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*J Immunol* 2013; 191:3347-3357; Prepublished online 16 August 2013; doi: 10.4049/jimmunol.1300699

http://www.jimmunol.org/content/191/6/3347

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Contributions of the Three CYP1 Monoxygenases to Pro-Inflammatory and Inflammation-Resolution Lipid Mediator Pathways

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All three cytochrome P450 1 (CYP1) monoxygenases are believed to participate in lipid mediator biosynthesis and/or their local inactivation; however, distinct metabolic steps are unknown. We used multiple-reaction monitoring and liquid chromatography-UV coupled with tandem mass spectrometry–based lipid-mediator metabololipidomics to identify and quantify three lipid mediator metabolomes in basal peritoneal and zymosan-stimulated inflammatory exudates, comparing Cyp1a1/1a2/1b1(−/−) C57BL/6J-background triple-knockout mice with C57BL/6J wild-type mice. Significant differences between untreated triple-knockout and wild-type mice were not found for peritoneal cell number or type or for basal CYP1 activities involving 11 identified metabolic steps. Following zymosan-initiated inflammation, 18 lipid mediators were identified, including members of the eicosanoids and specialized proresolving mediators (i.e., resolvins and protectins). Compared with wild-type mice, Cyp1 triple-knockout mice exhibited increased neutrophil recruitment in zymosan-treated peritoneal exudates. Zymosan stimulation was associated with eight statistically significantly altered metabolic steps: increased arachidonic acid–derived leukotriene B4 (LTB4) and decreased 55-hydroxyeicosatetraenoic acid; decreased docosahexaenoic acid–derived neuroprotectin D1/protectin D1, 17S-hydroxydocosahexaenoic acid, and 14S-hydroxydocosahexaenoic acid; and decreased eicosapentaenoic acid–derived 18R-hydroxyeicosapentaenoic acid (HEPE), 15S-HEPE, and 12S-HEPE. In neutrophils analyzed ex vivo, elevated LTB4 levels were shown to parallel increased neutrophil numbers, and 20-hydroxy-LTB4 formation was found to be deficient in Cyp1 triple-knockout mice. Together, these results demonstrate novel contributions of CYP1 enzymes to the local metabolite profile of lipid mediators that regulate neutrophil inflammation. The Journal of Immunology, 2013, 191: 3347–3357.

Lipid mediators (LMs) derived from polyunsaturated fatty acids (Fig. 7A) include >150 chemicals, many of which have potent bioactivity (1). The ω-6 fatty acid, arachidonic acid (AA), is converted to 12 known classes of LMs: PGs, prostacyclins, thromboxanes, leukotrienes (LTs), epoxidecyclostratrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), dihydroxyeicosatetraenoic acids (DHETEs), hydroperoxyeicosatetraenoic acids, ω- and ω-1 alcohols, lipoxins, heparoxins, and oxins (2–4). AA is well known to be converted by cytochrome P450 to EETs (5), some of which are active in inflammation (6). In contrast, the ω-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are converted to resolvins (E-series and D-series), neuroprotectins, and maresins (2, 3, 7–9). Many ω-6 and ω-3 LMs participate in inflammation. Some are more involved in the initiation phase, whereas others participate more in the resolution phase.

Oxidative biosynthesis of LMs is carried out in multiple steps by specific arachidonate lipoxigenases (encoded by six human genes [Alox5, Alox12, Alox12b, Alox15, Alox15b, and Alox3] and seven mouse genes [Alox5, Alox12, Alox12b, Alox12e, Alox15, Alox8, and Alox3]). In addition to the cyclooxygenases (COX1, COX2; official names PG G synthase-1 and -2) and arachidonic lipoxigenases, there is considerable evidence (2–4) suggesting that cytochrome P450 (CYP) monoxygenases participate in the oxidative biosynthesis and inactivation of LMs. Members of the CYP1, CYP2, CYP3, and CYP4 gene families, as well as CYP5A1 and CYP8A1, are involved in biosynthesis and further inactivation of LMs.

Received for publication March 18, 2013. Accepted for publication July 19, 2013.

This work was supported by Cystic Fibrosis Foundation Research Development Program Center Component II Grant (to C.L.K.), National Institutes of General Medical Sciences (Grant P01 GM095467 to C.N.S.), and National Institute of Environmental Health Sciences (Grant T32 ES016646 to M.G.-P. and Grants R01 ES008147, R01 ES014403, and P30 ES06096 to D.W.N.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AA, arachidonic acid; CYP, cytochrome P450; ddH2O, doubly distilled water; DHA, docosahexaenoic acid; DHETE, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EPA, eicosapentaenoic acid; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LC-MS-MS, liquid chromatography coupled with tandem mass spectrometry; LM, lipid mediator; LTA4, leukotriene A4; LTB4, leukotriene B4; LXα, lipoxin A4; MRM, multiple-reaction monitoring; PD1, protectin D1; RvE2, resolvin E2; TKO, Cyp1a1/1a2/1b1(−/−) triple-knockout on C57BL/6J background; WT, C57BL/6J wild-type.
LMs (10). Although some P450-mediated specific reactions of bioactive LMs have been described (reviewed in Ref. 4), the vast majority remains to be determined, largely because of the technical challenges involved in identifying LMs, particularly with regard to stereochemistry. The CYP1 gene family encodes three enzymes (CYP1A1, CYP1A2, and CYP1B1) in both human and mouse that are evolutionarily highly conserved, suggesting that mouse CYP1A may likely be extrapolated to human CYP1 functions. In contrast, the CYP2, CYP3, and CYP4 families are far more complex due to multiple gene-duplication events followed by “genetic drift” during the past 65 million years, because humans and mice had a common ancestor. This resulted in the human genome having 16 functional CYP2 protein–coding genes, 4 CYP3 protein–coding genes, and 12 CYP4 protein–coding genes compared with the mouse genome, which has 50 functional Cyp2 protein–coding genes, 9 Cyp3 protein–coding genes, and 20 Cyp4 protein–coding genes (11).

Lipoxigenases insert both atoms (12), whereas P450 monoxygenases insert one atom (13–15), of diatomic oxygen into substrates to form the products (Fig. 7B). Another important distinction between lipoxigenase and P450 monoxygenase reactions is that, although lipooxygenases occasionally produce epoxides (e.g., leukotriene A₄ [LTA₄] formation by ALOX5), the major product is a fixed-chirality hydroperoxide; in contrast, P450 monoxygenases can generate racemic mixtures of internal-monohydroxy products, terminal-monohydroxy products, and epoxides that (following hydrolysis) often proceed to form racemic mixtures of dihydroxy products (Fig. 7B).

Ultimately, among many other functions (4, 10), AA-derived LMs are more likely to be involved in the proinflammatory phase, whereas the DHA-derived bioactive metabolome and EPA-derived bioactive metabolome are LMs that orchestrate the resolution phase of self-limited inflammatory responses. In addition, the location of many of these metabolic reactions usually is highly tissue and/or cell type specific (2–4).

One approach to resolving the challenging problem of identifying which CYP enzyme participates in which step(s) of the LM cascade involved in acute inflammation is to use Cyp-knockout mouse lines in combination with the most advanced metabololipidomics (16) for separating and identifying as many unique LM metabolites as possible. The present study capitalizes on this approach. The Cyp1a1/1a2/1b1(−/−) triple-knockout (TKO) mouse (4, 17, 18) has all three highly conserved members of the mammalian Cyp1 gene family genetically deleted. We compared TKO mice with C57BL/6J wild-type (WT) mice during zymosan-induced peritonitis. In this study, we did not investigate the classical EET products produced by P450 from AA in the liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) profiles; rather, we focused on their roles in lipoxigenase and cyclooxygenase pathways. Results from the current study indicate that CYP1 monoxygenases play a highly significant role in the regulation of at least eight key steps during LM biosynthesis and their further metabolism; thus, these findings provide potentially useful new therapeutic targets for treating inflammation and its natural resolution.

Materials and Methods

Chemicals

Zymosan A was obtained from Sigma-Aldrich (St. Louis, MO). Liquid chromatography–grade solvents were purchased from Fisher Scientific. Agilent Eclipse Plus C18 columns (50 × 4.6 × 1.8 μm) and C18 SPE columns were bought from Waters. Synthetic standards for LC-MS-MS quantitation and deuterated internal standards were procured from Cayman Chemicals. All chemicals and reagents represented the highest available grades.

Animals

Generation of the TKO mouse line was described (17). TKO animals were backcrossed for >20 generations onto C57BL/6J to ensure a genetic background that was >99.9% similar (19) to WT C57BL/6J mice. All TKO mice used in the experiments were age matched with WT controls. We found that mice provided with regular laboratory chow gave highly variable results. Therefore, both WT and TKO mice were maintained, from prior to conception, on flavone-free Laboratory Chow Diet 5001. This diet is designed to assure minimal inherent biological variation in long-term studies because it is enriched with ω-3 essential fatty acids (http://www.labdiet.com); indeed, the 5001 diet provided us with highly reproducible data. Results appeared to be more consistent in males than in females; subsequently, for all experiments described in this article, we studied males only, between 50 and 100 d of age. All animal experiments were approved by and conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and the University of Cincinnati Medical Center Institutional Animal Care and Use Committee.

Zymosan challenge

An acute inflammatory response was induced with 1 mg zymosan/mouse (20). Zymosan (insoluble carbohydrate from yeast cell wall) was freshly prepared (2 mg/ml) in sterile 0.9% NaCl, and 0.5 ml was injected i.p. into each mouse; zero-time controls were not treated. At the appropriate time points (0, 6, and 9 h), each peritoneal cavity was washed twice with 5 ml PBS (Cellgro); all baseline peritoneal cells at time zero, as well as all peritoneal exudates at 6 and 9 h following zymosan challenge, were harvested and centrifuged (560 × g for 6 min). Supernatant fractions were collected and mixed (2:1) with cold methanol (Sigma-Aldrich); samples were kept at −80°C until metabololipidomics analyses were performed (a fixed-chirality hydroperoxide; in contrast, P450 monoxygenases can generate racemic mixtures of internal-monohydroxy products, terminal-monohydroxy products, and epoxides that (following hydrolysis) often proceed to form racemic mixtures of dihydroxy products (Fig. 7B).

Cell quantification

Following centrifugation (560 × g for 6 min), total cell counts were quantified from peritoneal lavage at baseline or lavage of peritoneal exudates following zymosan challenge, as well as from cells isolated from peripheral blood or bone marrow. Differential cell counts were quantified following cytospin analysis (on 80 μl total cell exudate at baseline, an additional 1:20 dilution for conditions involving zymosan challenge) and subsequent Diff-Quik staining (Siemens). We quantified 400 cells/slide to determine the percentages of neutrophils, monocytes/macrophages, mast cells, eosinophils, and lymphocytes.

Sample extraction and LM metabololipidomics

All samples for LC-MS–based analyses were extracted using SPE columns (16). Briefly, columns were equilibrated with one column volume of methanol and two volumes of doubly distilled water (ddH₂O). Prior to extraction, 500 pg deuterium-labeled internal standards δ₅–5S-HETE, δ₅-leukotriene B₅ (LTB₅), and δ₅-PGE₂ were added to facilitate quantification of sample recovery. Sample supernatant fractions were diluted with 10 volumes of ddH₂O, acidified (pH ~3.5), and immediately loaded onto a SPE column. After loading, columns were washed with one volume of neutral ddH₂O and one volume of hexane. Samples were eluted with 5% methanol/acetone and taken to dryness using Speedvac or nitrogen stream. Samples were subsequently suspended in methanol/water for LC-MS-MS. The liquid chromatography–UV coupled with tandem mass spectrometry system includes QTrap 3200 equipped with a Shimadzu SIL-20AC auto-injector and LC-20AD binary pump or QTrap 5500 (ABSciex), equipped with an Agilent HP1100 binary pump. An Agilent Eclipse Plus C18 column (30 mm × 4.6 mm × 1.8 μm or 100 mm × 4.6 mm × 1.8 μm) was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (v/v/v) to 100:0:0.01 at 0.5-ml/min flow rate.

To monitor and quantify the levels of the various LMs, we developed a multiple-reaction-monitoring (MRM) method with signature ion fragments for each molecule. Identification was conducted using published criteria (16), with at least six diagnostic ions. Calibration curves were obtained using a multiple-reaction-monitoring (MRM) method with signature ion fragments for each molecule. Identification was conducted using published criteria (16), with at least six diagnostic ions. Calibration curves were obtained using published criteria (16), with at least six diagnostic ions. Calibration curves were obtained using published criteria (16), with at least six diagnostic ions. Calibration curves were obtained using published criteria (16), with at least six diagnostic ions.
17-hydroxydocosahexenoic acid (HDHA), 14-HDHA, 7-HDHA, and 4-HDHA at 12.5, 25, 50, and 100 ng (Supplemental Table I). Linear calibration curves for each were obtained with r² values in the range of 0.98-0.99. Quantification was carried out based on peak area of the MRM transition and the linear calibration curve for each compound. Reverse-phase chiral LC-MS-MS was conducted, as described (21).

Human and mouse neutrophils

Neutrophils were isolated as described (22), with minor modifications. Specifically, human neutrophils were isolated from peripheral blood; mouse neutrophils were purified from femoral and tibial bone marrow, as described (23). Cells were collected in ice-cold Ca²⁺- and Mg²⁺-free HBSS (Life Technologies) supplemented with 0.1% BSA (Sigma-Aldrich); neutrophils were purified using a discontinuous Percoll (GE Healthcare) gradient. Subsequently, neutrophils were layered onto Histopaque 1119 (Sigma-Aldrich), centrifuged at 650 × g for 20 min, and stopped without brake, to separate RBCs. Purified neutrophils were collected and washed with HBSS, 0.1% BSA, centrifuged for 5 min at 400 × g, and resuspended in PBS with Ca²⁺ and Mg²⁺ prior to being stimulated with LTB₄.

The purity of isolated neutrophils was determined by cytospin analysis, followed by Diff-Quik stain (Siemens). Isolation of human neutrophils was performed at Cincinnati Children’s Hospital Medical Center; all participants provided written informed consent and the study was approved by the Cincinnati Children’s Hospital Medical Center Institutional Review Board.

Statistical analysis

All assays were performed in duplicate or triplicate, and average values from each mouse were considered one independent determination. Statistical differences were assessed by the Student pair-wise t test or χ² analysis. Data were normally distributed and are presented as mean ± SEM. All p values < 0.05 were regarded as statistically significant.

Results

Peritoneal cell numbers and cell types

In an earlier study, peritoneal immune cell numbers and types were found to be similar at baseline between WT and TKO mice (17). In contrast, following i.p. zymosan, an exaggerated increase in peritoneal exudate total cell number, neutrophils, and monocyte/macrophages was observed in TKO mice (17). In the current study, zymosan challenge also led to significant increases in total cell numbers in peritoneal exudates of TKO mice compared with WT mice at 6 h (>14-fold) and 9 h (~10-fold), with significantly increased neutrophil accumulation 6 and 9 h after challenge and significantly increased monocyte/macrophage accumulation at 6 h after challenge (Fig. 1). Significant differences between WT and TKO mice were not found for the numbers of eosinophils, mast cells, or lymphocytes.

Comparison of WT versus TKO levels of LMs at baseline

To determine the contributions of CYP1 monooxygenases in LM biosynthetic pathways and LM profiles in vivo, we initially quantified LM levels in untreated mice (Fig. 2). Illustrations of representative chromatography (Fig. 2A) unequivocally demonstrate that each of the bioactive LMs was identified using strict reported criteria (Materials and Methods). Of the 24 LMs screened, 11 were detected in baseline peritoneal cells of WT mice but not TKO mice: PGD₂, PGE₂, LTB₄, 12-HETE, 15-HETE derived from AA (Fig. 2B–D); PD₃, 14-HDHA, 17-HDHA derived from DHA (Fig. 2E, 2F); and 12-HEPE, 15-HEPE, and 18-HEPE derived from EPA (Fig. 2G). Statistically significant differences were not found between TKO and WT peritoneal cell LMs without zymosan challenge.

Comparison of WT versus TKO levels of LMs in exudate 6 h into inflammation

Of the LMs screened via targeted LC-MS-MS–based lipidomics, 17 were identified in peritoneal exudates from WT mice, but not...
TKO mice, treated with zymosan for 6 h (data not shown). In addition to those 11 identified in baseline peritoneal cells (Fig. 2), 6 additional LMs and pathway markers were found following 6 h of inflammation. These included PGF$_{2\alpha}$, LXA$_4$, 5-HETE, 4-HDHA, RvE2, and 5-HEPE. Moreover, compared with WT mice, zymosan-treated TKO mice revealed a trend toward increased levels of AA-derived PGs and LTB$_4$, as well as decreased levels of 12-HETE, DHA-derived PD1 and 14-HDHA, and EPA-derived 15-HEPE (data not shown).

Comparison of WT and TKO levels of LMs in exudate 9 h into inflammation

Of the LMs and pathways profiled, 16 were identified in the peritoneal exudate of WT mice, but not TKO mice, treated with zymosan for 9 h (Fig. 3). LXA$_4$, which was found at low levels at 6 h into inflammation, was no longer detectable at 9 h. Although LTB$_4$ levels increased dramatically between 6 and 9 h of zymosan treatment in TKO mice, seven other LMs were decreased to a statistically significant extent in TKO mice compared with WT mice (Fig. 3, Supplemental Table I).

Therefore, of the three functionally distinct LM metabolomes screened (i.e., AA-, DHA- and EPA-bioactive metabolomes), statistically significant alterations were obtained in eight LMs and pathway markers during inflammation in TKO mice compared with WT mice: TKO mice exhibited an $\sim$3.9-fold increase in LTB$_4$ levels; an $\sim$7.5-fold decrease in PD1 levels; 1.7- to 2.3-fold decreases in 5-HETE, PD1, 14-HDHA, 12-HEPE, 15-HEPE, and 18-HEPE levels; and a 1.3-fold decrease in 5-HETE levels (Fig. 3).

**LTB$_4$ metabolism ex vivo**

We chose to explore LTB$_4$ metabolism further in peritoneal and bone marrow neutrophils ex vivo. A proof-of-principle experiment was first carried out with human neutrophils. When the substrate LTB$_4$ was added to human peripheral blood neutrophils, $\sim$4-fold more 20-COOH-LTB$_4$ was produced compared with 20-OH-LTB$_4$ (Fig. 4A, 4E). The confirmed mass-to-charge ratio of the parent compound LTB$_4$ was $m/z$ 335 (Fig. 4B); it was $m/z$ 351 for 20-OH-LTB$_4$ (Fig. 4C) and $m/z$ 365 for 20-COOH-LTB$_4$ (Fig. 4D). Thus, this ex vivo experiment with human neutrophils validates the identification of LTB$_4$ and two additional metabolites that were reported earlier to be produced by a member of the human CYP4 family (24).

Peritoneal and bone marrow neutrophils were then isolated from TKO and WT mice that had received 4 h of zymosan treatment. Before incubation with the substrate LTB$_4$, peritoneal neutrophils from TKO mice displayed significant increases in LTB$_4$ levels compared with those from WT neutrophils (Fig. 5A, 5B). After incubation with the substrate LTB$_4$, TKO mice showed about two thirds as much 20-OH-LTB$_4$ in both peritoneal neutrophils (Fig. 5C) and bone marrow neutrophils (Fig. 5D) relative to WT mice. Curiously, although a significant difference in 20-OH-LTB$_4$ levels was found between TKO- and WT-elicited peritoneal neutrophils, the 20-COOH-LTB$_4$ (downstream oxidized metabolite) was not detected in either TKO or WT mice (Fig. 5A). In contrast, high levels of 20-COOH-LTB$_4$ were clearly demonstrable with human neutrophils (Fig. 4A, 4D, 4E); these data indicate that mice apparently do not have the CYP4 enzyme that is equivalent in...
function to the human CYP4 enzyme responsible for 20-COOH-LTB₄ formation (24). In fact, the amount of 20-COOH-LTB₄ in human neutrophils was ~4-fold greater than the amount of 20-OH-LTB₄ (Fig. 4E).

To determine the contribution of CYP1 enzymes to LM biosynthesis, we next assessed the production of LM precursors by peritoneal exudate leukocytes. We found that 14-HDHA, 17-HDHA, 12-HETE, and 15-HETE levels were significantly lower in TKO leukocyte incubations compared with those of WT leukocyte incubations (Fig. 6). Finally, using reverse-phase chiral LC-MS-MS (21), we investigated the levels of R/S-enantiomers for each of the monohydroxy acids, which were significantly decreased in TKO peritoneal leukocyte incubations. This analysis demonstrated an ~59 ± 9% decrease in the levels of 14-HDHA, 17-HDHA, 12-HETE, and 15-HETE; these four metabolites in TKO carrying the hydroxyl group in the R-enantiomeric position in TKO leukocyte incubations, when compared with that in WT leukocyte incubations. For these four metabolites carrying the hydroxy group in the S-enantiomeric position, we found an ~62 ± 14% diminution in the levels of 14-HDHA, 17-HDHA, 12-HETE, and 15-HETE. These results provide further evidence for the contribution of CYP1 enzymes to LM precursor biosynthesis.

Discussion

In the current study, we examined the combined contributions of the three CYP1 enzymes in LM pathways involved during acute inflammation (13). We started with the hypothesis that the absence of all three CYP1 enzymes would alter the LM metabololipidomics profile at precise steps during inflammation and its resolution. Specifically, if an LM was increased in TKO relative to WT, this suggests that one or more of the CYP1 enzymes might be critical in a downstream metabolic step in this pathway (i.e., absence of CYP1 would lead to a build-up in levels of the upstream LM). In contrast, if a specific LM was decreased in TKO relative to WT, this suggests that one or more of the CYP1 enzymes is critical in an upstream event involved in formation of the metabolite in this pathway (i.e., absence of CYP1 would lead to a diminution of the downstream LM). Elucidation of P450 enzyme specificity for any particular step in the initiation phase or resolution phase of inflammation should aid in developing novel drugs for treatment of various forms of acute inflammation, as well as chronic inflammatory disorders, such as cardiovascular disease, rheumatoid arthritis, periodontitis, and Alzheimer’s disease (1, 25).

Leukocyte trafficking within the site of inflammation is a dynamic process regulated by chemical cues produced at the site of injury and/or infection. In this study, we focused on the initiation phase of the inflammatory response, using metrics that map leukocyte-recruitment dynamics in response to a self-limited challenge (26). Our results demonstrate that TKO mice display altered leukocyte recruitment dynamics in response to a self-limited challenge, with elevated neutrophil recruitment following zymosan challenge and significantly higher exudate monocyte/
macrophage levels at the peak of inflammation compared with their WT littermates. These results, together with the finding that exudate proinflammatory LM levels (vide infra) were elevated in TKO mice, indicate that TKO mice display a proinflammatory phenotype. Our present findings are in agreement with those made in models of nonresolving (27) and delayed-resolving (28) inflammation; these data underscore the contribution of CYP1 enzymes to inflammation and its timely resolution.

Interestingly, statistically significant differences in LM levels between TKO and WT mice were not found in peritoneal cells at baseline; they were only found after zymosan stimulation. This observation is consistent with the well-known fact that CYP1 levels in immune tissues are very low or negligible under basal conditions (29). After zymosan stimulation, an endogenous inducer of CYP1, believed to be one or more LMs, or after treatment of the animal with a foreign chemical (e.g., PAH or dioxin), CYP1 levels in immune cells become dramatically increased.

After 6 h of zymosan challenge, TKO peritoneal exudates showed a trend of increased PGD$_2$, PGE$_2$, and PGF$_2\alpha$, along with a 2.3-fold increase in LTB$_4$ levels compared with WT mice; in contrast, relative to WT mice, TKO exudates showed an $\sim$6-fold decrease in PD1 levels and noticeable decreases in 12-HETE, 14-HDHA, and 15-HEPE. Following 9 h of zymosan-initiated inflammation, TKO exudates demonstrated statistically significant differences from WT exudates with regard to eight chemicals: strikingly increased LTB$_4$ levels and strikingly decreased PD1 levels, as well as decreased levels of 5-HETE, 14-HDHA, 17-HDHA, 12-HEPE, 15-HEPE, and 18-HEPE. The reason for the diminished levels of AA-derived 5-HETE in TKO inflammatory exudates after 9 h of zymosan (Fig. 3) is not known; the small
decrease in 5-HETE could be caused by various factors. One possibility is that decreased overall ALOX5 activity is coupled with more efficient conversion of 5-hydroperoxycosatetraenoic acid to LTB4; another possibility is that at least a portion of 5-HETE formation represents the direct metabolism of AA by CYP1 (30, 31).

These results suggest that, prior to challenge, the contribution of the three CYP1 enzymes to LM biosynthesis and/or further metabolism is not significant, indicating that resident peritoneal cells do not express functionally appreciable amounts of these enzymes at levels that would regulate LM levels in the absence of challenge. Upon challenge, leukocytes are recruited to the peritoneal cavity, in particular polymorphonuclear leukocytes, which possess the CYP1 enzymes and lead to alterations in the exudate LM profiles in WT and TKO mice. Future studies will need to address the contribution of the CYP1 enzymes and the role(s) of dynamic cellular traffic at the site of inflammation and their contribution to proresolving mediator biosynthesis during inflammation resolution.

We conclude that one or more CYP1 enzymes participate significantly in at least eight specific metabolic steps of LM pathways in the acute inflammatory response. Furthermore, our data indicate that CYP1 monoxygenases contribute to the overall balance of local autacoids (i.e., deletion of an inactivation pathway can give rise to accumulation of a specific LM during the time course of both the initiation and resolution phases of inflammation in the intact animal).

AA-derived LTB4 is known to be a potent chemoattractant, generated from activated innate immune cells, such as neutrophils, macrophages, and mast cells (32) with mice lacking either 5-LOX or LTA4 hydrolase; these two enzymes are responsible for biosynthesis of this potent mediator, displaying decreased leukocyte recruitment following zymosan challenge (33). Thus, our finding of zymosan-elicited increases in the number of these three cell types in TKO exudates compared with WT exudates is consistent with the subsequent observation of increased LTB4 levels in TKO mice following zymosan challenge.

TKO peritoneal and bone marrow neutrophils ex vivo show a diminished capacity to metabolize LTB4, but this effect seems to be small relative to the ∼4-fold increase in LTB4 observed in peritoneal exudates from TKO mice. It is not known with certainty whether this increase reflects a requirement of CYP1 for LTB4 oxidation in vivo in these mice or whether it reflects some compensatory decreased level of a mouse enzyme equivalent in function to that of the human CYP4 monoxygenase that is known to metabolize LTB4 in neutrophils (24). Curiously, CYP4A14 and CYP4A10 mRNA expression was found to be 2–3-fold lower in TKO liver than in WT liver (34); however, when TKO liver was compared with WT liver under more rigorous microarray conditions in a later study (17), no mouse CYP4 mRNA was found to be significantly upregulated or downregulated.

Alternatively, increased LTB4 levels in TKO mice might be explained by the increase in neutrophils present in TKO exudates rather than by LTB4 catabolism. Nevertheless, there appears to be an intriguing CYP1-mediated effect on TKO cellular dynamics in the peritoneal cavity as a result of inflammation; the current study did not determine the mechanism of this effect with certainty. In any event, CYP1 participation in the step from LTB4 to 20-OH-LTB4 appears to be very likely.

It is not clear that differences in LTB4 metabolism in neutrophils ex vivo (Fig. 5) can account for the increased LTB4 observed in the intact mouse; this is only one of several possible explanations. For example, LTB4 could be higher because the higher number of neutrophils present in the TKO exudate might produce greater amounts of LTB4. Another possibility is that LTB4 levels are greater as the result of compensatory upregulation of lipoxygenases or effects secondary to the clearly altered state of inflammation that exists in the TKO peritoneal cavity. Even in
isolated neutrophils ex vivo, it remains to be determined with absolute certainty that any of the CYP1 enzymes is directly responsible for altered LM metabolism.

Conversion of 20-OH-LTB₄ to 20-COOH-LTB₄ in human neutrophils is the main route of LTB₄ inactivation (24); this finding was also corroborated in Fig. 4, whereas this enzymatic reaction does not appear to occur in mouse neutrophils (Fig. 5). It has been known for >2 decades that even one altered amino acid residue can dramatically modify P450 substrate specificity (35); this is the major reason why substrate specificities of various P450 enzymes differ between mouse and human, especially among members of the CYP2, CYP3, and CYP4 families (11), in which orthologs between the two species cannot be determined conclusively. In addition to ω-oxidation pathways, an alternative dehydrogenation pathway was described for the inactivation of LTB₄ that involves the dehydrogenation of the hydroxyl group on C-12 by 12-hydroxydehydrogenase (PGR/LTB₄DH) to produce 12-oxo-LTB₄ (36, 37).

The role of CYP1 enzymes (Fig. 7B) in the inactivation of LTB₄ in mouse neutrophils is of interest and the main point of the experiments described in this article. We first investigated the pathway known for LTB₄ inactivation in human neutrophils that express the human orthologs of these enzymes (24). In this study, we showed that human neutrophils converted LTB₄ to the P450-mediated metabolites 20-OH-LTB₄ and 20-COOH-LTB₄, as reported elsewhere (24). Formation of these two metabolites are described first, to follow sequential metabolic steps regulated by distinct enzymes. The formation of 20-OH-LTB₄ occurs first and is primarily regulated by CYP1 enzymes, which, in humans, is then converted by CYP4 enzymes to 20-COOH-LTB₄ (24).

FIGURE 6. Contribution of CYP1 enzymes to LM biosynthesis following zymosan challenge. The four most significant changes in the generation of LM precursors by peritoneal exudate leukocytes included 14-HDHA, 17-HDHA (A), and 12-HETE and 15-HETE (B) levels; these metabolites were significantly lower in TKO mice compared with WT mice. *p < 0.05, **p < 0.01 versus WT.

FIGURE 7. Introduction to polyunsaturated fatty acids, lipoxygenase versus monooxygenase reactions, and LM biosynthesis pathways. (A) Origin of ω-6 and ω-3 LMs. (B) Diagram showing the mechanisms for lipoxygenase versus P450-monooxygenase reactions.
to zymosan challenge) and bone marrow polymorphonuclear leukocytes; each converted LTB₄ to 20-OH-LTB₄. Of note, 20-COOH-LTB₄ was not produced in these incubations from two mouse neutrophil populations, unlike human neutrophils. Quantification of both LTB₄ and 20-OH-LTB₄ levels in these incubations demonstrated that, in the absence of the CYP1 enzymes (in TKO mice), there was an accumulation of LTB₄ with a higher recovery rate. These results indicate that CYP1 enzymes are important in the inactivation of this initiating signal, which is also proinflammatory via ω-hydroxylation in mice. This is in line with the elevated LTB₄ levels found within inflammatory exudates from TKO mice, in which we also found elevated polymorphonuclear leukocyte recruitment to the peritoneum upon zymosan challenge.

Although LTB₄ biosynthesis in self-limited inflammatory exudates is not restricted to neutrophils, in this model LTB₄ production coincides with increased exudate neutrophil levels (38); in the absence of CYP1 enzymes, LTB₄ levels accumulated at the site of inflammation, which led to the exaggerated inflammatory response (Fig. 1). Together, these findings suggest that CYP1 enzymes in phagocytes exert a pivotal role in inflammation resolution.

The absence of CYP1 in TKO mice results in decreased formation of the DHA-derived 17S-HDHA. It is noteworthy that, in human polymorphonuclear leukocytes (39), double dioxygenation via a second lipoxigenase produces 10,17-diH₂DHA, which is then reduced by peroxidase activity to form 10S,17S-dihydroxy-9Z,11E,13E,15Z,19Z-hexaenoic acid, an isomer of neuroprotectinPD1 (40). It also is possible to produce this PD1 isomer via a second sequential P450 step (e.g., P450-mediated 17S-HDHA conversion to the 10,17-dihDHA isomer of PD1). The double geometry of this isomer is different from an enzymatic epoxide-dependent pathway and epoxide-hydrolase reaction (25, 40), which can then produce PD1 and other specialized pro-resolving mediators as their main route in human leukocytes. Also, during the resolution phase of inflammation, the absence of CYP1 in TKO mice leads to decreased formation of DHA-derived PD1.

Finally, the current study shows that CYP1 contributes to the levels of EPA-derived 15-HEPE and 12-HEPE, as well as involvement in the oxidative step from EPA-derived 18–hydroperoxyeicosapentaenoic acid to 18R-HEPE. This latter step is a bottleneck to the generation of resolvins of the E-series. Thus, CYP1 ablation might also affect production of a number of pro-resolving LMs, in addition to the ones mentioned above that include the AA-derived lipoxins, as well as the D-series resolvins. CYP1 ablation appears to supply the required precursors (i.e., 17-HDHA and 15-HETE) that are then converted by lipoxigenases to D-series resolvins and lipoxins, respectively (41–43).

Comparing WT and TKO mice, metabololipidomic analysis of the products made ex vivo by exudate leukocytes demonstrated a significant reduction in TKO LM precursors (i.e., 17-HDHA, 14-HDHA, 15-HETE, 12-HETE, and 5-HETE) (Fig. 6). These results are in line with the hypothesis that deletion of CYP1 enzymes is responsible, at least in part, for the differences found in exudate LM levels between WT and TKO mice. Although we found a significant decrease in 18-HEPE production in TKO mice, this did not correlate with a parallel decrease in RvE2 levels, an observation that may reflect a difference in the further distal conversion of RvE2 in TKO mice, which leads to an apparent accumulation of this mediator in the inflammatory exudates. Although we did not see any detectable differences between TKO and WT resolin levels in the current study, this may reflect issues related to detection limits for these mediators.

Compared with WT mice, TKO mice exhibited ~2-fold lower levels of LXA₄ at 6 h, but not at 9 h (Fig. 3), of zymosan challenge. This finding suggests that LXA₄ formation from 15S-hydroxy-5(S)-epoxy-ETE, at least initially, might involve contributions from CYP1 enzyme(s); subsequently, other oxidative enzymes might compensate to continue producing sufficient LXA₄ levels after 9 h of zymosan-initiated inflammation. Of possible relevance, LXA₄ itself (44) as well as two downstream metabolites of LTA₄, 55,6R-DHETE and 55,6S-DHETE (45), were suggested to act as endogenous ligands for aryl hydrocarbon receptor, a transcription factor involved in regulating all three CYP1 genes (4, 46). Aryl hydrocarbon receptor–dependent activity is likely to be required during the acute inflammatory response in these peritoneal exudate cells.

In addition to the TKO mouse described in this article, numerous P450-knockout mouse lines have been generated, including Cyp1b1(−/−) (47), Cyp2e1(−/−) (48), Cyp2j5(−/−) (49), and Cyp4a10(−/−) (50) single knockouts; Cyp1a1/1a2(−/−), Cyp1a1/1b1(−/−), and Cyp1a2/1b1(−/−) double knockouts (51); and, more recently, ablation of the entire Cyp2c (52), Cyp2d (53), and Cyp3a (54, 55) gene subfamily clusters. Experiments similar to the current study should now be possible to identify participation of the specific CYP1A1, CYP1A2, CYP1B1, CYP2D, CYP2E1, and CYP4 enzymes in distinct steps of the LM metabolic pathways during inflammation. Most likely, as we found with the CYP1 enzymes, numerous LM steps will be altered in the absence of the CYP2C, CYP3A, and CYP4 monooxygenases. Moreover, we predict that redundancy of CYP enzymes for many of these LM biosynthesis steps will likely be found during the acute inflammatory response.

Although mouse and human CYP1 enzymes display quite similar substrate specificities and inducers that upregulate CYP1 expression (46), substrate specificities vary to a much greater degree in the CYP2C, CYP3, and CYP4 families because many gene-duplication events have occurred such that one cannot assign orthologs between the mouse and human genomes (11). Hence, we predict that “humanized” P450 mouse lines, in which the “knocked-in” human-specific CYP2C, CYP3A, or CYP4 gene is expressed in place of the mouse ortholog, will provide the ultimate tool for further understanding the role of each human CYP2C, CYP3A, and CYP4 monooxygenase during LM biosynthesis/inactivation. Furthermore, the use of such tools would directly confirm the clinical importance of each P450 enzyme in a specific LM biosynthetic step. Identification of such steps should be useful in the future for drug targeting and, as such, promise a strong therapeutical potential.

Humanized P450 mouse lines include hCYP1A1/1A2 (56–58), hCYP1B1 (F. Gonzalez, personal communication), hCYP2C9 (52), hCYP2D6 (59), hCYP2E1 (60), and hCYP3A4 (61). Of note, the first humanized hCYP2D6 (59) and hCYP3A4 (61) mouse lines had the human gene inserted into the mouse genome that included all of the mouse Cyp2d and Cyp3a genes.

It should be mentioned that, in the current study, we screened for changes in the profiles and levels of several novel LMs and pathway markers from the AA metabolome (PGs, leukotrienes, lipoxins) and the DHA and EPA metabolomes (resolvins, protectins, and maresins), as well as their pathway markers relevant during the acute inflammatory response and its resolution. Whether CYP1 monooxygenases participate in metabolic steps (Fig. 7) involving any of the >125 other LMs during inflammation have not been assessed in these TKO mice. Moreover, the current study monitored the levels of basal peritoneal cells versus zymosan-initiated inflammatory exudates. Dozens of other cell types, plus numerous LM-mediated physiological and pathological stimuli (38, 39, 44),
in addition to zymosan-initiated inflammation, also remain to be scrutinized via this approach in future studies.

In summary, our hypothesis was that global ablation of all three CYP1 enzymes combined, and comparison of genetically modified TKO mice with WT mice, would uncover a number of disruptions in the signature profiles of LM pathways during an acute inflammatory response. Hence, to this end, we compared basal CYP1 activities, as well as zymosan-stimulated CYP1 activities. Although no statistically significant differences were found between untreated baseline TKO and WT peritoneal cells, eight statistically significant alterations were uncovered between TKO and WT peritoneal exudates during inflammation. Specifically, compared with WT mice, TKO mice revealed statistically significant large increases in LTβ, and decreases in PD1 levels; TKO mice also showed significantly decreased amounts of 5-HETE, 14-HDHA, 17-HDHA, 12-HEPE, 15-HEPE, and 18R-HEPE. Because of CYP1’s participation in these metabolic steps and the absence of CYP1 in TKO mice, the current study shows that one or more of the CYP1 enzymes play(s) a role in polyunsaturated fatty acid metabolism, in addition to further LM metabolism by cyclooxygenases and ALOXs. The possibility of direct CYP1 involvement exists. Further experiments will be required to determine the precise step at which each CYP1 monooxygenase participates to effect the eight changes in LM levels observed in the current study.

Compared with WT mice, TKO mice also show substantial differences in cell population response to zymosan. It is conceivable that, at one or more of these metabolic steps, CYP1 enzymes might clear metabolites related to the zymosan challenge rather than metabolizing LMs directly; failure to clear those metabolites in the absence of CYP1 might lead to an alteration in the local cellular response that could, in turn, produce a change in LM mediator production by cyclooxygenases and ALOXs.

Therefore, future experiments in zymosan-challenged mice genetically lacking only one of the Cyp1a1, Cyp1a2, or Cyp1b1 genes will be necessary to further dissect which specific CYP1 monooxygenase participates in each particular metabolic step. Because of the high degree of conserved functions and substrate specificities known to exist between the three mouse and three human CYP1 enzymes, such a reductionist approach should lead to elucidation of clinically important novel drug targets.

Acknowledgments

We thank colleagues, especially Larry Marnett (Vanderbilt University, Nashville, TN), for valuable discussions and/or careful reading of the manuscript. We are indebted to Dr. Frank J. Gonzalez for sharing the manuscript. We are indebted to Dr. Frank J. Gonzalez for sharing the Pharmacogenetics: beneficial as well as deleterious effects.

Disclosures

C.N.S. is an inventor on patents (resolvas) assigned to the Brigham and Women’s Hospital and licensed to Resolvyx Pharmaceuticals. C.N.S. is scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the scientific founder of Resolvyx Pharmaceuticals and owns equity in the company.

References

EHP: Toxicogenomics 111(1T): 45–51.


Table S1

Targeted MRM transitions used for LM quantification and LM values for identified AA-, DHA-, and EPA-derived LMs (summary of basal plus the two zymosan-treated levels)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q3</th>
<th>Zero hours</th>
<th>6 hours Zymosan</th>
<th>9 hours Zymosan</th>
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<td>LXA(_4)</td>
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<td>LXB(_4)</td>
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<td>221</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>LTB(_4)</td>
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<td>0.7</td>
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<td>282.4</td>
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<td>PGD(_2)</td>
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<td>233</td>
<td>8.3</td>
<td>82.6</td>
<td>24.5</td>
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<td>PGE(_2)</td>
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<td>189</td>
<td>31.2</td>
<td>74.2</td>
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<td>PGF(_{2\alpha})</td>
<td>353</td>
<td>193</td>
<td>*</td>
<td>86.9</td>
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<td>5-HETE</td>
<td>319</td>
<td>115</td>
<td>*</td>
<td>−9.2</td>
<td>−23.5</td>
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<tr>
<td>12-HETE</td>
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<td>12.6</td>
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<td>−1.7</td>
<td>−37.5</td>
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<td>*</td>
<td>*</td>
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<td>27.2</td>
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<td>*</td>
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<td>*</td>
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<tr>
<td>RvE2</td>
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<td>0.9</td>
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\(^a\) TKO values are expressed as percent (increase or decrease) of that in WT.