Deactivation of 12(S)-HETE through (ω-1)-hydroxylation and β-oxidation in alternatively activated macrophages^E

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Abstract  Polarization of macrophages to proinflammatory M1 and to antiinflammatory alternatively activated M2 states has physiological implications in the development of experimental hypertension and other pathological conditions. 12/15-Lipoxygenase (12/15-LO) and its enzymatic products 12(S)- and 15(S)-hydroxyicosatetraenoic acid (HETE) are essential in the process since disruption of the gene encoding 12/15-LO renders the mice unsusceptible to hypertension. The objective was to test the hypothesis that M2 macrophages catabolize 12(S)-HETE into products that are incapable of promoting vasocostriction. Cultured M2 macrophages metabolized externally added [14C]12(S)-HETE into more polar metabolites, while M1 macrophages had little effect on the catabolism. The major metabolites were identified by mass spectrometry as (ω-1)-hydroxylation and β-oxidation products. The conversion was inhibited by both peroxisomal β-oxidation inhibitor, thioridazine, and cytochrome P450 inhibitors. Quantitative PCR analysis confirmed the identified 12,19-dihydroxy-5,8,10,14-eicosatetraenoic acid and 8-hydroxy-6,10-hexadecadienoic acid metabolites were tested on abdominal aortic rings for biological activity. While 12(S)-HETE enhanced vasoconstriction, the metabolites did not. These results indicate that M2, but not M1, macrophages degrade 12(S)-HETE into products that no longer enhance the angiotensin II-induced vascular constriction, supporting a possible antihypertensive role of M2 macrophages.—— Tamas Kriska, M. J. Thomas, J. R. Falck, and W. B. Campbell

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Among the cells of innate immunity, macrophages are a heterogeneous and dynamic population that represent a spectrum of phenotypes (1). For example, lipopolysaccharide (LPS) and IFNγ (LPS/IFNγ) induce macrophages to the proinflammatory M1 phenotype, whereas interleukin (IL)-4 and -13 induce macrophages into the proresolving M2 phenotype. Macrophages are involved in the development of many pathological conditions, such as diabetes (2), atherosclerosis (3), and hypertension (4, 5). Depletion of macrophages can reverse these processes or prevent these conditions from developing (5, 6). An emerging body of data indicates the importance of the polarization state of macrophages in disease development (7, 8); however, this phenomenon is not well understood.

One of the consequences of macrophage polarization is a change in metabolic orientation that is essential for their function (9). Classically activated M1 macrophages display a shift toward the glycolytic pathway, while the alternatively activated M2 macrophages change toward fatty acid oxidation (10). Macrophages are also capable of metabolizing the oxidized forms of fatty acids, including HETEs, by β-oxidation (11). The physiological role of the resulting products has not been described.

12/15-Lipoxygenase (12/15-LO) is encoded by the Alox15 gene and is expressed in large quantities by macrophages. Its enzymatic products are 12(S)- and 15(S)-HETE that are essential in several forms of experimental hypertension (5). Deletion of Alox15 and the resulting loss of 12- and 15-HETE synthesis block the formation of experimental hypertension in mice. An R261Q polymorphism in 12-LO is associated with essential hypertension and increased urinary 12(S)-HETE excretion (12, 13).

Angiotensin II and endothelial dysfunction are involved in human and experimental hypertension (14). As
a consequence, inhibitors of the renin–angiotensin system lower blood pressure (15). Oxidized arachidonic acid (AA) products such as PGE2 or 12(S)-HETE enhance the constriction effect of angiotensin II (16, 17).

In the present study, we used M1/M2-polarized, cultured mouse macrophages as a model to investigate the influence of macrophage polarization on the metabolism of 12-HETE. Our findings indicate that alternatively activated M2 macrophages catalyze 12(S)-HETE into products that no longer enhance the angiotensin II-induced constriction of isolated blood vessels.

MATERIALS AND METHODS

Chemicals

Thioridazine and LPS were purchased from Sigma (St. Louis, MO). 4-phenyl-5-methyl-1,2,3-dithiadiazole (PMT) and 12(S),20-diHETE were obtained from Santa Cruz Biotechnology. 10 μM sodium pyruvate, 25 mM HEPES, 6 mM glucose, pH 7.4). Nonelicited peritoneal macrophages were harvested from isoflurane-euthanized mice by peritoneal lavage using sterile RPMI 1640 (Gibco catalog no. 22400-089). Neutrophils were purified from the bone marrow of isoflurane-euthanized mice. Macrophages were isolated using IV-Diol (Cayman Chemical Co. All solvents were HPLC grade and purified by reverse-phase HPLC (RP-HPLC). The [14C]15(S)-HETE was synthesized in the presence of sodium borohydride and then separating the products on reverse-phase HPLC (RP-HPLC). The [14C]15(S)-HETE was synthesized in the same manner, but by using 15LO (Biomol). 12(S)-19-dihETE was synthesized from 19(S)-HETE by using porcine 12-LO (Cayman Chemical Co.) in the presence of sodium borohydride at 37°C and then separating the products on reverse-phase HPLC (RP-HPLC).

Animals

Male C57BL6 mice (~ 25 g) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin, and procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011).

Tissue preparation

Aortas were dissected from isoflurane-anesthetized mice and cleaned of connective tissue in ice-cold HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 6 mM glucose, pH 7.4). Nonelicited peritoneal macrophages (PMs) were isolated from the bone marrow of isoflurane-euthanized mice by peritoneal lavage using sterile RPMI 1640 (Gibco catalog no. 22400-089) medium supplemented with 2 mM l-glutamine, 100 μM nonessential amino acids, 10 μM sodium pyruvate, 25 mM HEPES, 50 mM 2-mercaptoethanol, and 50 μg/ml gentamicin. Cells were cultured in poly-L-lysine (0.01 %)-coated dishes in RPMI medium supplemented with 10 ng/ml macrophage colony-stimulating factor (M-CSF). Following the 4 h of initial attachment period, the medium was changed, and the cells were incubated with either 20 ng/ml IFNγ plus 1 ng/ml LPS or 20 ng/ml IL-4 overnight to polarize the resting M0 macrophages to M1 and M2, respectively.

Isoflurane-anesthetized mice were disinfected with 70% ethanol. Femur and tibia from both hind legs were excised. The ends of the bones were cut, and the bone marrow was slowly flushed out with RPMI medium using a 1 ml syringe and a 25-gauge needle. The bone marrow cells were repeatedly pipetted up and down and passed through a 7 μm nylon cell strainer to produce a single cell suspension, and the suspension was centrifuged at 200 g for 10 min. The pellet was resuspended with fresh medium and seeded into 60 mm dishes at a concentration of 2.5 × 106 cells per dish. Cells were cultured for 6 days in the presence of 10 ng/ml M-CSF to induce differentiation into bone marrow-derived macrophages (BMDMs).

RT-PCR

Macrophages were scraped from the dishes and pelleted, and total RNA was isolated by using the RNeasy Mini Kit (Qiagen) and subjected to reverse transcription with the SuperScript III First-Strand cDNA Synthesis System (Invitrogen). cDNA samples were analyzed by quantitative PCR (qPCR; CFX96 iCycler; Bio-Rad) using the iQTM SYBR® Green Supermix reagent (Bio-Rad). PCR primers are listed in Table 1. Macrophage M1 polarization markers were IL-1β, inducible NO synthase (iNOS), and IL-6. Macrophage M2 polarization markers were arginase 1, Chi3l3/Ym1, and Mcr1. Mitochondrial β-oxidation markers were glucose transporter 1 (GLUT1) and hexokinase 2 (HK2). Peroxisomal β-oxidation markers were hydroxysteroid 17β dehydrogenase 4 (Hsd17b4) and ATP binding cassette subfamily D member 2 (ABCD2). The expressions of seven cytochrome P450 (CYP450) enzymes (Cyp2U1, Cyp1A2, Cyp4A12, Cyp2A5, Cyp4F18, Cyp2E1, and Cyp1B1) were checked in M0, M1, and M2 macrophages. Mouse 18S was used as a housekeeping gene. The PCR program consisted

| Primer | Sequence |
|--------|----------|
| IL-1β Fw | 5′-CAC ACA ACA AGT GAT ATT CTC CAT G-3′ |
| IL-1β Rev | 5′-GAT CCA CAC TCT CCA GCT GCA-3′ |
| iNOS Fw | 5′-GAG ACA GGA AAG TCT GAAGCCA C-3′ |
| iNOS Rev | 5′-CCA GCA GAG TTG GCT CCT CTT G-3′ |
| IL-6 Fw | 5′-CAA AGG CAG AGT CCT TCA GAG-3′ |
| IL-6 Rev | 5′-CAG TCC TTC TGT GAC TCC AGC-3′ |
| Arginase 1 Fw | 5′-CAC AAG AAT GGA AGA AGA GTG-3′ |
| Arginase 1 Rev | 5′-ACA TTA TGG CTA AGA GCT A-3′ |
| Chi3l3 Fw | 5′-GCA GAA GAT CAG CCA GCC TCC-3′ |
| Chi3l3 Rev | 5′-ATT GCC CCT TCT TCC GTA GCC CTA-3′ |
| Mrc1 Fw | 5′-TTT CTA TCC AGA CTG CTC G-3′ |
| Mrc1 Rev | 5′-CAC AAA GCC ACT TCC CTC C-3′ |
| GLUT1 Fw | 5′-GCTT CTTCC AACCT GGA CTC TACT CAG-3′ |
| GLUT1 Rev | 5′-ACG AGG AGC ACC TGT AAG ATG A-3′ |
| HK2 Fw | 5′-CCC TTG GAA GAT GTT GCC CAC TCC-3′ |
| HK2 Rev | 5′-CCT CTC CTT GGC ATT ACG CAG G-3′ |
| Hsd17b1 Fw | 5′-TGG CTT CTG CAG GAC TGG G-3′ |
| Hsd17b1 Rev | 5′-CTG ATT CCG CTT TCT GAC GAG G-3′ |
| ABCD2 Fw | 5′-CTG ATT CCG CCT TCG TGC GAC GTG-3′ |
| ABCD2 Rev | 5′-CAT TTC GCC CCG TGG TAT AAG T-3′ |
| S18 Fw | 5′-AAA TCA GTG ATG GTC CTT TCT GG-3′ |
| S18 Rev | 5′-GCT CTA GAA TTA CCA CAG TAT TAA AA-3′ |
| Cyp2U1 Fw | 5′-TGG CTT CTC TCC ACA TGA TCC C-3′ |
| Cyp2U1 Rev | 5′-CGA TGA GGA CAG AAG TCG TCT C-3′ |
| Cyp1A2 Fw | 5′-CAT CAC AAG TGC CTT GAT CAA-3′ |
| Cyp1A2 Rev | 5′-AAT GCT CCA GGT GAT GGC TGT G-3′ |
| Cyp4A12 Fw | 5′-CAG ATG GTC CTC TAA TGA GTC C-3′ |
| Cyp4A12 Rev | 5′-GAT GTC GAC GAA ATC TCA TGG CCA-3′ |
| Cyp2A5 Fw | 5′-CCT CTC TCA GTC GAA TGC TGG A-3′ |
| Cyp2A5 Rev | 5′-TGA CGG CTC TGT TGC CAG CAA-3′ |
| Cyp1A18 Fw | 5′-GGA AAG GCT CTG TGT GAT GGG-3′ |
| Cyp1A18 Rev | 5′-TGG GTG TCT TGC CAG GTT CTA-3′ |
| Cyp2E1 Fw | 5′-AGG CTT CAG AGG AGG TGC CAT T-3′ |
| Cyp2E1 Rev | 5′-AAA ACC TCC GCA GGT CCT CTC A-3′ |
| Cyp1B1 Fw | 5′-GCC ACT ATT ACG GAC ATC TCC GGG-3′ |
| Cyp1B1 Rev | 5′-ACA ACC TGG TCC AAC TCA GCC C-3′ |

Fw, forward primer; Rev, reverse primer.
of 33 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL).

12(S)- and 15(S)-HETE metabolism and RP-HPLC analysis

Dishes with M1- or M2-polarized macrophages were rinsed twice with PBS and overlaid with nonsupplemented RPMI 1640 medium containing 100 nM 12(S)-HETE plus 25 nCi [1-14C]12(S)-HETE. After 1, 2, or 4 h of incubation at 37°C, the reactions were stopped by adding ice-cold ethanol to a final concentration of 15%. Cells were pelleted, and the buffer was acidified (pH < 3.5) with glacial acetic acid and extracted on Bond Elut octadecylsilyl liquid-solid-phase extraction columns (Agilent Technology) as previously described (18). Extracted metabolites were dried under a stream of nitrogen and stored at −40°C until analysis. Polarized macrophages were also given medium containing 100 nM 15(S)-HETE plus 25 nCi [1-14C]15(S)-HETE or 100 nM trioxilin plus 25 nCi [1-14C]trioxilin. In some experiments, cells were pretreated with 50 µM peroxisomal β-oxidation inhibitor thioridazine or 20 µM mitochondrial β-oxidation inhibitor (+)-etomoxir for 1 h before the [1-14C]12(S)-HETE was added. In one set of experiments, macrophages were pretreated with 100 µM CYP1B1 inhibitor TMS or 100 µM CYP2E1 inhibitor PMT for 1 h before the [1-14C]12(S)-HETE was added. For mass spectrometric analysis, 1 µM unlabeled 12(S)-HETE or vehicle was incubated with macrophages for 2 h, followed by solid phase extraction as described above.

Metabolites were resolved by RP-HPLC using a Nucleosil-C18 column (5 µ, 4.6 × 250 mm). The following solvent system was used: Solvent A was water, and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program consisted of a 40 min linear gradient from 50% solvent B in A to 70% solvent B. Flow rate was 1 ml/min. Column eluate was collected in 2 ml fractions. Absorbance was also monitored at 235 nm, and column fraction radioactivity was determined by liquid scintillation spectrometry. For samples from unlabelled 12(S)-HETE and vehicle incubations, fractions corresponding to the elution of radioactive metabolites were collected, pooled, and liquid-liquid extracted by using a 1:1 mixture of cyclohexane:ethyl acetate. The organic phase containing the metabolites was collected, evaporated under a stream of nitrogen gas, and stored at −40°C until analyzed.

Mass spectrometric analysis

The unknown metabolites of 12(S)-HETE and 15(S)-HETE that were separated with RP-HPLC were identified by LC/MS as previously described (19, 20). Analysis was performed on an Agilent 6460 triple quadrupole mass spectrometer interfaced to the ESI source. Data were acquired in the negative ion mode from m/z 12 to 300 with a scan time of 200 ms. M2 scans were conducted in the negative ion mode from m/z 12 to 500 with a scan time of 300 ms. Metabolites observed in the reconstructed total ion current chromatograms of the 12(S)-HETE incubations but absent in the vehicle incubations were further analyzed by MS/MS. Product ion analysis was performed by using a scan range from m/z 20 to 500 with a scan time of 500 ms, fragmentor at 135 V, a collision energy of 12 V, and a cell acceleration of 7 V. Samples were separated on a Kromasil 150 × 2.0 mm column packed with 5µ particles having 100 Å pores. The flow rate was 0.3 ml/min with the following profile: 0–1 min, 35% B; 1–40 min, 35% B to 80% B; then reequilibrate to 35% A over 17 min. Solvent A was water containing 0.1% acetic acid while solvent B was pure acetonitrile.

High mass accuracy for the m/z 335 ion and its fragments was obtained by using a Thermo Fisher Orbitrap Velos mass spectrometer. Overall, the fragmentation was similar to that produced by the Agilent triple quadrupole. The samples were injected onto a PLRP-S Michrom column, 0.3 x 150 mm, packed with 3 µm particles having 100 Å pores, at a flow rate of 5 µl/min. Gradient elution used water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B. The profile was as follows: hold at 5% B for 3 min, then to 90% B at 33 min, hold at 90% B for 5 min, back to 5% B in 5 min, and then reequilibrate at 5% B for 20 min. The effluent was interfaced to the ESI source. Data were acquired in the negative ion mode.

Isometric tension in mesenteric arteries

Abdominal aortic sections (∼1.5 mm in length) were mounted in a four-chamber wire myograph (model 610M; Danish Myo Technology A/S) and maintained at 37°C in physiological saline solution: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 24 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.926 mM EDTA, and 5.5 mM glucose, gassed with O₂ containing 5% CO₂ as previously described (21). The aortic rings were stretched to a resting tension of 1.5 mN. The vessels were stimulated three times with 60 mM KCl plus 100 nM U46619 for 8–10 min at 10 min intervals. Aortic rings were pretreated with 100 nM 12(S)-HETE, 15(S)-HETE, 8-Br-cAMP, 12(S),19-diHETE, or 12(S),20-diHETE for 8 min, followed by addition of cumulative concentrations of angiotensin II (100 nM to 1 µM). Results are expressed as percent contraction of the aortas, with 100% representing the maximum U46619/KCl contraction.

Statistics

Data are presented as means ± SEM. The two-tailed Student’s t-test or ANOVA was used for determining the significance of observed differences between experimental values, with P < 0.05 considered statistically significant.

RESULTS

Polarization of PMs and BMDMs to M1 or M2 phenotype

Incubation of cultured nonelicited PMs or BMDMs with IFNγ/LPS or IL-4/IL-13 resulted in robust morphological differences. IFNγ/LPS-induced M1 cells were flattened and rounded with a cobblestone or fried-egg shape (Fig. 1A). In contrast, the IL-4/IL-13-induced M2 cells were thin and elongated with a spindle shape (Fig. 1B). There were no morphological differences between the polarized PMs and BMDMs. In PMs, the morphological changes that were induced by IFNγ/LPS and IL-4/IL-13 were accompanied by dramatic shifts of polarization markers toward the M1 (Fig. 1C) and M2 (Fig. 1D) phenotypes, respectively. The measured markers included iNOS, IL-1β, and IL-6 for M1 polarization and arginase-1, Chi3L3/Ym1, and Mrcl for M2 polarization. Similar shifts were observed with BMDMs (Fig. 1E, F). M1 marker changes were identical between PMs and BMDMs; however, M2 marker changes differed. In PMs, Chi3L3 and Mrcl were upregulated, while arginase-1 remained unchanged (Fig. 1B). In contrast, arginase-1 showed the greatest upregulation in BMDMs (Fig. 1F).

Metabolism of 12(S)- and 15(S)-HETE by M1- and M2-polarized macrophages

PM or BMDM cultures were polarized to the M1 or M2 phenotype and incubated with 100 nM 12(S)-HETE and
absent. However, similar to 12(S)-HETE, M2 macrophages metabolized the 15(S)-HETE to a much greater extent than M1 macrophages.

**Mass spectrometric identification of the major macrophage 12(S)- and 15(S)-HETE metabolites**

All three major metabolites detected by RP-HPLC were more polar than parental 12(S)- or 15(S)-HETE, suggesting further oxidation or hydrocarbon chain shortening. To identify the P1 and P2 12(S)-HETE metabolites, as well as the P3 15(S)-HETE metabolite, the incubation of M2-polarized PMs was repeated with unlabeled 12(S)- or 15(S)-HETE, and the metabolites were extracted and separated on RP-HPLC as described for radioactive-labeled products. RP-HPLC fractions corresponding to P1, P2, and P3 were analyzed by LC/MS/MS.

A total ion chromatogram of P1 showed that the major component had a M-1 ion of m/z 335.3 for all occurrences. This mass could correspond to the molecular mass of a diHETE (Fig. 3B, C, inset). MS/MS fragmentation of the m/z 335.3 ion produced fragments of m/z 317 [M-H-H2O], m/z 299 [M-H-2H2O], and m/z 255 [M-H-2H2O-CO2], indicating the presence of two hydroxyl groups and a carboxyl group (Fig. 3A). Fragment ions of m/z 179.2 and 208.3 indicated scission at C11–C12 and C12–C13, respectively, and were consistent with a hydroxyl group at C12. High-resolution MS analysis that can predict chemical formulas of ions gave an exact mass for the M-1 ion of m/z 335.2212 and indicated C20H33O4. The ion fragment m/z 208.11026 contained C17H31O4, while ion fragment m/z 273.22192 was C17H29O from the loss of CO2 (43.99 Da). Ion m/z 229.19586 represented the C17H29 fragment of m/z 273 from the loss of C2H4O (44.0262 Da) and indicated the presence of a second hydroxyl group in the ω-1 position.

To confirm this exact position of the second hydroxyl group, we analyzed the fragmentation pattern of both 12,19-dihydroxy-5,8,10,14-eicosatetraenoic acid (12,19-diHETE) and 12,20-diHETE standards (Fig. 3B, C). Fig. 3B shows the MS/MS fragmentation pattern of 12,19-diHETE. The m/z 229 fragment represented scission at C18–C19 and is a signature fragment revealed by the high-resolution MS analysis. In contrast, repositioning the second hydroxy group in the ω position forced the scission at C19–C20 (22–24), resulting in a characteristic fragment of 243 (Fig. 3C). The MS/MS spectrum for P1 contained m/z 229, but not m/z 243. Thus, the structure of P1 was identified as the (ω-1)-hydroxylation product 12,19-diHETE.

The major component of P2 consisted mostly of a M-1 ion of m/z 267.3 that corresponds to a hydroxylated C16:2 structure. MS/MS fragmentation produced ions of m/z 249.2, 155.1, and 127.0 that were consistent with loss of H2O, scission at C8–C9, and scission at C7–C8, respectively, and indicated a hydroxyl group at C8. Ion fragments of m/z 110.9 [m/z 155, M-H-CO2] and m/z 137.0 [m/z 155, M-H2O] suggest the presence of one carboxyl and one hydroxyl group in the m/z 155 fragment. Thus, P2 was consistent with the β-oxidation product 8-HHDD, a tetranor 12(S)-HETE metabolite (Fig. 3D). The ion m/z...
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265.2 was also present in P2, indicating the presence of 8-hydroxy-4,6,10-hexadecatrienoic acid.

The major component of P3 consisted mostly of a M-1 ion of m/z 265.1 that corresponds to a hydroxylated C16:3 structure. MS/MS fragmentation produced ions of m/z 247.4 and 165.0 that are consistent with loss of H₂O and scission at C10–C11, respectively, and indicated a hydroxyl group at C11. Ion fragments of m/z 220.8 [M-H-CO₂], m/z 247.4 [M-H₂O], and m/z 203.2 [M-H₂O] suggest the presence of one carboxyl and one hydroxy group on the structure. Thus, P3 was consistent with the β-oxidation product 11-hydroxy-4,7,9-hexadecatrienoic acid (Fig. 3E). The ion m/z 266.9 was also present in P3, indicating the presence of 11-hydroxy-7,9-hexadecadienoic acid.

Fig. 3F summarizes the metabolite pathways observed with 12(S)-HETE and 15(S)-HETE. The most noticeable difference between the metabolism of these two HETEs is the absence of (ω-1)-hydroxylation of 15(S)-HETE.

(ω-1)-Hydroxylation β-oxidation of 12(S)-HETE by M2-polarized macrophages

To test the hypothesis that P2 and P3 are β-oxidation products, mRNA of M1- and M2-polarized PMs or BMDMs were subjected to qPCR analysis for mitochondrial markers (Glut1 and Hk2) and peroxisomal β-oxidation markers (Hsd17b4 and ABCD2). The results are summarized in a clustergram in Fig. 4A. M1 macrophages showed an increase in mitochondrial markers, while M2 macrophages had an increase in peroxisomal markers of β-oxidation. The M0 macrophages were similar to M2-polarized macrophages. To determine the impact of these mRNA changes on 12(S)-HETE metabolism, M2-polarized macrophages were pretreated with either mitochondrial [(+)-etomoxir] or peroxisomal (thioridazine) β-oxidation inhibitors and incubated with [14C]12(S)-HETE for 2 h. The (+)-etomoxir had no effect on 12(S)-HETE metabolism (Fig. 4B), while the thioridazine inhibited the formation of both P1 and P2 metabolites by 93.3 ± 3.8% and 71.5 ± 5.2%, respectively.
These data with inhibitors suggest that peroxisomal oxidation processes may be involved in the formation of both P1 and P2 metabolites of 12(S)-HETE. Because the structure of P1 metabolite indicated formation through a (ω-1)-hydroxylation pathway, we analyzed the mRNA of M0-, M1-, and M2-polarized macrophages for possible upregulation of CYP450 enzymes that have been reported in macrophages. The results are summarized in a clustergram on Fig. 4D. Of the seven CYP450 enzymes included in the analysis, only Cyp2E1 and Cyp1B1 were upregulated in M2-polarized macrophages. To test whether these enzymes were responsible for the P1 metabolite formation, we pretreated the M2-polarized macrophages with a specific Cyp2E1 inhibitor (PMT) or Cyp1B1 inhibitor (TMS). Both CYP1B1 and CYP2E1 inhibitors decreased P1 by 38.6 ± 5.6% and 17.6 ± 8.8%, respectively. At the same time, the CYP1B1 and CYP 2E1 inhibitors increased the formation of P2 by 96.5 ± 29.4% and 48.1 ± 13.6%, respectively (Fig. 4E, F).

Loss of the enhanced angiotensin II vasoconstrictor effect of 12(S)-HETE by MΦ metabolism

In abdominal aortic rings, angiotensin II (10 and 100 nM) caused constriction of 4.5 ± 0.9% and 15.1 ± 2.9%, respectively. These constrictions were significantly enhanced by 3.5% and 10.0%, respectively, after preincubating the rings with 100 nM 12(S)-HETE (Fig. 5A). To assess the biological activity of the 12(S)-HETE-derived metabolites 12(S),19-DiHETE in P1 and 8-HHDD in P2, synthetic standards were tested individually in the same fashion and concentration as the 12(S)-HETE. Unlike 12(S)-HETE, 12(S),19-diHETE (Fig. 5B) failed to significantly alter the angiotensin II-induced constrictions. To test whether the position of the second hydroxyl group was crucial for activity, we also examined synthetic 12(S),20-diHETE (Fig. 5C). It also failed to enhance the angiotensin II-induced vasoconstriction. The β-oxidation product 8-HHDD was also inactive (Fig. 5D). These data indicate that M2-polarized macrophages oxidize 12(S)-HETE into (ω-1)- and β-oxidized products that no longer enhance the vasoconstrictor effect of angiotensin II. Thus, (ω-1)- and β-oxidation represent inactivation pathways for 12(S)-HETE. Interestingly, preincubating the rings with 100 nM 15(S)-HETE, the second most abundant metabolite of 12/15-LO, did not enhance the angiotensin II constrictions (Fig. 5E).

DISCUSSION

Macrophages are activated in response to a variety of stimuli. Depending on the nature of the stimulus, the macrophage response can be antimicrobial, immunomodulatory, phagocytic, angiogenic, or promoting wound healing and involves polarization toward opposite phenotypes, proinflammatory M1 and proresolving M2 (25). In actuality, macrophage polarization represents a spectrum of phenotypes, and M2 macrophages were further divided into subpopulations (26). LPS and IFNγ induce M1 polarization that is characterized by inflammatory cytokine secretion and NO production. IL-4 and -13 are commonly used to polarize macrophages to the M2 state that can be defined by upregulation of arginase-1, Chi3l3, and Mrc1. Since M2 macrophages have much greater heterogeneity in morphology and marker expression, their identification require the use of multiple markers. We used both nonelicited PM and BMDM cultures to produce M1 and alternatively
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activated M2 macrophages. We found that these two subsets differed in term of polarization marker upregulation. In resident PMs, arginase-1 is constitutively expressed (27) and is therefore not a good indicator of polarization state. The same is true for Chi313 and Mrc1 for BMDMs (25). Our qPCR results were consistent with this expression pattern. While arginase-1 in PMs and Chi313 and Mrc1 in BMDMs showed very little upregulation beyond their high background expression, other markers clearly confirmed M2 polarization.

Despite all these differences in the expression of markers, PM and BMDM M2 cultures were morphologically identical and catabolized 12(S)-HETE to the same extent. On the other hand, M1-polarized cultures of both origins showed very little 12(S)-HETE catabolic activity. Evidence suggests that cellular energy metabolism is one of the major functional changes that macrophages undergo in response to a polarization signal. M1-polarized macrophages shift metabolism toward aerobic glycolysis, while M2 macrophages use fatty acid oxidation as their main metabolic pathway (9). This difference is consistent with our findings that M2-polarized macrophages catabolized 12(S)-HETE to a greater extent than M1 macrophages. The catabolic rate was similar to a previous report (11), taking several hours for the cells to consume the available 12(S)-HETE. This can be described as a relatively slow process with unclear physiological consequences. Because metabolism of externally supplied 12(S)-HETE was determined, the rate-limiting step could have been the uptake. Macrophage catabolism of endogenously synthetized 12(S)-HETE could be a much faster process. When the recovery of radiolabeled 12(S)-HETE was determined following incubation with macrophages, more than 75% was recovered from the medium. The loss included both cell uptake and medium remaining in the culture dish. Importantly, we found no difference in the recoveries between cultures of M1- vs. M2-polarized macrophages. Thus, our studies focused on the metabolites that were released from the M1 and M2 macrophages into the medium. The cell-associated radioactivity was not analyzed further.

The observed difference in metabolism by M1- and M2-polarized macrophages is not unique to 12(S)-HETE. M2 macrophages metabolized 15-HETE through \( \beta \)-oxidation to a much greater extent as compared with M1 cells. Interestingly, the \((\omega-1)\)-hydroxylation product of 15(S)-HETE was completely absent. While CYP450-catalyzed 15,20-diHETE formation is possible (28), \((\omega-1)\)-hydroxylation of 15(S)-HETE has not been reported.

\( \beta \)-oxidation can proceed through two alternative pathways linked to mitochondria and peroxisomes. Peroxisomes are ubiquitous organelles involved in lipid metabolism. They are responsible for the cellular metabolism of very-long-chain, highly unsaturated fatty acids such as docosahexaenoic acid, cholesterol, bile acids, eicosanoids, and hydrogen peroxide (29). The most well-characterized metabolic pathway in peroxisomes is fatty acid \( \beta \)-oxidation, which plays a critical role in lipid catabolism (30). Peroxisome-deficient cells fail to convert 12(S)-HETE into more

Fig. 4. \( \beta \)-oxidation and \((\omega-1)\)-hydroxylation of 12(S)-HETE by M2 macrophages. A: qPCR marker analysis summarized in a clustergram indicates strong correlation between M1 macrophages and mitochondrial \( \beta \)-oxidation as well as M2 macrophages and peroxisomal \( \beta \)-oxidation. Expression results were obtained from nonpolarized macrophages labeled with M0. In the clustergram, green color indicates low, while red color indicates elevated normalized expression levels. Mitochondrial \( \beta \)-oxidation inhibitor (+)-etomoxir (Mito Inh) had no effect on 12(S)-HETE metabolite formation (B), while the peroxisomal \( \beta \)-oxidation inhibitor (Perox Inh) thioridazine inhibited the formation of both metabolites, P1 and P2 (C). D: qPCR expression analysis of different CYP450 isoforms is summarized in a clustergram. E shows the effect of specific CYP2E1 inhibitor (CYP2E1 Inh) on metabolite formation by M2 macrophages. F: Effect of specific CYP1B1 inhibitor (1B1 Inh) on metabolite formation by M2 macrophages. Representative traces of three experiments are shown.
polar products, suggesting a peroxisomal ω-oxidation pathway (31, 32). Also, purified liver and kidney peroxisomes metabolize 12(S)-HETE to 8-OH 16:3, an apparent endpoint of the 12(S)-HETE ω-oxidation (33, 34). In the present study, 8-HHDD was one of the major macrophage metabolites of 12(S)-HETE, and the formation of this metabolite was blocked by the selective peroxisomal inhibitor, thioridazine (35). Our qPCR results confirmed that peroxisomal, but not mitochondrial, ω-oxidation markers were upregulated in M2-polarized macrophages. In stark contrast, the peroxisomal ω-oxidation markers were downregulated in M1-polarized macrophages, providing a possible explanation for the observed low level of 12(S)-HETE metabolism.

Mitochondrial ω-oxidation is also implicated in 12(S)-HETE metabolism. The conversion of 12(S)-HETE by isolated mitochondria is carnitine-dependent (36). This was surprising, since earlier reports asserted that peroxisome-deficient cells are unable to metabolize 12(S)-HETE (31, 32). It is still unclear why different cell types may use different metabolic pathways. However, in the present study, both downregulation of mitochondrial oxidation markers and the failure of mitochondria-specific ω-oxidation inhibitor, (+)-etomoxir, to inhibit the metabolism of 12(S)-HETE indicated that mitochondrial oxidation may not be involved.

Unexpectedly, peroxisomal inhibitor thioridazine also inhibited P1 metabolite production. The P1 metabolite is formed by (ω-1)-hydroxylation that most likely requires a CYP450. Some microsomal CYP450s such as CYP2D6 or CYP2B metabolize thioridazine, and other reactions by these CYP450s are inhibited by it (37, 38). Thioridazine is a poor inhibitor of CYP2E1 and has not been tested on CYP1B1 (39, 40). Coincidentally, one study showed the presence of CYP2E1 in peroxisomes (41). Our pharmacological findings raise the possible, but do not provide proof, for the involvement of peroxisomes in macrophage (ω-1)-hydroxylation of 12(S)-HETE. However, there is certainly the possibility that thioridazine acts through a different mechanism. Our current knowledge about CYP450-mediated (ω-1)-hydroxylation in peroxisomes is very limited. Until proven otherwise, macrophage (ω-1)-hydroxylation is likely due to microsomal or mitochondrial CYP450s.

Previously, only nonpolarized macrophages were used to study the metabolism of 12(S)-HETE. It was postulated that the 12(S)-HETE conversion occurs in peroxisomes by ω-oxidation. Extensive analysis of the products by HPLC and GC/MS was reported (11, 42). Our goal here was to determine the impact of different polarization modes on 12(S)-HETE metabolism. Strikingly, M1 polarization prevented most of the 12(S)-HETE metabolism. M2-polarized macrophages, on the other hand, produced two distinct

Fig. 5. Effect of 12(S)-HETE and macrophage metabolites P1 and P2 on angiotensin II (AngII)-induced vasoconstriction in mouse abdominal aortic rings under a resting tension. A: The constrictions to angiotensin II were significantly enhanced by preincubating the rings with 100 nM 12(S)-HETE. Synthetic P1 and P2 compounds were added in the same fashion and concentration as 12(S)-HETE. Neither P1 (12,19-diHETE) (B), its ω-hydrolyzed regioisomer (12,20-diHETE) (C), nor P2 (8-HHDD) (D) significantly altered basal tension or the angiotensin II-induced constrictions. E: Preincubating the rings with 100 nM 15(S)-HETE had no effect on angiotensin II-induced constrictions. The bars are representing the mean ± SEM of 12 rings. * P < 0.05; ** P < 0.01 compared with non12(S)-HETE pretreated control.
products that were detectable by HPLC. Both of these products were stable, and no transient products were observed during the time-course measurement. On RP-HPLC, the metabolites eluted earlier than 12(S)-HETE, indicating that they were more polar. Using the LC/MS/MS technique, we identified the two peaks as 12,19-diHETE and 8HHDD. The latter metabolite was the major product identified in previous studies. To date, formation of 12,20-diHETE was reported in polymorphonuclear leukocytes (43–45). However, formation of an (ω-1)-hydroxylated metabolite of 12(S)-HETE was not previously observed in macrophages or any other cell types.

We tried to identify the enzyme responsible for formation of the P1 metabolite. Earlier PCR-based expression analysis revealed the presence of only two CYP450 enzymes in macrophages (46). Here, we found seven macrophage CYP450s, but only two were upregulated in M2-polarized macrophages, CYP2E1 and CYP1B1. Both of these enzymes are considered (ω-1)-hydroxylases, with the CYP2E1 rather specific (47). CYP1B1 has a wider range of terminal hydroxylase activity (48), but with unknown reactivity toward 12(S)-HETE. Inhibition of these two CYP450 (ω-1)-hydroxylases shifted the metabolite formation toward the β-oxidation product. The same effect was observed with 15(S)-HETE metabolism. In the absence of ω-hydroxylation of 15(S)-HETE, the β-oxidation product P3 was in great abundance. It is not clear why the inhibition of P1 formation by CYP2E1 and CYP1B1 inhibitors was not complete. There are the possibilities that more CYP450 isoforms participate in the process or that the inhibitors have low efficacy. CYP4F3 was responsible for ω-hydroxylation of the proinflammatory eicosanoids such as 12(S)-HETE in human tissues (49); however, its murine analog, CYP4F18, was downregulated in M2 macrophages and thus, cannot be responsible for P1 metabolite formation.

The processes of β- and (ω-1)-oxidation may play an important role in the resolution of inflammation, since 12(S)-HETE is a potent proinflammatory mediator. 12(S)-HETE constricts blood arteries and also contributes to the vascular response to angiotensin II (17). We tested the biological activity of the individual 12(S)-HETE metabolites, 8HHDD and 12,19-diHETE, on isometric tension of aortic rings. While 12(S)-HETE significantly increased the angiotensin II-induced constriction of aortic rings, its major oxidation products, 12,19-diHETE and 8HHDD, failed to do so. The observed difference in 12(S)-HETE metabolism between M1 and M2-polarized macrophages is consistent with the accepted proresolving properties of the latter phenotype. Thus, M1 polarization is associated with inflammation and M2 with antiinflammatory conditions.

In conclusion, M1- and M2-polarized macrophages exhibited striking differences in their ability to metabolize exogenous 12(S)-HETE in vitro. Only alternatively activated M2 macrophages transformed the 12(S)-HETE into products that no longer enhanced the angiotensin II-induced vasoconstriction. Two major pathways were identified, β-oxidation and CYP450-mediated (ω-1)-oxidation, that resulted in the formation of 8HHDD and 12,19-diHETE, respectively. These results indicate the ability of alternatively activated M2 macrophages to degrade 12(S)-HETE and contribute to their antiinflammatory functions.

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