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5-Lipoxygenase plays an essential role in 4-HNE-enhanced ROS production in murine macrophages via activation of NADPH oxidase

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Abstract

4-Hydroxynonenal (HNE) mediates oxidative stress-linked pathological processes; however, its role in the generation of reactive oxygen species (ROS) in macrophages is still unclear. Thus, this study investigated the sources and mechanisms of ROS generation in macrophages stimulated with HNE. Exposure of J774A.1 cells to HNE showed an increased production of ROS, which was attenuated by NADPH oxidase as well as 5-lipoxygenase (5-LO) inhibitors. Linked to these results, HNE increased membrane translocation of p47phox promoting NADPH oxidase activity, which was attenuated in peritoneal macrophages from 5-LO-deficient mice as well as in J774A.1 cells treated with a 5-LO inhibitor, MK886 or 5-LO siRNA. In contrast, HNE-enhanced 5-LO activity was not affected by inhibition of NADPH oxidase. Furthermore, leukotriene B_4, 5-LO metabolite, was found to enhance NADPH oxidase activity in macrophages. Altogether, these results suggest that 5-LO plays a critical role in HNE-induced ROS generation in murine macrophages through activation of NADPH oxidase.

Keywords: *4-Hydroxynonenal, 5-lipoxygenase, reactive oxygen species, NADPH oxidase*

Abbreviations: *AA, arachidonic acid; COX, cyclooxygenase; DPI, diphenyleneiodonium; HNE, 4-hydroxynonenal; 5-LO, 5-lipoxygenase; LT, leukotriene; NAC, N-acetylcysteine; NDGA, nordihydroguaiaretic acid; ROS, reactive oxygen species.*

Introduction

An increasing amount of evidence indicates that endogenous aldehydes generated during lipid peroxidation are causally involved in the pathogenesis of a large number of inflammatory and degenerative processes including atherosclerosis [1–3]. Among the lipid-derived aldehydes, 4-hydroxynonenal (HNE) is a major by-product of oxidative breakdown of membrane polyunsaturated fatty acids [4]. Because of its stability and high reactivity, HNE has been proposed to be responsible for the pathophysiological consequences of oxidative stress [5].

In phagocytes, large amounts of ROS are produced during the oxidative burst by plasma membrane NADPH oxidase which consists of a membrane-associated cytochrome b558, composed of p22phox and gp91phox, and cytosolic sub-units, p47phox, p67phox, and rac [6,7]. In addition, lipoxygenase (LO) is known as a key enzyme in the generation of ROS as a by-product during the oxidation step of arachidonic acid (AA) [8,9] and 5-LO activity is also involved in the production of intracellular ROS [10,11]. Although it has been suggested that HNE provides a link between oxidative stress and vascular pathophysiology, the source and mechanisms involved in HNE-induced ROS generation in macrophages are unknown.

NADPH oxidase is activated by a variety of stimuli such as AA and tumour necrotic factor-α [12,13], as

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well as G-protein coupled receptor ligands [14]. Products of the phospholipase A_2 (PLA_2)/LO pathway such as leukotrienes (LT) may also participate in ROS generation [15–17], which may be mediated through NADPH oxidase activity [18]. In addition, LTB_4 was reported to activate the NADPH oxidase through enhanced phosphorylation with subsequent membrane translocation of p47phox in rat alveolar macrophages [19,20]. On the basis of our previous report showing that HNE activated 5-LO in murine macrophages through p38MAPK-mediated translocation of 5-LO from the cytosol to the nuclear membrane [21], it was postulated that HNE might activate NADPH oxidase and subsequently generate ROS in murine macrophages through activation of 5-LO pathways. Thus, in the present study, we investigated the roles of HNE on NADPH oxidase activation with subsequent ROS production in J774A.1 macrophages. Furthermore, the interaction of NADPH oxidase with 5-LO and 5-LO metabolites in HNE-stimulated macrophages was also determined.

Methods

Chemicals and antibodies

HNE, U-75302, REV-5901, MK571, LTB_4, LTC_4 and LTD_4 were purchased from Cayman Chemical Co. (Ann Arbor, MI). 2',7'-Dichlorofluorescein diacetate (DCFH-DA), polyethylene (PEG)-catalase, protein A agarose, lucigenin, diphenyleneiodonium (DPI), NADPH, nordihydroguaiaretic acid (NDGA) and arachidonic acid (AA) were from Sigma Chemical Co. (St. Louis, MO). Apocynin was supplied by Calbiochem (La Jolla, CA). MK886, baicalein and anti-phosphoserine were obtained from Biomol (Plymouth Meeting, PA). Anti-p47phox and anti-CD11b (Integrin *a*M) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and isolation of mouse peritoneal macrophages

J774A.1 macrophages (a murine macrophage cell line; ATCC, Rockvill, MD) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and antibiotic–anti-mycotic at 37°C in 5% CO_2. After reaching confluence, the cells were detached from the surface of T75 culture flasks by gentle scraping. The detached cells were then washed and resuspended in complete medium. Cells between passages 2–5 were used for experiments.

All animal procedures were performed in accordance with the institutional guidelines for animal research and were approved by the institutional animal care and use committee. At 4 days after intraperitoneal injection of 3% thioglycollate, mouse peritoneal macrophages (MPM) were harvested from 5-LO deficient mice (B6.129S2-Alox5tm1Fun/J; Jackson Laboratories, Bar Harbor, ME) and C57BL/6J wild-type controls (C57BL/6J; Jackson Laboratories) and confirmed by CD11b staining and morphology. MPM were maintained in RPMI 1640 medium supplemented with 10% FBS until the experiments were performed.

Quantitating ROS generation

Changes in intracellular ROS levels were evaluated by measuring the oxidative conversion of DCFH-DA to fluorescent DCF as described previously [22]. Cells grown in 12-well plates were loaded with 10 μM DCFH-DA for 30 min at 37°C and then incubated with HNE under the indicated conditions. The cells were washed with PBS and harvested by gentle scraping. DCF fluorescence in 10 000 cells was detected by a FACScaliber flow cytometer (Becton Dickinson, San Jose, CA). The results were obtained as histogram plots of cell number vs fluorescence intensity (FL-1) and the mean fluorescence for each sample within an experiment was analysed using the CellQuest Software (ver 3.3, Becton Dickinson). To exclude the potential non-specific fluorescence of DCF, we tested the effect of PEG-catalase (500 U/ml) to blunt the HNE-induced DCF signal. The PEG-catalase-inhibitable fluorescence was regarded as a ROS-evoked signal and results were expressed as percentage change of control.

Quantitating NADPH oxidase activity

NADPH oxidase activity was measured using a lucigenin-enhanced chemiluminescence assay as described previously [23]. Briefly, cells were washed with PBS and harvested. After low spin centrifugation, the pellet was lysed in phosphate buffer (20 mM monobasic potassium phosphate [pH 7.0], 1 mM EGTA, 10 μM aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin and 0.5 mM phenylmethlysulphonyl fluoride [PMSF]). The cellular lysates were centrifuged for 10 min at 13 000 rpm and the supernatant was used for the assay. The total protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma Chemical Co.). The reaction mixture comprised a Krebs/HEPES buffer, pH 7.0, lucigenin (5 μM) as the electron acceptor and NADPH (100 μM) as substrate. The reaction was initiated by the addition of 25 μg protein and photon emission was measured every second for 10 min in a microtiterplate luminometer (Microlumat LB96P, EG and G Berthold, Germany). The activity was expressed as relative light units (RLU) per second per milligram of total protein.

Preparation of siRNA and in vitro transfection

We designed 5-LO siRNA based on the sequence of Mus musculus arachidonate 5-LO mRNA (PubMed, GenBank™; accession number: NM-009662). p47phox siRNA was the same as oligonucleotide of p47 phox siRNA published by Friis et al. [24]. 5-LO siRNA;

5'-CAU ACU CGC AGA UAA GCU GUU CCC G-3' (sense) and 5'-CGG GAA CAG CUU AUC UGC GAG UAU G-3' (anti-sense) and p47 phox siRNA; 5'-UAA CGU AGC UGA CAU CAC A-3' (sense) and 5'-UGU GAU GUC AGC UAC GUU A-3' (anti-sense) were synthesized by Invitrogen (Carlsbad, CA). The siRNA negative control duplex (Invitrogen) was used as the control oligonucleotide. The siRNA or negative control oligonucleotide was transfected into J774A.1 macrophages using Lipofectamine^{TM} 2000 (Invitrogen).

Quantitation of LTB_4 production

LTB_4 production was measured in cell-free supernatants using a commercially available LTB_4 assay kit (R&D Systems, Minneapolis, MN). Briefly, after macrophages were stimulated with HNE (10 μM) in the presence of exogenous AA (40 μM), the conditioned media was harvested. LTB_4 in the conditioned media was quantified using ELISA (Bio-Tek Instrument Inc., Winooski, VT) following the manufacturer's instructions.

Preparation of cellular fractionation and immunoprecipitation

To determine membrane translocation of p47phox, J774A.1 macrophages were washed with PBS and collected by scrapping. After centrifugation at 1000 g for 5 min, the pellets were lysed in lysis buffer (12.5 mM Tris, 2 mM EGTA, 25 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 μM PMSF, 10 μM aprotinin, 0.5 μg/mL leupeptin) and centrifuged at 100 000 g for 60 min at 4°C. The resulting pellets were resuspended with lysis buffer containing 1% Triton X-100 and used as the membrane fraction. The supernatants were used as the cytosolic fraction.

To determine phosphorylation of p47phox, immunoprecipitation was performed as described by Serezani et al. [19] with some modification. Protein A agarose was incubated with p47phox antibody overnight at 4°C. The cytosolic fractions were mixed with p47phox antibody (conjugated Protein A agarose) and incubated with rotation for 6 h at 4°C. Immunoprecipitates were washed with lysis buffer three times and added with sample buffer. Samples were immunoblotted using anti-phosphoserine.

Immunoblot analysis

Cell lysates containing equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (Hybond, Amersham Biosciences), which was then incubated with specific antibodies. Horseradish peroxidase (HRP)-conjugated IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Visualization of the blot was performed with the Supersignal west dura extended duration substrate kit (Pierce Chemical, Rockford, IL). The blots were scanned by ScanJet 4C (Hewlett-Packard, Palo Alto, CA) and analysed using UN-SCAN-IT GEL^{TM} (Version 5.1, Silk Scientific Inc., UT).

Immunofluorescence analysis

Macrophages plated onto glass coverslips were fixed with methanol and non-specific binding sites were blocked with 10% normal donkey serum. The fixed cells were incubated with specific antibodies. Cells were washed in PBS and then incubated with Cy3-conjugated IgG (Zymed Laboratries; Invitrogen, Carlsbad, CA). The DNA was stained with 0.1 μg/ml diamidino-2-phenylindole (DAPI) in PBS for 3 min at room temperature. The cells were mounted in carbonate-buffered glycerol and evaluated using a laser scanning confocal microscope (LSM 510, Carl Zeiss Inc., Germany).

Statistical analysis

The results were expressed as means ± SEM. Statistical significance was estimated by Student's *t*-test for unpaired observations between two groups or by ANOVA with Bonferroni correction for comparisons of multiple groups. $p < 0.05$ was regarded as significant.

Results

NADPH oxidase and 5-LO were required for HNE-induced ROS generation

To examine the dependence of an increased DCF fluorescence in HNE-stimulated macrophages on ROS, we determined the role of PEG-catalase, a cell membrane-permeable catalase, to blunt the HNE-enhanced DCF fluorescence. The increased DCF fluorescence in HNE-stimulated cells was markedly attenuated by treatment with PEG-catalase (500 U/ml) (Figures 1A and B), supporting a contribution of ROS to HNE-evoked DCF fluorescence.

To determine the enzymatic source of HNE-induced ROS production in murine macrophages, the effects of various inhibitors on HNE-induced ROS production were investigated. Our data showed that pre-treatment with DPI, apocynin, NDGA and MK886, but not inhibitors for other pro-oxidant enzymes including xanthine oxidase (100 μM allopurinol), cyclooxygease (100 μM indomethacin) and mitochondrial oxidases (1 μM rotenone plus stigmatellin) (data not shown), completely abolished ROS production by HNE (Figure 1C). These results suggested that both NADPH oxidase and 5-LO were essential for HNE-induced generation of ROS in macrophages.

Identification of HNE as an NADPH oxidase activator

Because p47phox was phosphorylated and subsequently translocated to the membrane during the

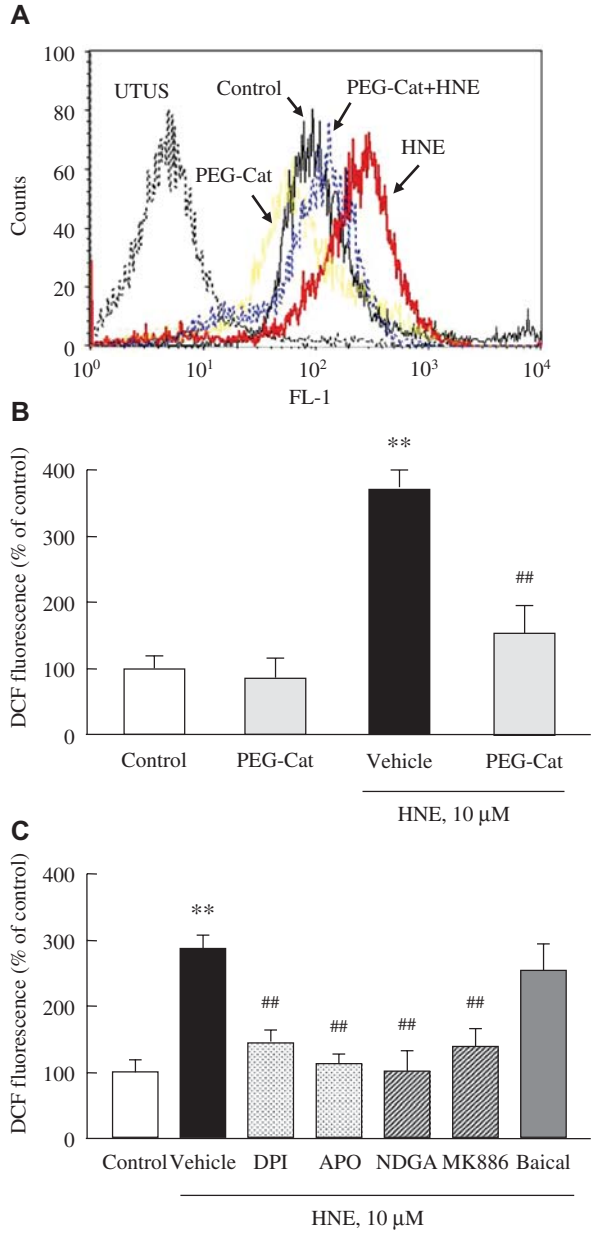

Figure 1. ROS production by HNE in J774A.1 macrophages. (A) The histograms are representatives of HNE-enhanced DCF fluorescence analysed by flow cytometer (FACS). Cells were treated with 10 μM HNE for 45 min in the absence or presence of PEG-catalase (Cat, 500 U/ml) and PEG-catalase-inhibitable DCF fluorescence was regarded as ROS-evoked signal. FL-1, the fluorescence intensity of DCF; UTUS, untreated and unstimulated. (B) The PEG-catalase-inhibitable DCF fluorescence of A was represented as percentage changes of control. (C) Cells were pre-treated with indicated inhibitors (apocynine (APO, 100 μM), nordihydroguaiaretic acid (NDGA, 10 μM), MK886 (10 μM) or baicalein (Baical, 10 μM)) and then stimulated with HNE (10 μM) for 45 min. DCF fluorescence was determined by FACS and the PEG-catalase-inhibitable DCF fluorescence was represented as percentage changes of control. Data were presented as means ± SEM from six independent experiments. $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs vehicle.

initiating step of NADPH oxidase activation, we investigated the effects of HNE on phosphorylation and translocation of 47phox from the cytosol to the membrane. Immunofluorescence and Western blot analyses demonstrated that p47phox was localized mainly in the cytosol of control cells. In cells stimulated with HNE, an increase in the membrane fraction of p47phox was detectable at 15 min and maintained high up to 60 min in association with an increased phosphorylation of p47phox (Figures 2A and B). In line with these results, NADPH oxidase activity in HNE-treated macrophages was also markedly increased in a time-dependent manner (Figure 2C).

Role of 5-LO in HNE-enhanced NADPH oxidase activity

When macrophages were stimulated with HNE in the presence of MK886, a 5-LO inhibitor, both HNE-induced membrane translocation of the p47phox and NADPH oxidase activity were markedly attenuated (Figures 3A and C). Furthermore, NADPH oxidase activity with a concomitant increase in membrane translocation of the p47phox was significantly reduced in cells transfected with 5-LO siRNA (Figures 3B and D). Our results suggested that 5-LO played a pivotal role in HNE-induced activation of NADPH oxidase.

HNE-enhanced ROS generation and NADPH oxidase activity were abolished in 5-LO-deficient macrophages

In a FACS analysis, HNE significantly increased intracellular ROS levels in MPM from wild type mice, which was reduced by apocynin, a NADPH oxidase inhibitor as well as by MK886, a 5-LO inhibitor. However, although PMA enhanced ROS generation in 5-LO-deficient cells, HNE failed to increase ROS levels in MPM from 5-LO-deficient mice. These results suggested that the HNE-enhanced production of ROS was largely dependent on the activity of 5-LO (Figure 4A). Furthermore, in contrast to the dose-dependent increase in NADPH oxidase activity by HNE in MPM from wild type mice, NADPH oxidase was not activated by HNE in MPM from 5-LO-deficient mice (Figure 4B).

Role of NADPH oxidase in HNE-enhanced 5-LO activity

As shown in Figure 5A, HNE enhanced LTB_4 production, a marker for 5-LO activity, in murine macrophages. Although the HNE-enhanced LTB_4 production was markedly reduced by MK886 in a concentration-dependent manner, apocynin, an NADPH oxidase inhibitor, failed to attenuate the increase in HNE-enhanced LTB_4 production. Furthermore, HNE-induced 5-LO product formation was not affected by transfection of cells with p47phox

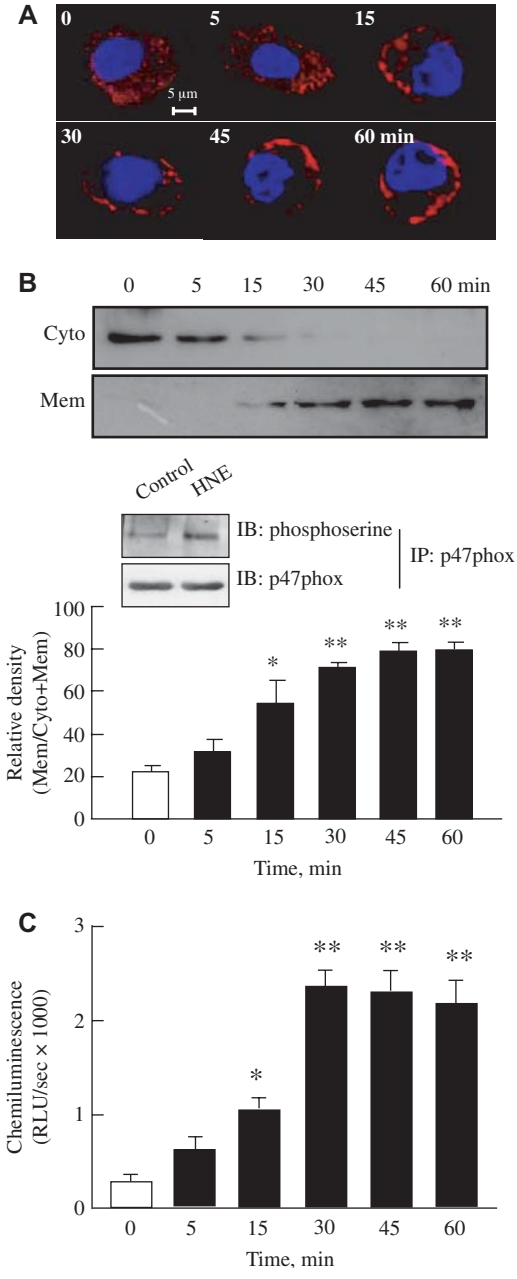

Figure 2. Time course of NADPH oxidase activity by HNE in J774A.1 macrophages. Cells were stimulated with 10 μM HNE for the indicated time. (A) Representative photographs for membrane translocation of p47phox. p47phox (red) and DAPI (blue). Scale bars = 5 μm. (B) Representative immunoblots (upper) and averaged data (lower) of p47phox in cytosolic (Cyto) and membrane (Mem) fractions. Relative density was presented as means ± SEM from five independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ vs value at time 0. Inset: Representative immunoblots for phosphorylated p47phox using anti-phosphoserine in immunoprecipitated protein (p47phox) of cytosolic fraction. Cells were stimulated with 10 μM HNE for 5 min. (C) NADPH oxidase activity was determined in cell lysates by lucigenin-enhanced chemiluminescence assay. Relative light units (RLU) were presented as means ± SEM from six independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ vs value at time 0.

siRNA (Figure 5B). These results suggest that NADPH oxidase was not involved in the regulation of 5-LO activity induced by HNE.

LTB_4 activated NADPH oxidase in J774A.1 macrophages

To verify the role of 5-LO metabolites in the regulation of NADPH oxidase activity, we used various LT receptor antagonists such as U-75302 as a LTB_4 receptor antagonist, REV-5901 as a CysLTs receptor antagonist and MK-571 as a LTD_4 receptor antagonist. Pre-treatment of the cells with MK-571 failed to prevent the increase in HNE-enhanced NADPH oxidase activity, whereas U-75302 and REV-5901 significantly attenuated the increase in NADPH oxidase activity by HNE (Figure 6A). In line with these results, LTB_4 and LTC_4, but not LTD_4, directly activated NADPH oxidase in murine macrophages in a concentration-dependent manner. Interestingly, the effects of LTB_4 on the activation of NADPH oxidase were greater than that of LTC_4 (Figure 6B).

Discussion

The present study demonstrated that HNE increased the activity of NADPH oxidase in J774A.1 macrophages in association with an increased generation of ROS, which was attenuated by inhibition of 5-LO. In addition, whereas HNE-enhanced 5-LO activity was not affected by inhibition of NADPH oxidase, HNE-induced NADPH oxidase activity was significantly attenuated in peritoneal macrophages from 5-LO-deficient mice as well as in cells treated with 5-LO inhibitors. This suggested that 5-LO metabolites played a critical role in mediating ROS generation in HNE-stimulated macrophages through the activation of NADPH oxidase.

Consistent with other reports showing that lipid peroxidation products including HNE and other reactive aldehydes stimulated ROS formation in various types of cells [25–27], a significant increase in ROS generation in macrophages was observed at 45 min after HNE treatment. Although ROS in macrophages is reportedly generated by a variety of enzymatic sources [28], our study showed that HNE-induced generation of ROS was attenuated by inhibitors of either NADPH oxidase or 5-LO. Although DPI, an NADPH oxidase inhibitor, has non-specific actions such as inhibition of xanthine oxidase and mitochondrial complex I [29,30], it was suggested that HNE-induced ROS generation in macrophages occurred exclusively through activation of both NADPH oxidase and 5-LO because inhibitors for xanthine oxidase and mitochondrial complexes had no effects on the action of HNE. Furthermore, considering the facts that both these enzyme inhibitors had similar magnitudes of inhibition during ROS generation, it was also suggested that there was an interaction between these two enzymes in ROS generation in macrophages.

Previously, it was reported that a deficiency of NADPH oxidase reduced 5-LO expression and decreased LT

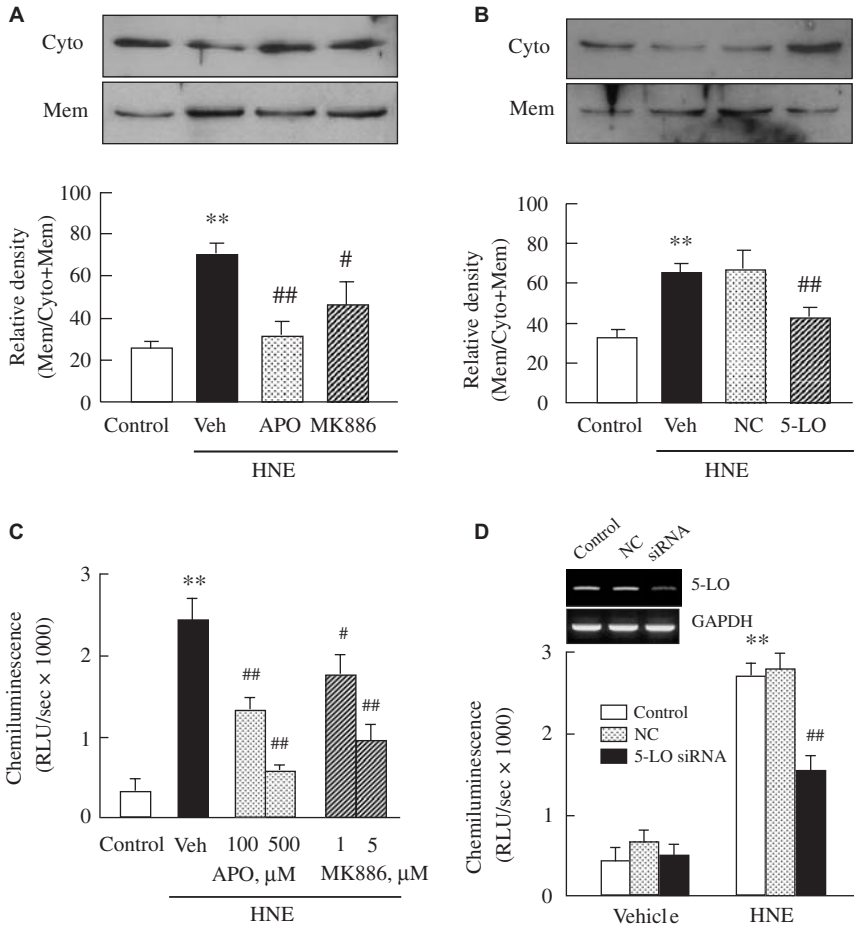

Figure 3. Role of 5-LO on HNE-enhanced NADPH oxidase activity in J774A.1 macrophages. Cells were pre-treated with apocynine (APO, 100 μM in (A), 100 and 500 μM in (C)) or MK886 (1 μM in (A), 1 and 5 μM in (C)) or transfected with 5-LO or negative control (NC) siRNA oligonucleotides and then stimulated with HNE (10 μM) for 30 min. (A) and (B) Representative immunoblots (upper) and averaged data (lower) of p47phox in both cytosolic (Cyto) and membrane (Mem) fractions. Relative density was presented as means ± SEM from five-to-six independent experiments. $^{**}p < 0.01$ vs control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs vehicle (Veh). (C) and (D) NADPH oxidase activity was quantified by chemiluminescence assay. Relative light units (RLU) were presented as means ± SEM from five-to-six independent experiments. $^{**}p < 0.01$ vs control (C) or control in vehicle (D), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs vehicle (C) or corresponding vehicle (D). Inset in (D) shows representative immunoblots for 5-LO in cells transfected with 5-LO siRNA.

synthesis in alveolar macrophages [31]. However, in the present study using J774A.1 macrophages, whereas the HNE-enhanced 5-LO activity was not affected by regulation of NADPH oxidase activity, HNE-enhanced NADPH oxidase activity was markedly attenuated by inhibition of 5-LO in macrophages. Thus, we asked if HNE activated NADPH oxidase by itself or via formation of 5-LO metabolic products. To address this question, we inhibited the formation of 5-LO metabolites using a pharmacological inhibitor such as a FLAP inhibitor as well as a molecular approach using siRNA oligonucleotides. We observed an inhibition in HNE-induced membrane translocation of p47phox with a subsequent inhibition of NADPH oxidase activity. Furthermore, in line with the report by Serezani et al. [19] showing that stimulation of neutrophils from 5-LO-deficient mice with AA showed a reduced level of ROS production compared to wild type mice, our results showed that HNE failed to increase ROS generation in peritoneal macrophages from 5-LO-deficient mice. Based on the previous reports in which angiotensin II increases 5-LO activity with a subsequent production of LTB_4, which then activates NADPH oxidase, leading to ROS generation in VSMC [18,32], it was therefore suggested that HNE increased ROS generation in macrophages, which resulted from NADPH oxidase activation via 5-LO metabolites.

AA is converted to LT by 5-LO and the major products formed in the neutrophils are 5-HETE and LTA_4, the precursor of LTB_4 and cysteinyl LT (CysLT) such as LTC_4, LTD_4 and LTE_4 [33–35]. In contrast to prostaglandin F_{2a} (PGF_{2a}), a COX metabolite, which stimulate NADPH oxidase in vascular smooth muscle cells through transcriptional upregulation of NOX-1, a sub-unit of NADPH oxidase [36,37], LTB_4 activated NADPH oxidase in neutrophils and macrophages through translocation of p47phox to the membrane, a process that was dependent on PKC activity [19,20]. These observations suggest a short-term and delayed activation of

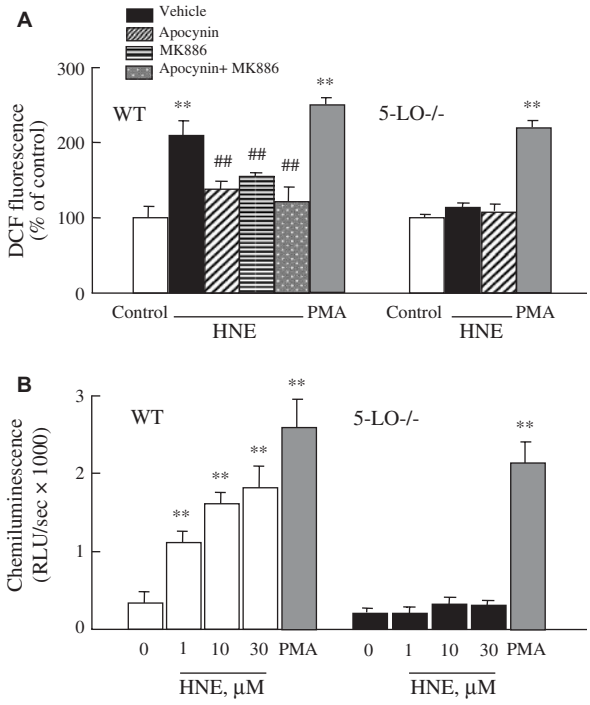

Figure 4. Attenuation of HNE-enhanced ROS production and NADPH oxidase activity in MPM from 5-LO-deficient mice. (A) MPM from wild type (WT) or 5-LO deficient mice (5-LO$^{-/-}$) were stimulated with HNE (10 μM) for 45 min in the absence and presence of apocynin (100 μM), MK886 (5 μM) or a mixture of apocynin and MK886. DCF fluorescence was determined by FACS and the PEG-catalase-inhibitable DCF fluorescence was represented as percentage changes of control. Data were presented as means ± SEM from seven independent experiments. $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs vehicle. (B) MPM was stimulated with the indicated concentrations of HNE for 30 min. NADPH oxidase activity was quantified by chemiluminescence assay. Data were presented as means ± SEM from six independent experiments. PMA (100 nM) was used as a positive control. $^{**}p < 0.01$ vs values at concentration 0.

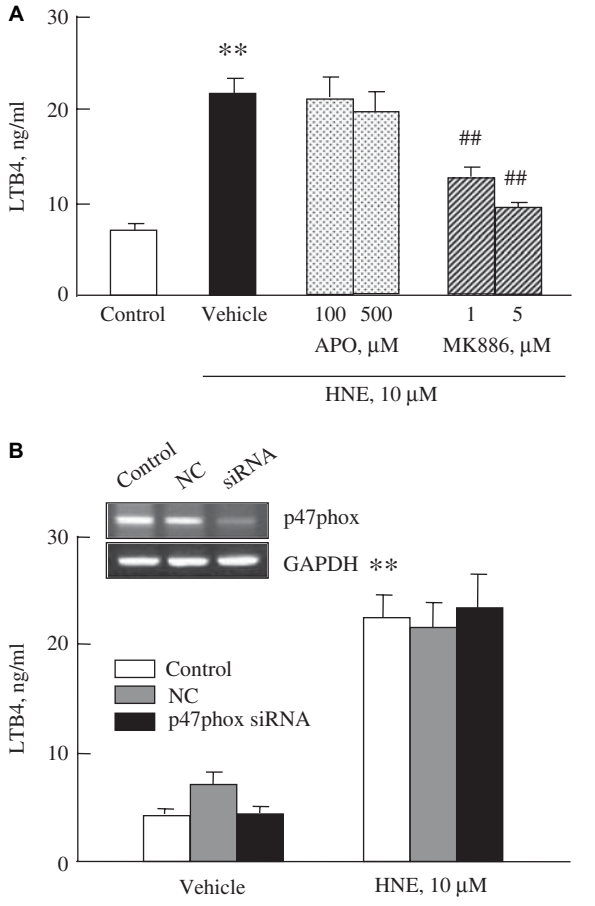

Figure 5. Effect of NADPH oxidase inhibition on HNE-induced LTB_4 production in J774A.1 macrophages. Cells were pre-treated with apocynine (100 and 500 μM) or MK886 (1 and 5 μM) or transfected with p47phox or negative control (NC) siRNA oligonucleotides and then stimulated with HNE (10 μM) for 30 min. The levels of LTB_4 in medium were presented as means ± SEM from five-to-six independent experiments. (A) $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs vehicle. (B) $^{**}p < 0.01$ vs control in vehicle. Inset in (B) shows representative immunoblots for p47phox in cells transfected with p47phox siRNA.

the NADPH-oxidase system by eicosanoids. The short-term activation may involve membrane translocation of cytosolic sub-units by 5-LO-derived LTB_4 formation while the delayed long-term activation may involve a transcriptional up-regulation of the NADPH oxidase sub-units by PGF_{2a}. In this regard, our present study is of particular interest as it shows the HNE-enhanced synthesis and release of LTB_4 in murine macrophages. However, since the non-specific COX inhibitor indomethacin had no influence on ROS formation by HNE, it was suggested that PGF_{2a} formation might not be involved in the early phase of NADPH oxidase activation.

Reportedly, 5-LO metabolites are necessary for NADPH oxidase activation in neutrophils stimulated by platelet-activating factor, formyl-Met-Leu-Phe, PMA and A23187 [38]. Previous reports showed that neutrophils responded predominantly to LTB_4, but not cysLT [39,40]. Consistent with these reports, when macrophages were pre-treated with the BLT_1 or cysLT receptor antagonists followed by stimulation with HNE, an increased NADPH oxidase in HNE-stimulated macrophages was markedly attenuated by a BLT_1 receptor antagonist. Likewise, LTB_4, but not LTD_4, was capable of stimulating NADPH oxidase activity in macrophages. Considering our experimental results with other reports in which LTB_4 activated NADPH oxidase through posphorylation and translocation of p47phox to the membrane [19,20], it has been suggested that HNE enhanced phosphorylation with subsequent translocation of p47phox because LTB_4 was considered as a major activator of NADPH oxidase in HNE-stimulated macrophages.

In conclusion, our experiments showed that HNE activated NADPH oxidase in murine macrophages and that this activation was dependent on 5-LO activity, LTB_4 synthesis and BLT_1 receptor signalling.

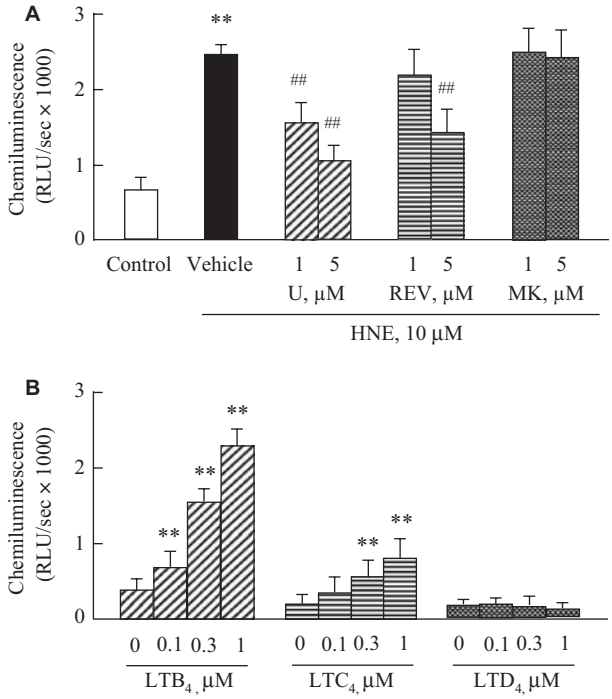

Figure 6. Effect of various leukotrienes on NADPH oxidase activity in J774A.1 macrophages. (A) Macrophages were stimulated with HNE for 30 min in the absence and presence of U-75302 (U), REV-5901 (REV) or MK571 (MK). (B) Cells were incubated with LTB_4, LTC_4 or LTD_4 at the indicated concentrations for 30 min. NADPH oxidase activity was quantified by chemiluminescence assay. Relative light units (RLU) were presented as means ± SEM from five-to-seven independent experiments. (A) $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs vehicle. (B) $^{**}p < 0.01$ vs value at concentration 0.

Indeed, we demonstrated that LTB_4 was required for an essential aspect of NADPH oxidase activation, namely the phosphorylation and translocation of p47phox to the membrane, in macrophages stimulated by HNE. This study presents a new model of NADPH oxidase activation by HNE. Moreover, the identification of a role for LTB_4 in the activation of NADPH oxidase by HNE suggests possible therapeutic interventions targeting this lipid mediator for inflammatory conditions in which ROS production is responsible for vascular pathophysiology.

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