membrane translocation in AA-stimulated PMNs.

PUFAs are involved in inflammatory conditions, such as modulation of vascular contraction, chemotaxis, cell adhesion and diapedesis, cell activation, and cell death [44]. AA is a pleiotropic lipid involved in inflammatory and physiological situations. A role for AA in inflammation has been suggested by studies showing that AA increases chemotaxis, granule release, and effector functions such as phagocytosis, microbicidal activity, and release of ROI in PMNs [45]. The role of AA as a second messenger [2] and its ability to activate NADPH oxidase are well-known [4, 5, 12–19]. In addition, AA is the precursor of bioactive lipids including prostanoids and LTs, which are themselves able to induce cell activation. Upon activation, the major AA metabolites produced by PMNs are LTB_4 and 5-hydroxy-5-HETE [32], but the respective roles of AA and its metabolic products in cell signaling are incompletely understood. Thus, we sought to evaluate the role of these 5-LO products in AA and LA stimulation of NADPH oxidase activation.

We first asked if PUFAs activate NADPH oxidase by themselves or via formation of metabolic products. As expected [18, 19], both PUFAs were able to induce H_2O_2 release dose-dependently to a similar extent at concentrations similar to those observed previously. To address the role of 5-LO metabolites in AA- or LA-stimulated PMNs, we inhibited 5-LO or FLAP (both essential for LT synthesis [46]), and we observed an inhibition in H_2O_2 production of ~40% when compared with the untreated control. This effect was confirmed using cells obtained from genetically 5-LO-deficient mice. Although ROI can be generated by a variety of enzymatic sources [47], the ability of the NADPH oxidase-like flavoprotein inhibitor DPI to completely attenuate ROI production in AA- or LA-stimulated PMNs suggests that PUFA effects occurred exclusively through activation of NADPH oxidase.

Our results strongly suggest that AA and LA act to increase 5-LO activity, which results in NADPH oxidase activation. These results are consistent with studies in AA-stimulated guinea pig eosinophils pretreated with the AA congener eicosatetraynoic acid (ETYA), which prevents the further metabolism of AA [48]. By contrast, Pompeia et al. [38] demonstrated that O_2^- production by HL-60 cells stimulated with AA was independent of 5-LO metabolites. Regarding the dependence on AA metabolites observed with exogenous LA stimulation, Alzoghaibi et al. [49] showed that LA-induced interleukin-8 production in human intestinal smooth muscle cells was dependent on 5-LO and cyclooxygenase metabolites. To our knowledge, however, this is the first report showing that LA induction of NADPH oxidase activity depends on 5-LO metabolites. That endogenous 5-LO activity is important for activating NADPH oxidase has been demonstrated in PMNs stimulated by myriad agonists. Maridonneau-Parini et al. [50] showed that opsonized zymosan, but not PMA, induced O_2^- generation in a manner dependent on 5-LO and cyclooxygenase products in human PMNs. Furthermore, it was observed that 5-LO metabolites are necessary for NADPH oxidase activation in PMNs stimulated by platelet-activating factor, formyl-Met-Leu-Phe, PMA, and A23187 [51]. Although the role of 5-LO metabolites in NADPH oxidase activation is recognized, the importance of individual LTs in PUFA-induced NADPH oxidase activation is not. When we pretreated PMNs with BLT1 or cysLT1 receptor antagonists followed by stimulation with AA or LA, we observed that only the BLT1 antagonist abolished the H_2O_2 secretion in PUFA-stimulated PMNs. Likewise, only LTB_4, but not cysLTs, was capable of stimulating secretion of H_2O_2 in PMNs. To confirm that the effects of PUFAs were dependent on LTB_4 interaction with BLT1, we performed "add back" experiments. The first approach was to pretreat cells with AA-861 and then add AA with or without LTB_4. Our experiments clearly show that in 5-LO-inhibited cells, the addition of LTB_4 restored the PMN ability to secrete H_2O_2. We confirmed that the LTB_4 effects are mediated by interaction with BLT1, as LTB_4 addition was ineffective in BLT1 antagonist-treated cells. At lower (i.e., 5 μM) AA concentrations more relevant to those produced endogenously, which might function as second messengers, we observed an even more prominent dependence on 5-LO products and BLT1 in AA-stimulated PMNs. This could reflect the ability of lower doses of AA to activate classical PKC [11] or Ca^{++} release [9], both of which are known to be important in the activation of 5-LO [46].

Results with 5-LO inhibitors and 5-LO KO cells as well as a BLT1 antagonist implicate 5-LO metabolism/BLT1 signaling in NADPH oxidase activation by AA and LA. However, we could only measure immunoreactive LTB_4 production in PMNs stimulated with AA and not with LA. These results are consistent with an alternative LT derivative of LA other than LTB_4 interacting with BLT1 to affect NADPH oxidase, but we are aware of no precedent regarding such a possibility. It will be of interest to clarify this in future studies.

That PMNs respond predominantly to LTB_4, but not cysLTs, is in agreement with previous findings from our laboratory and others. Mancuso et al. [52] showed that LTB_4, but not cysLTs, increased phagocytosis of opsonized *Klebsiella pneumoniae* in human PMNs. In addition, Palmblad et al. [53] showed that only LTB_4 induced NADPH oxidase activation in eosinophils and PMNs. However, Larfars et al. [42] demonstrated that LTB_4 and cysLTs were able to induce nitric oxide production in human PMNs. In our experimental system, a role for 5-LO

products in PUFA stimulation of NADPH oxidase was confined to LTB_4 and its interaction with BLT1.

Although AA promotes p47phox translocation in human monocytes, its ability to enhance phosphorylation of this molecule has been the subject of conflicting reports [19]. In eosinophils, LTB_4 stimulated H_2O_2 production independent of AA release [29], and this effect was dependent on PKC, PLC, and Ca^{++} [30]. The molecular mechanism by which LTB_4 activates NADPH oxidase activation in PMNs is unclear. Thus, we sought to determine the effects of LTB_4 on p47phox phosphorylation and translocation in AA-stimulated PMNs. Our results show that inhibition of 5-LO metabolism or antagonism of BLT1 decreased phosphorylation and translocation of p47phox in AA-stimulated cells and that exogenous LTB_4 was able to increase its phosphorylation and translocation. We have observed similar effects of LA. Together, our results suggest that LTB_4 contributes to activation of the p47phox cytosolic component of NADPH oxidase in PUFA-stimulated PMNs.

Activation of p47phox is regulated by the action of a variety of kinases, including PKC [43]. PUFAs act as second messengers, in part, by stimulating these kinases [2]. Our present data are the first to implicate 5-LO products in PUFA-induced p47phox activation. The plausibility of such a role for LTB_4 is emphasized by our recent demonstration in macrophages that LTB_4 elicits activation of p47phox via effects on the upstream kinase PKC-δ [31].

In conclusion, our experiments show that n-6 PUFAs activate NADPH oxidase in glycogen-elicited PMNs, and this activation is partially dependent on 5-LO activity, LTB_4 synthesis, and BLT1 signaling. Indeed, we demonstrated that LTB_4 is required for two essential aspects of NADPH oxidase activation, namely, phosphorylation and translocation of p47phox, in PMNs stimulated by AA. This study presents a new model of NADPH oxidase activation by PUFAs, and the identification of a role for LTB_4 suggests possible therapeutic interventions targeting this lipid mediator for inflammatory conditions in which ROI production is responsible for tissue injury.

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