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Original Contribution

Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation

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ABSTRACT

Acute inflammation is a common feature of many life-threatening pathologies, including septic shock. One hallmark of acute inflammation is the peroxidation of polyunsaturated fatty acids forming bioactive products that regulate inflammation. Myeloperoxidase (MPO) is an abundant phagocyte-derived hemoprotein released during phagocyte activation. Here, we investigated the role of MPO in modulating biologically active arachidonic acid (AA) and linoleic acid (LA) metabolites during acute inflammation. Wild-type and MPO-knockout (KO) mice were exposed to intraperitoneally injected endotoxin for 24 h, and plasma LA and AA oxidation products were comprehensively analyzed using a liquid chromatography–mass spectrometry method. Compared to wild-type mice, MPO-KO mice had significantly lower plasma levels of LA epoxides and corresponding LA- and AA-derived fatty acid diols. AA and LA hydroxy intermediates (hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids) were also significantly lower in MPO-KO mice. Conversely, MPO-deficient mice had significantly higher plasma levels of cysteinyl-leukotrienes with well-known proinflammatory properties. In vitro experiments revealed significantly lower amounts of AA and LA epoxides, LA- and AA-derived fatty acid diols, and AA and LA hydroxy intermediates in stimulated polymorphonuclear neutrophils isolated from MPO-KO mice. Our results demonstrate that MPO modulates the balance of pro- and anti-inflammatory lipid mediators during acute inflammation and, in this way, may control acute inflammatory diseases.

Acute inflammation is a common sign of many acute life-threatening pathologies, including septic shock and multiple organ failure [1]. Polymorphonuclear neutrophils (PMNs) play an important role in the pathogenesis of sepsis, producing a wide range of inflammatory mediators. However, the exact mechanisms by which these cells participate in sepsis remain incompletely characterized. Activated PMNs release myeloperoxidase (MPO), an abundant hemoprotein that represents up to 5% of total PMN proteins. MPO is thought to mediate primarily host defense reactions [2–4]. Recent evidence suggests that, aside from its role in host defense, reactive intermediates formed by MPO-catalyzed reactions may modify signaling mediators, leading to alterations in cellular signaling [3,5,6]. During acute inflammation, MPO contributes to vascular dysfunction by nitric oxide catalytic consumption and suppression of nitric oxide bioavailability, an effect that disrupts nitric oxide-dependent signaling pathways [7]. Further, MPO catalyzes posttranslational modification of proteins (e.g., chlorination, nitration, and dityrosine bridge formation), in a way that may affect their structure and function [2,3,8–10]. The ability of MPO to modulate redox-sensitive signaling pathways may point to a role for this protein in regulating the inflammatory process [5,7].

Lipid peroxidation is a characteristic feature of acute inflammation, including inflammation associated with septic shock [11]. Oxidative metabolites of arachidonic acid (AA) and linoleic acid (LA) are potent inflammatory mediators, and an increase in their synthesis generally correlates with the severity of sepsis or trauma [11]. Epoxides of AA and LA are epoxyeicosatrienoic acids (EETs) and epoxyoctadecenoic

Abbreviations: AA, arachidonic acid; DHETE, dihydroxyeicosatrienoic acid; DHOME, dihydroxyoctadecenoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecenoic acid; H(P)ETE, hydroxyeicosatrienoic acid and hydroperoxyeicosatrienoic acid; H(P)ODE, hydroxyoctadecadienoic acid and hydroperoxyoctadecadienoic acid; LA, linoleic acid; LOX, lipoxygenase; LPS, lipopolysaccharide; MPO, myeloperoxidase; MPO-KO, MPO-deficient mice; oxo-EET, oxoepoxyeicosatrienoic acids; oxo-ODE, oxo-octadecadienoic acid; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil; SEM, standard error of the mean.

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acids (EpOMEs). EETs have the ability to decrease inflammatory responses, including fever [12,13]. EETs and EpOMEs are further metabolized by soluble epoxide hydrolase to their corresponding diols, dihydroxyeicosatrienoic acids (DHETEs) and dihydroxyoctadecenoic acids (DHOMEs) [12–16]. In contrast to epoxides, DHETEs possess fewer anti-inflammatory properties and DHOMEs are mostly proinflammatory [13,17]. Other initial products of AA and LA metabolism are hydroperoxyeicosatrienoic acids (HPETEs) and hydroperoxyoctadecadienoic acids (HPODEs), respectively, and their corresponding reduced forms, hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs) [13,18]. H(P)ETEs and H(P)ODEs stimulate both pro- and anti-inflammatory pathways depending on the positional isomer of the metabolite, cell type, and bioactivity levels [18]. Hydroperoxy fatty acids can be converted to specific epoxy leukotrienes (LTs). Primarily, 5-hydroperoxy arachidonate (5-HPETE) is metabolized to the highly unstable epoxide intermediate LTA4. Further, LTB4 is formed from LTA4 or LTA4 conjugates with glutathione to yield LTC4, which is additionally metabolized to LTD4 and LTE4 [18,19]. LTs have long been recognized as potent inducers of the inflammatory response [19].

Cytochrome P450 mono-oxygenases, cyclo-oxygenases, and lipoxygenases (LOXs) are widely recognized as the primary enzymes participating in the formation of biologically active lipid mediators [11,13,18]. However, various heme peroxidases that are structurally similar to the family of cytochrome P450 enzymes may also participate in the metabolism of biologically active lipids [20]. Among these is MPO. The MPO/hydrogen peroxide system or hypochlorous acid (a MPO-derived oxidant) promotes the formation of epoxides, peroxides, and chlorohydrins from polyunsaturated fatty acids and cholesterol in isolated PMNs, high-density lipoprotein particles, or chemical systems [21–33]. Recent studies of MPO-KO mice also show that MPO catalyzes the initiation of lipid peroxidation and lipoprotein oxidation in vivo [8,9,34,35]. However, whether MPO participates in the formation of AA- and LA-derived lipid mediators that are involved in the regulation of the inflammatory response is unclear.

Here, we investigated MPO-dependent modulation of selected biologically active AA and LA metabolites in mice with acute inflammation induced by intraperitoneal (ip) application of lipopolysaccharide (LPS), a noninfectious model of systemic sepsis in mice. We focused on AA and LA metabolites that are generally considered products of cytochrome P450 mono-oxygenase and lipoxygenase pathways. Alterations in a wide spectrum of AA- and LA-derived lipid oxidation products were simultaneously monitored in the plasma of MPO-KO and wild-type mice and isolated PMNs using a unique liquid chromatography–mass spectrometry method [14,15]. Our findings show that MPO modulates the formation of pro- and anti-inflammatory lipid mediators and suggest that this enzyme is a direct systemic regulator of the acute inflammatory response.

Material and methods

Materials

The following AA and LA metabolites were purchased from Cayman Chemicals (Ann Arbor, MI, USA): (±)9(10)-epoxy-12*Z*-octadecenoic acid [9(10)-EpOME], (±)12(13)-epoxy-9*Z*-octadecenoic acid [12(13)-EpOME], (±)5(6)-epoxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid [5(6)-EET)], (±)8(9)-epoxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid [8(9)-EET], (±)14(15)-epoxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid [14(15)-EET], (±)11(12)-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid [11(12)-EET], (±)13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), (±)9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE), 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 19-HETE, 20-HETE, 5,6-DHETE, 8,9-DHETE, 11,12-DHETE, 14,15-DHETE, 9-oxo-10*E*,12*Z*-octadecadienoic acid (9-oxo-ODE), 13-keto-9*Z*,11*E*-octadecadienoic acid (13-oxo-ODE), 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), 15-oxo-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-oxo-ETE), leukotriene B4 (LTB4), LTC4, LTD4, LTE4, *N*-acetylleukotriene E4 (Na-LTE4), and 6-oxo-9α,11α,15*S*-trihydroxyprost-13*E*-en-1-oic-3,3,4,4-d4 acid (6-keto prostaglandin F1α–d4). Lordan Fine Lipids provided (±) 9,10-dihydroxy-12(*Z*)-octadecenoic acid (9,10-DHOME) and (±) 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOME). The remainder of the metabolites were synthesized in-house [12-(3-cyclohexylureido)dodecanoic acid, 10(11)-epoxyheptadecanoic acid, and 10(11)-dihydroxynonadecanoic acid] as described previously [36]. Omni-Solv acetonitrile and methanol were from EM Science (Gibbstown, NJ, USA). All other chemical reagents of a high-performance liquid chromatography grade or better were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Animal experiment

All animal protocols were approved by the Animal Research Committee of the University of California at Davis. MPO-KO mice backcrossed on a C57BL/6J background have been comprehensively characterized elsewhere [37–39]. Age- and sex-matched wild-type C57BL/J6 mice and 4-month-old male MPO-KO mice weighing 22–28 g were given a single ip injection of *Escherichia coli* LPS (10 mg/kg), which was freshly prepared in sterile phosphate-buffered saline (pH 7.4). Control mice received an equivalent amount of sterile phosphate-buffered saline. The LPS murine model of acute systemic inflammation was selected because we have previously observed extensive activation of the P450-epoxygenase/soluble epoxide hydrolase and LOX lipid metabolism pathways in this model [14,15]. Twenty-four hours after LPS administration, the mice received an overdose of pentobarbital (ip), and blood was collected via cardiac puncture with an ethylenediaminetetraacetic acid-rinsed syringe. The plasma was immediately separated, and a combination of triphenylphosphine, ethylenediaminetetraacetic acid (1 mM), and butylated hydroxytoluene (0.2% w/w) was added to stabilize the samples. All samples were stored at less than −70°C until analysis.

In vitro experiments with isolated PMNs

Isolation of mouse neutrophils was performed as described previously, with modifications [40,41]. A sterile solution of casein (2% w/v in phosphate-buffered saline) was injected into the peritoneal cavity of the mouse (1 ml per mouse) to enrich the exudate of PMNs. The mice were euthanized by carbon dioxide 24 h later, and peritoneal lavage was performed using repeated applications of phosphate-buffered saline. The resulting cells were washed twice with ice-cold phosphate-buffered saline (200 *g* for 10 min at 4°C), and contaminating erythrocytes were lysed in 0.45% NaCl. PMNs were purified using Histopaque-1077 and -1119 (800 *g* for 30 min at room temperature), washed three times with ice-cold phosphate-buffered saline (200 *g* for 10 min at 4°C), and resuspended in Hanks' balanced salt solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid. All samples were >93% pure as determined by Diff-Quik staining and had >96% viable PMNs as determined by trypan blue dye exclusion. For determination of AA and LA metabolites, PMN samples were pooled from four or five wild-type or MPO-KO mice to obtain a sufficient amount of PMNs. PMNs (2×10^6) in 1 ml of Hanks' balanced salt solution were preincubated with or without AA (50 μM) and LA (50 μM) at 37°C for 5 min. Stock solutions of AA and LA were in ethanol and the final concentration of ethanol in cell suspension was <0.05%. A subset of samples was incubated for an additional 30 min (control PMNs). Another subset (activated PMNs) was stimulated first with PMA (phorbol 12-myristate 13-acetate; 100 nM) for 5 min and then with the calcium ionophore A23187 (2 μM) for 25 min. Stimulation of samples was

terminated with 0.5 ml ice-cold methanol. A combination of triphenylphosphine, ethylenediaminetetraacetic acid (1 mM), and butylated hydroxytoluene (0.2% w/w) was then immediately added to stabilize the samples. All samples were stored at less than −70°C until analysis.

Oxylipin profile analysis

The analysis was performed as described previously [42–44]. Immediately before solid-phase extraction, 250 μl serum or 1.5 ml PMN suspension was diluted 1:1 (v/v) with 2.5 mM phosphoric acid. Each sample was then spiked with the surrogates 26.7 nM 6-keto prostaglandin F1α–d4, 26.7 nM 10(11)-epoxyheptadecanoic acid, and 26.7 nM 10(11)-dihydroxynonadecanoic acid. Oasis HLB 60-mg cartridges (Waters, Milford, MA, USA) were preconditioned with 2 ml of methanol and 2 ml of solution containing 2.5 mM phosphoric acid and 10% methanol (pH 3.8). After the samples were applied, the cartridges were washed with 2 ml of the 2.5 mM phosphoric acid–10% methanol mixture. The analytes were eluted with 2 ml of ethyl acetate. The ethyl acetate residue was evaporated under nitrogen gas, and the samples were resuspended in 100 μl of methanol containing 26.7 nM internal standard 12-(3-cyclohexylureido)dodecanoic acid. The samples were then vortexed for 5 min, transferred to autosample vials, and stored at −80 °C until analysis. A Waters 2790 separation module equipped with a Luna C18 column (2.0×150 mm, 5 μm; Phenomenex, Torrance, CA, USA) and a Quattro Ultima tandem-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source were used for high-performance liquid chromatography separation and electrospray tandem mass spectrometry, respectively. Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile:methanol (84:16) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 400 μl/min. Chromatography was optimized to separate all analytes in 21 min. The Quattro Ultima tandem-quadrupole mass spectrometer was operated in multiple reaction monitoring modes, and both negative and positive ion electrospray was used as the means of ionization.

Data collection and processing were performed using MassLynx software version 4.0, as described previously [14,15,43,44]. Surrogates were added to samples before extraction to mimic the extraction of AA and LA metabolites. Internal standard was used to verify surrogate recovery and to monitor instrument response. The analytes were linked to their corresponding surrogates for the purpose of quantification. In addition, if the surrogate recoveries, as monitored by internal standards, were not between 80 and 120%, the sample was considered invalid and not included in the dataset. Calibration curves were prepared to generate a series of six calibration points spanning a concentration range from 0.1 to 100 nM for all analytes, with the surrogates and internal standards at a constant concentration of 26.7 nM.

Statistical analysis

Data are expressed as the means ± standard error of the mean (SEM). Groups were compared using an unpaired Student *t* test. Values of *p* less than 0.05 were considered significant.

Results

MPO deficiency suppresses endotoxemia-induced formation of LA epoxides and vicinal dihydroxy metabolites of AA and LA

To model acute inflammation associated with systemic endotoxemia/sepsis, we injected mice ip with LPS and measured AA and LA metabolites 24 h later. In wild-type animals, LPS-induced endotoxemia greatly increased the plasma levels of LA epoxides (EpOMEs) as well as vicinal dihydroxy metabolites of AA and LA (DHETEs and DHOMEs, respectively), confirming that the P450-epoxygenase/soluble epoxide hydrolase lipid metabolism pathways are activated in our noninfectious model of systemic sepsis (Table 1). Interestingly, after induction of sepsis MPO-KO mice had significantly lower plasma levels of 12(13)-EpOME, 9,10-DHOME, and 12,13-DHOME. Septic MPO-KO mice also had significantly lower levels of 5,6-DHETE, 8,9-DHETE, 11,12-DHETE, and 14,15-DHETE. The plasma concentration of another LA epoxide, 9(10)-EpOME, was not significantly different in WT and MPO-KO mice. The legend to Table 1 lists other metabolites belonging to these metabolic pathways that fall below the detection limit in both wild-type and MPO-KO mice.

MPO deficiency suppresses endotoxemia-induced increases in HETEs and HODE

In wild-type mice, endotoxin challenge also significantly increased the formation of lipid metabolites from LOX-catalyzed pathways (Table 2). However, induction of these AA (5-HETE, 11-HETE, 12-HETE, and 15-HETE) and LA (9-HODE and 13-HODE) metabolites was significantly suppressed in MPO-KO mice. The 12-HETE plasma concentrations were approximately 2 orders higher compared to other metabolites and associated with high variability. This was most likely due to contamination from platelets in the isolated plasma samples. The legend to Table 2 lists other metabolites belonging to these metabolic pathways that fall below the detection limit in both wild-type and MPO-KO mice.

Table 1
MPO deficiency suppresses endotoxemia-induced increases in EpOMEs, DHOMEs, and DHETEs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
(±)9(10)-Epoxy-12*Z*-octadecenoic acid [9(10)-EpOME]	3.5 ± 1.0	4.3 ± 0.7	5.5 ± 1.1	3.0 ± 0.5
(±)12(13)-Epoxy-9*Z*-octadecenoic acid [12(13)-EpOME]	6.0 ± 0.9	6.9 ± 1.1	13.0 ± 2.13†	7.4 ± 0.8*
(±)9,10-Dihydroxy-12(*Z*)-octadecenoic acid (9,10-DHOME)	2.7 ± 0.4	4.0 ± 1.0	19.4 ± 4.4†	11.0 ± 1.4*,‡
(±)12,13-Dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOME)	9.1 ± 2.1	12.1 ± 3.8	50.6 ± 9.4†	30.4 ± 3.1*,‡
(±)5,6-Dihydroxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid (5,6-DHETE)	2.3 ± 0.5	1.6 ± 0.5	6.4 ± 1.7†	3.0 ± 0.6*
(±)8,9-Dihydroxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid (8,9-DHETE)	5.0 ± 1.3	4.9 ± 0.3	12.2 ± 1.2†	7.7 ± 1.1*,‡
(±)11,12-Dihydroxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid (11,12-DHETE)	3.2 ± 0.5	3.5 ± 0.3	10.6 ± 1.8†	6.0 ± 0.6*,‡
(±)14,15-Dihydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid (14,15-DHETE)	5.4 ± 0.7	4.5 ± 0.3	15.8 ± 2.1†	7.9 ± 0.8*,‡

Plasma levels (nM) of EpOMEs (epoxides of LA), DHOMEs (dihydroxy metabolites of LA), and DHETEs (dihydroxy metabolites of AA) at 24 h after induction of endotoxemia are shown. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of other determined metabolites including epoxides of AA (EETs) and the degradation products 9-oxo-ODE, 13-oxo-ODE, 5-oxo-ETE, and 15-oxo-ETE were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

* $p<0.05$ MPO-KO vs wild type.

† $p<0.05$ control wild type vs LPS-treated wild type.

‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

Table 2
MPO deficiency suppresses endotoxemia-induced increases in HETEs and HODEs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
(±)5-Hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE)	3.1 ± 0.4	2.2 ± 0.2*	18.4 ± 2.8†	8.0 ± 1.3*,‡
(±)11-Hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid (11-HETE)	1.38 ± 0.34	1.01 ± 0.27	2.39 ± 0.21†	0.89 ± 0.25*
(±)12-Hydroxy-5*E*,8*Z*,10*Z*,14*Z*-eicosatetraenoic acid (12-HETE)	3485 ± 786	3202 ± 374	4555 ± 665	3869 ± 746*
(±)12-Hydroxy-5*E*,8*Z*,10*Z*,14*Z*-eicosatetraenoic acid (15-HETE)	1.24 ± 0.18	1.19 ± 0.08	2.03 ± 0.31†	0.97 ± 0.08*,‡
(±)9-Hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE)	5.5 ± 1.2	5.2 ± 0.9	10.6 ± 0.7†	5.8 ± 1.0*
(±)13-Hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE)	8.4 ± 1.3	7.6 ± 1.3	22.3 ± 2.2†	11.8 ± 1.2*,‡

Plasma levels (nM) of HETEs (AA-derived lipid metabolites) and HODEs (LA-derived lipid metabolites) generated by LOX-catalyzed pathways. HETEs and HODEs were analyzed 24 h after induction of endotoxemia. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of other determined metabolites, including 8-HETE, 9-HETE, 19-HETE, and 20-HETE, were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.
* $p<0.05$ MPO-KO vs wild-type.
† $p<0.05$ control wild type vs LPS-treated wild-type.
‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

MPO deficiency increases endotoxemia-induced formation of LTs

The involvement of the enzyme 5-LOX in AA and LA metabolism is complex, because its primary metabolite 5-HPETE feeds multiple biosynthetic pathways, including pathways for the synthesis of LTs and 5-HETE. In an alternate metabolic pathway, 5-HPETE can undergo stereospecific dehydration to LTA4 by a second 5-LOX-catalyzed step. LTA4 can then be converted to LTB4 or by LTC4 synthase into the cysteinyl-LTs LTC4, LTD4, and LTE4. In contrast to other AA and LA mediators, cysteinyl-LTs were revealed to be higher in the plasma of MPO-KO mice than in the plasma of wild-type mice (Table 3). Levels of LTA4 were not detectable in this study and LTB4 did not differ significantly among WT and MPO-KO mice. However, particularly interesting were significantly higher levels of LTC4, LTD4, LTE4, and LTE4-NA in the plasma of septic MPO-KO mice. LTC4 and LTE4 levels were also higher in control MPO-KO mice than in wild-type mice, suggesting that these cysteinyl-LTs accumulate in MPO-KO mice even in the absence of an acute inflammatory response. This could due to a disturbance of the peritoneum by the ip application of sterile saline in control mice.

Activated PMNs from wild-type mice contain significantly higher levels of AA and LA epoxides, vicinal dihydroxy metabolites, HETEs, and HODEs than those from MPO-KO mice

Knowing that the MPO deficiency affects systemic fatty acid metabolism in septic mice, we evaluated AA and LA mediator profiles in PMNs isolated from MPO-KO and wild-type mice. In these experiments, purified AA (50 μM) and LA (50 μM) were added to a suspension of isolated PMNs, which were then activated by successive exposure to PMA and a calcium ionophore. Samples containing nonactivated PMNs and lacking exogenous AA and LA served as controls. The AA and LA concentrations selected, as well as the use of the combination of PMA and calcium ionophore, were intended to maximize the production of AA and LA mediators by isolated PMNs and were based on preliminary data and literature [41,45,46].

Consistent with our in vivo findings, PMNs isolated from MPO-KO mice formed, in the presence of exogenous AA and LA, significantly lower levels of AA epoxides [5(6)-EET, 8(9)-EET, and 11(12)-EET] and LA epoxides [9(10)EpOME and 12(13)-EpOME)] than wild-type PMNs (Fig. 1). Correspondingly, levels of vicinal diols of AA and LA were significantly lower in MPO-KO PMNs in the presence of exogenous AA and LA (Fig. 2). Concentrations of AA metabolites (5-HETE, 8-HETE, 9-HETE) and LA metabolites (9-HODE and 13-HODE) were significantly lower in PMNs isolated from MPO-KO mice (Fig. 3). Oxo-octadecadienoic acids (9-oxo-ODE and 13-oxo-ODE), which are formed by degradation of unstable LA-derived hydroperoxides (HPODE), were significantly decreased in MPO-KO PMNs in the presence of exogenous AA and LA (Figs. 4A and 4B). The same was true of the AA metabolites oxo-epoxyeicosatrienoic acids (5-oxo-EET and 15-oxo-EET; Figs. 4C and 4D). The complex profile of LTs was not analyzed in the homogenates of PMNs. However, a determination of cysteinyl-LTs in the supernatants of PMNs isolated from wild-type and MPO-KO mice incubated in the absence and presence of PMA for 10, 30, and 120 min showed no significant differences (data not shown).

Table 3
MPO deficiency enhances endotoxemia-induced increases in cysteinyl LTs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
Leukotriene B4 (LTB4)	0.4 ± 0.5	0.5 ± 0.7	16.2 ± 6.8†	20.6 ± 4.3‡
Leukotriene C4 (LTC4)	2.1 ± 1.4	9.9 ± 0.6*	9.2 ± 1.4†	16.3 ± 1.8*,‡
Leukotriene D4 (LTD4)	3.3 ± 2.1	4.1 ± 1.9	6.6 ± 1.6	24.9 ± 4.9*,‡
Leukotriene E4 (LTE4)	0.9 ± 0.3	4.2 ± 0.8*	3.1 ± 0.6†	14.3 ± 1.5*,‡
N-acetylleukotriene E4 (LTE4-NA)	0.1 ± 0.0	0.1 ± 0.0	6.4 ± 0.5†	14.6 ± 4.2*,‡

LT plasma levels (nM) were analyzed 24 h after endotoxemia induction. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of LTA4 were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.
* $p<0.05$ MPO-KO vs wild type.
† $p<0.05$ control wild type vs LPS-treated wild type.
‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

Wild-type and MPO-KO mice have comparable numbers of blood leukocytes and differentiation counts

The numbers of peripheral blood leukocytes and PMNs were determined in wild-type and MPO-KO mice before sepsis and 24 h after induction of sepsis. Both wild-type and MPO-KO mice exhibited a drop in peripheral blood PMNs after endotoxin challenge. However, no significant differences in total leukocyte blood count and differentials were observed (control wild type, $4.31 \pm 0.54 \times 10^9$/L with 22 ± 9% neutrophil granulocytes; control MPO-KO, $4.05 \pm 0.62 \times 10^9$/L with 25 ± 11% neutrophil granulocytes; LPS-treated wild type, $3.22 \pm 0.32 \times 10^9$/L with 35 ± 10% neutrophil granulocytes; and LPS-treated MPO-KO, $3.48 \pm 0.46 \times 10^9$/L with 34 ± 12% neutrophil granulocytes). Eosinophilic polymorphonuclear granulocytes were under 2% and basophilic polymorphonuclear granulocytes were not found. Similarly, other authors have not found any significant differences in the numbers of leukocytes and PMNs between wild-type and MPO-KO mice in various inflammatory models [35,39,47].

Discussion

Our results illustrate the significance of MPO in the formation of biologically active metabolites of AA and LA. Data suggest that during acute inflammation, MPO plays an important role in the formation of

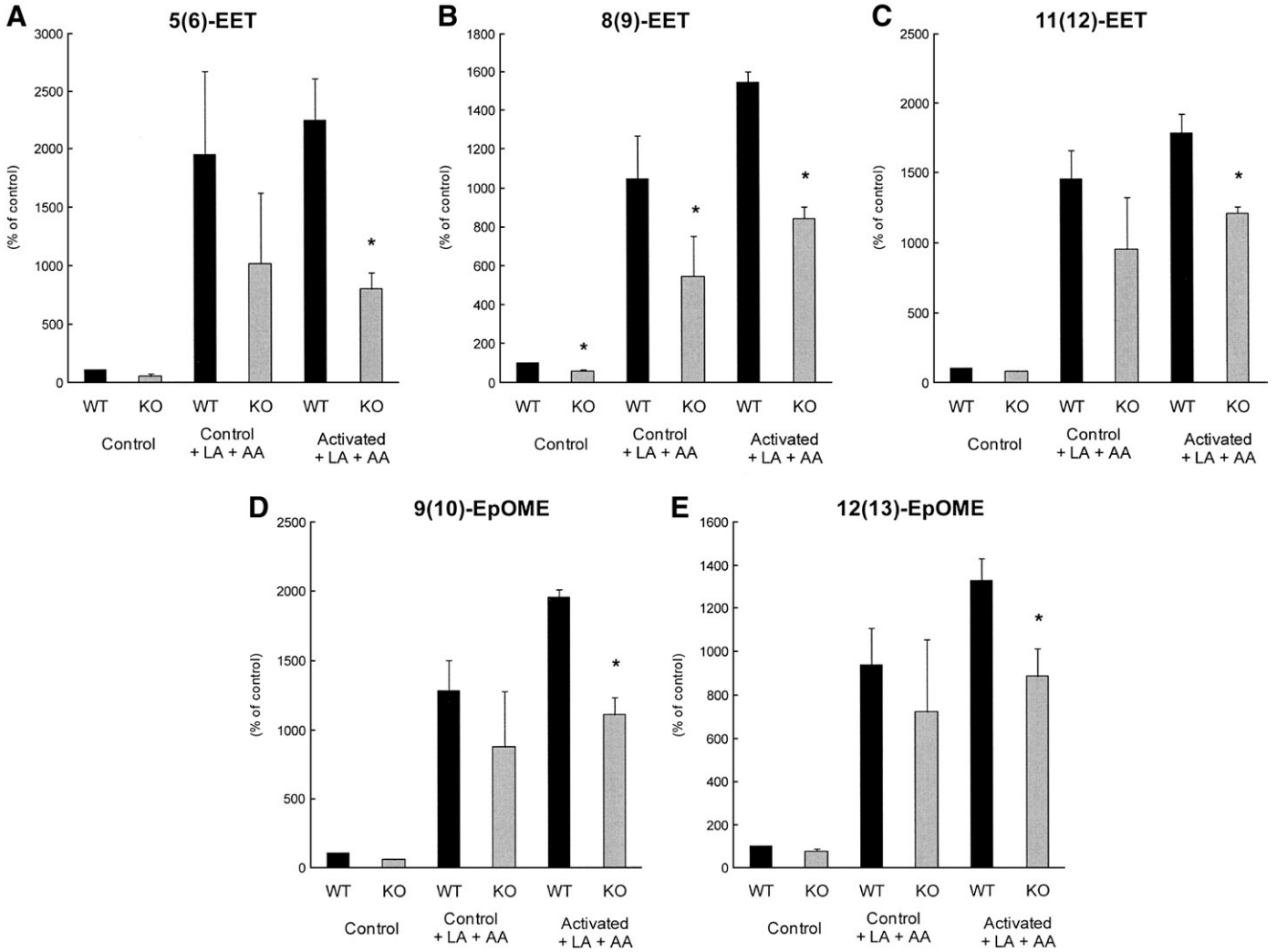

Fig. 1. Increased production of EET and EpOME by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of EETs (AA epoxides) and EpOMEs (LA epoxides) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five animals). *$p<0.05$ MPO-KO vs wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 5(6)-EET, 4.72 nM; 8(9)-EET, 2.33 nM; 11(12)-EET, 2.02 nM; 9(10)-EpOME, 2.22 nM; and 12(13)-EpOME, 2.97 nM. Concentrations of 14(15)-EET were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

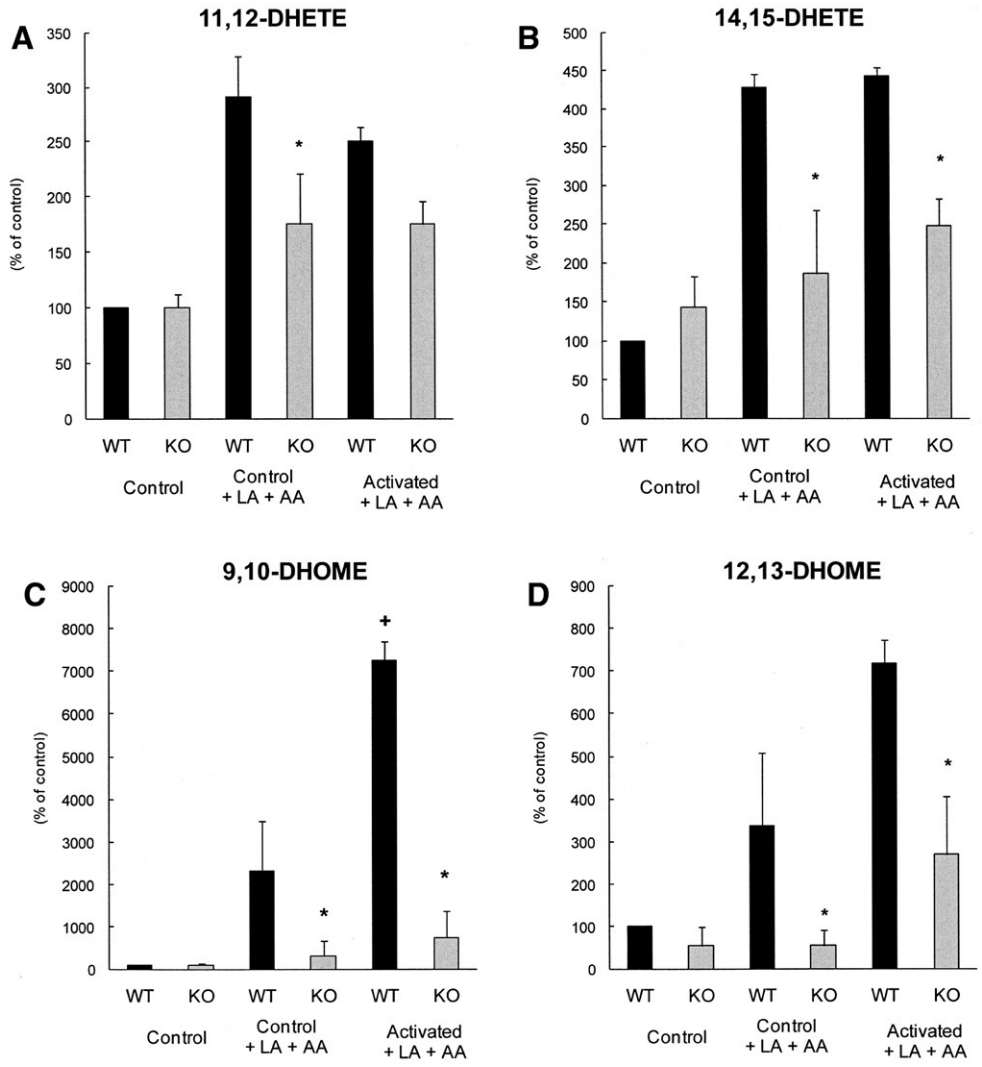

Fig. 2. Increased production of DHETE and DHOME by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of DHETEs (AA dihydroxy metabolites) and DHOMEs (LA dihydroxy metabolites) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA. All results are derived from at least three samples (pooled from four or five animals). *$p<0.05$ MPO-KO vs wild-type, +$p<0.05$ control wild type vs LPS-treated wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 11,12-DHETE, 0.10 nM;14,15-DHETE, 0.09 nM; 9,10-DHOME, 0.10 nM; 12,13-DHOME, 0.22 nM. Concentrations of 5,6-DHETE and 8,9-DHETE were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

AA and LA epoxides and hydroxy intermediates together with the catabolism of cysteinyl-LTs. Accordingly, the formation of AA and LA lipid mediators (HETEs, HODEs, and H(P)ODEs) was suppressed in MPO-KO mice and PMNs isolated from these animals. Interestingly, the difference between AA and LA metabolites formed in PMNs from wild-type and MPO-KO mice was also observed in the absence of any direct oxidative burst stimulation. However, PMNs elicited into the peritoneum by casein injection are partially activated although some responsiveness to proinflammatory stimuli remains [40,41]. Thus the activation of PMNs with casein together with the activation of PMNs by the isolation procedure can partly trigger PMNs to produce reactive oxygen species, including hydrogen peroxide. However, this observation suggests that metabolic pathways not directly related to the activation of MPO can also be involved in the observed phenomenon.

Several mechanisms may underlie the initiation of lipid peroxidation by MPO-catalyzed reactions. A wide range of oxidized lipid intermediates were shown to be formed in reactions of cholesterol and unsaturated fatty acids with MPO in the presence of low-molecular-weight intermediates (such as chloride, nitrite, or tyrosine) through MPO-generated diffusible radical species [2,3,8–10]. An alternative diffusible radical species formed by MPO that may participate in lipid peroxidation is nitrogen dioxide [28,29,48,49]. Epoxides can be formed from chlorohydrins that undergo HCl elimination [21,22,50]. Further, MPO as a member of the family of heme peroxidases can also be suggested to directly function as a fatty acid epoxygenase and lipoxygenase [20,51]. However, the epoxidation mechanisms catalyzed by heme proteins are not well understood. The putative mechanism for the epoxidation of alkenes by chloroperoxidases, also known as the ferryl–oxygen transfer mechanism [20,52], is comparable to the oxygen transfer mechanism of cytochrome P450.

The nearly uniform distribution of various isomers of AA and LA lipid peroxides formed during endotoxemia is consistent with a free radical, rather than a regioselective (e.g., involving LOXs and cyclooxygenases), mechanism of AA and LA oxidation. However, the employed methodological approach did not allow the analysis of stereoisomer specificity in our study. It was not possible to compare the activity of all enzymes involved in AA and LA in MPO-KO and wild-type mice. Moreover, other studies show that the cellular and enzymatic components capable of affecting lipid peroxidation are comparable between MPO-KO and wild-type mice under acute inflammatory conditions. Zhang et al. showed that cyclo-oxygenase-1, cyclo-oxygenase-2, and 12-LOX levels are similar in isolated

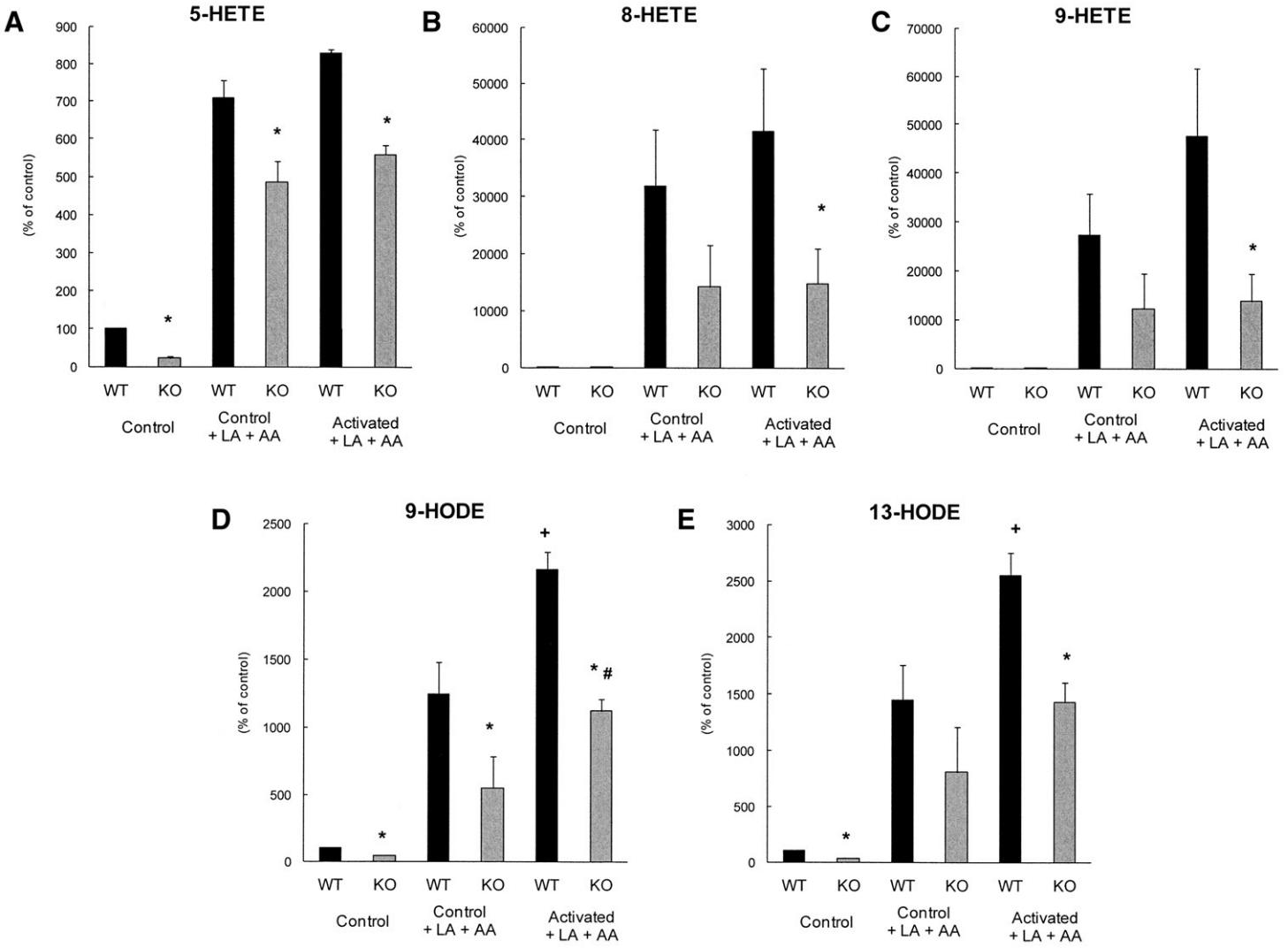

Fig. 3. Increased production of HETE and HODE by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of AA- (HETEs) and LA-derived (HODEs) lipid metabolites in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five animals). *$p<0.05$ MPO-KO vs wild-type, +$p<0.05$ control wild-type vs LPS-treated wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 5-HETE, 17.72 nM; 8-HETE, 0.1 nM; 9-HETE, 0.1 nM; 9 HODE, 73.07 nM; and 13 HODE, 65.76 nM. Concentrations of 11-HETE, 12-HETE, 15-HETE, 19-HETE, and 20-HETE were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

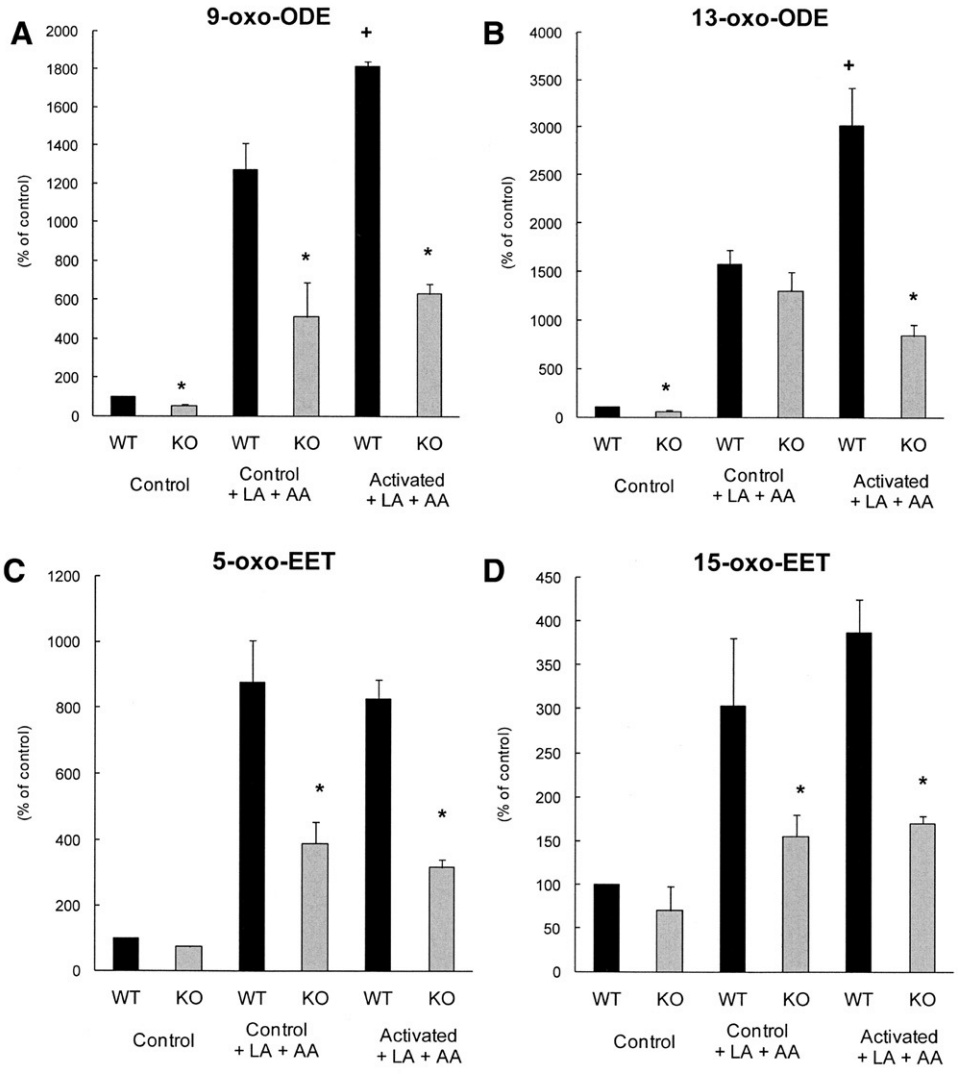

Fig. 4. Increased production of oxo-ODEs and oxo-EETs by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of oxo-ODEs (LA degradation products) and oxo-EETs (AA degradation products) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five mice). *$p<0.05$ MPO-KO vs wild-type, +$p<0.05$ control wild-type vs LPS-treated wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 9-oxo-ODE, 10.27 nM; 13-oxo-ODE, 20.62 nM; 5-oxo-EET, 2.11 nM; 15-oxo-EET, 5.75 nM. Limits of quantification are in Supplementary Table 1.

peritoneal leukocytes from MPO-KO and wild-type mice [35]. Likewise, another two in vivo studies of MPO-KO mice have suggested that initiation of lipid peroxidation is dependent upon MPO [34,35]. Interestingly, MPO-KO mice under septic conditions induced by *Candida* revealed significantly reduced plasma levels of isolevuglandins, a family of ketoaldehydes generated by free radical-induced peroxidation of arachidonate-containing lipids [34]. Similarly, activated PMNs isolated from MPO-deficient human subjects form 9-HETE and 9-HODE only after addition of exogenous MPO [30]. Nevertheless, the possibility that MPO-KO alters the activities of various AA and LA metabolic enzymes by changing their tissue-specific expression, posttranslational modification, or cellular localization cannot be excluded.

Our data demonstrate for the first time that MPO deficiency leads to the accumulation of cysteinyl-LTs in systemic circulation during the course of acute inflammation. The finding that these important proinflammatory mediators are deactivated by MPO in an in vivo model of acute inflammation is supported by several in vitro studies [53–55]. MPO is thought to degrade cysteinyl-LTs by their oxidation [53–55]. Previously, the MPO-dependent inactivation of LTs and cysteinyl-LTs in particular was demonstrated in cell-free systems and in activated human phagocytes. In the latter case, human MPO-deficient PMNs and monocytes were unable to degrade LTs unless MPO was added back into the system [53–55]. However, in our experiments we were not able to confirm these findings using PMNs isolated from wild-type and MPO-KO mice.

Interestingly, metabolites that were under the detection limit in plasma of both wild-type and MPO-KO mice and in PMNs isolated from wild-type and MPO-KO mice differ (legends to Tables 1 and 2 and Figs. 1, 2, and 3). For example, epoxides of AA were under the detection limits in the plasma of mice, similar to our other studies [14,15], which is probably related to their low half-life in vivo. Undetectable levels of various degradation products of unstable LA- and AA-derived hydroperoxides in vivo in contrast to in vitro could also be coupled with their short half-life in the blood circulation. On the other hand, the presence of detectable levels of some dihydroxy metabolites and hydroperoxides of AA and LA only in vivo can be connected to a limited range of enzymes involved in AA and LA metabolic pathways present in PMNs in contrast to the complex metabolism of AA and LA by various cell types in vivo. This raises the question of the importance of various cell types and their enzymatic accessories in the formation of biologically active AA and LA

metabolites during the course of acute inflammation. It is not possible to exclude differences in the activity of various enzymes in cells of different origin between wild-type and MPO-KO mice as discussed above; however, our data strongly support the importance of MPO in this process.

The MPO-dependent modulation of AA and LA mediators could significantly affect the physiological functions of the organism. It is interesting to speculate about the role of MPO-dependent formation of polyunsaturated fatty acid epoxides with mostly anti-inflammatory effects during acute inflammation, which could theoretically compensate for the decreased activity of cytochrome P450 enzymes due to inflammation [56]. Simultaneously, MPO can catalyze the degradation of the cysteinyl-LTs LTC4, LTD4, and LTE4, which stimulate various leukocyte functions, including chemotactic movement and tissue infiltration [13,18,19]. Nevertheless, the MPO-dependent formation of lipid mediators with anti-inflammatory properties and the catabolism of proinflammatory LTs may have positive effects on the inflammatory process. In this study, no significant effects of MPO deficiency on mortality or physiological functions such as blood pressure (data not shown) were observed up to the time of blood collection. However, several studies using various types of inflammatory models recently showed an increased level of the inflammatory process in MPO-deficient mice [37,47,57–59]. Thus the specific modulation of AA and LA metabolites by MPO can be included among other previously suggested anti-inflammatory effects, such as clearance of noxious stimuli, oxidative inactivation of proinflammatory mediators, inactivation of proteases, catabolism of NO, and inhibition of inducible nitric oxide synthase expression [60].

This study provides evidence that MPO plays an important role in the formation of both pro- and anti-inflammatory AA- and LA-derived lipid mediators in our a noninfectious model of systemic sepsis in mice. Our findings invite a reappraisal of the enzymatic participants in lipid peroxidation in vivo, with MPO added to the ranks of enzymes like LOXs, cyclo-oxygenases, and cytochrome P450 complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.02.010.

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