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TITLE PAGE

Asymmetric Acetylation of the Cyclooxygenase-2 Homodimer by Aspirin and Its Effects on the Oxygenation of Arachidonic, Eicosapentaenoic and Docosahexaenoic Acids

Narayan P. Sharma, Liang Dong, Chong Yuan, Kathleen R. Noon and William L. Smith

Departments of Biological Chemistry and Pharmacology,

University of Michigan, Ann Arbor, MI 48109 USA,

Author Affiliations: Biological Chemistry—NPS, LD, CY, WLS; Pharmacology--KRN

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Running Title: Cyclooxygenase-2 (COX-2) and aspirin

Corresponding author: William L. Smith, Department of Biological Chemistry, University of Michigan Medical School, 1150 W. Medical Center Dr., 5301 MSRB III, Ann Arbor, MI 48109-0606. Tel: (734) 647-6180; Fax: (734) 763-4581, E-mail: smithww@umich.edu

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Tables: 4

Figures: 4

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Words in Abstract: 198

Words in Introduction: 610

Words in Results: 1663

Words in the Discussion: 985

Nonstandard Abbreviations: PGHS, prostaglandin endoperoxide H synthase; PG, prostaglandin; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle medium; MEM, Minimum Essential medium; AA, arachidonic acid; TLC, thin layer chromatograpy; hu, human; mu, murine; nsNSAIDs, nonspecific nonsteroidal anti-inflammatory drugs; EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid; PBS, phosphate buffered saline; TCA, tricholoracetic acid; 11-HETE, 11-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 11-HEPE, 11-hydroxyeicosapentaenoic acid; 12-HEPE, 12-hydroxyeicosapentaenoic acid; 15-HEPE, 15- hydroxyeicosapentaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; 13-HDHA, 13-hydroxydocosahexaenoic acid, 17-HDHA, 17-hydroxydocosahexaenoic acid; RvE1, Resolvin E1 (5*S*, 12*R*, 18*R*-trihydroxy-

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6Z,8E,10E,14Z,16E-EPA); 17*R*-RvD1, 17*R*-Resolvin D1 (7*S*, 8*R*, 17*R*-trihydroxy-4Z,9E,13Z,15E,19Z-docosahexaenoic acid)

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Abstract

Prostaglandin endoperoxide H synthases (PGHSs)-1 and -2, also called cyclooxygenases (COXs), convert arachidonic acid (AA) to prostaglandin H_2 (PGH_2) in the committed step of prostaglandin biosynthesis. Both enzymes are homodimers, but the monomers often behave asymmetrically as conformational heterodimers during catalysis and inhibition. Here we report that aspirin maximally acetylates one monomer of human (hu) PGHS-2. The acetylated monomer of aspirin-treated huPGHS-2 forms 15-hydroperoxyeicosatetraenoic acid from AA while the non-acetylated, partner monomer forms mainly PGH_2 but only at 15-20% of the rate of native huPGHS-2. These latter conclusions are based on the findings that the nonsteroidal anti-inflammatory drug diclofenac binds a single monomer of native huPGHS-2 having an unmodified Ser-530 to inhibit the enzyme and that diclofenac inhibits PGH_2 but not 15-hydroperoxyeicosatraenoic acid formation by acetylated huPGHS-2. The 18*R*- and 17*R*-resolvins putatively involved in resolution of inflammation are reportedly formed via aspirin-acetylated PGHS-2 from eicosapentaenoic acid and docosahexaenoic acid, respectively, so we also characterized the oxygenation of these omega-3 fatty acids by aspirin-treated huPGHS-2. Our *in vitro* studies suggest that 18*R*- and 17*R*-resolvins could be formed only at low rates corresponding to less than 1% and 5%, respectively, of the rates of formation of PGH_2 by native PGHS-2.

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Introduction

Prostaglandin endoperoxide H synthases (PGHSs[1])-1 and -2, also called cyclooxygenases (COXs), catalyze the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2) in the committed step in prostaglandin and thromboxane biosynthesis (Rouzer and Marnett, 2003; Rouzer and Marnett, 2008; Schneider et al., 2007; Smith, 2008; van der Donk et al., 2002). The enzymes have two catalytic activities: (a) a cyclooxygenase (COX) activity responsible for oxygenating AA to PGG_2 and (b) a peroxidase activity that catalyzes a two electron reduction of PGG_2 to PGH_2. PGHSs are homodimers that exhibit half of sites COX activity with AA as the substrate (Yuan et al., 2006). That is, only one monomer is able to catalyze a reaction at a given time. The non-catalytic monomer functions as an allosteric regulator of the catalytic monomer (Kulmacz and Lands, 1985; Yuan et al., 2006; Yuan et al., 2009). This is of potential importance *in vivo* where certain fatty acids can function as allosteric regulators of PGHSs inhibiting PGHS-1 and stimulating PGHS-2 (Yuan et al., 2009).

Nonspecific nonsteroidal anti-inflammatory drugs (nsNSAIDs) inhibit the COX activities of both PGHS-1 and PGHS-2 whereas COX-2 inhibitors called coxibs are more selective for huPGHS-2 (Grosser et al., 2006). nsNSAIDs and coxibs fall into three general categories based on their mechanisms of action, and their mechanisms are related in part to the way in which they interact with the dimeric structures of PGHSs (Blobaum and Marnett, 2007; DeWitt, 1999; Smith and DeWitt, 1999; Smith et al., 2000; Walker et al., 2001). One category of inhibitors, which includes ibuprofen and mefenamic acid, are freely reversible competitive inhibitors. Binding of these inhibitors to the COX sites of both monomers comprising a dimer is required for inhibition of AA oxygenation by PGHS-2 (Prusakiewicz et al., 2009). This mechanism of inhibition may also underlie the actions of inhibitory fatty acids acting on PGHS-1 (Yuan et al., 2009). A second

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group of inhibitors is comprised of time-dependent, non-covalent inhibitors including the nsNSAIDs flurbiprofen, indomethacin, meclofenamate and diclofenac. Flurbiprofen, meclofenamate and indomethacin are allosteric inhibitors that bind to one monomer of PGHSs to inhibit PGHSs (Kulmacz and Lands, 1985; Yuan et al., 2006). We provide evidence in this report that inhibition by diclofenac also involves binding to one monomer of PGHSs and that an intact Ser-530 is required in at least one of the monomers. Finally, aspirin is unique to a third group of inhibitors that cause a time-dependent, covalent inhibition. Binding of aspirin by PGHS-1 or PGHS-2 leads to an irreversible acetylation of a highly conserved Ser-530 (Rouzer and Marnett, 2003; Schneider et al., 2007; Smith, 2008; van der Donk et al., 2002). Aspirin acetylates one monomer of PGHS-1 to cause a temporally correlated loss of COX activity (Rimon et al., 2010). However, the situation with PGHS-2 is more complex. Treatment of PGHS-2 with aspirin converts the enzyme to a form that generates 15*R*-hydroxyeicosapentaenoic acid (15*R*-HETE) from AA (Holtzman et al., 1992; Lecomte et al., 1994). We report here an investigation of the stoichiometry of PGHS-2 acetylation by aspirin and the consequences of acetylation on product formation from arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). We investigated the oxygenation of EPA and DHA because bioactive, tri-hydroxylated compounds called resolvin E1 (RvE1) and 17*R*-resolvin D1 (17*R*-RvD1) are reportedly formed at least in part via aspirin-acetylated PGHS-2 from the ω3 fatty acids EPA and DHA, respectively (Serhan et al., 2008; Serhan et al., 2000), and there is incomplete quantitative information on the oxygenation of EPA or DHA by purified PGHS-2. RvE1 is derived from 18*R*-hydroxy-eicosapentaenoic acid (18*R*-HEPE) formed from EPA and 17*R*-RvD1 is derived from 17*R*-hydroxy-docosahexaenoic acid (17*R*-HDHA) formed from DHA by aspirin-treated preparations containing PGHS-2.

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Materials and Methods

Materials. Flag-affinity resin, hemin and acetylsalicylic acid (aspirin, ASA) were purchased from Sigma. Arachidonic acid (AA), 11-hydroxyeicosatetraenoic acid (11-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE), 15-hydroxyeicosatetraenoic-d8 acid (15-HETE-d8), 11-hydroxyeicosapentaenoic acid (11-HEPE), 12-hydroxyeicosapentaenoic acid (12-HEPE), 15-hydroxyeicosapentaenoic acid (15-HEPE), 18- hydroxyeicosapentaenoic acid (18-HEPE), 13-hydroxydocosahexaenoic acid (13-HDHA), 17-hydroxydocosahexaenoic acid (17-HDHA), were from Cayman Chemical Co. [1-^{14}C]-AA (55 mCi/mmole), [1-^{14}C]-EPA (55 mCi/mmole) and [1-^{14}C]-acetylsalicylic acid (55 mCi/mmole) were from American Radiolabeled Chemicals. The non-ionic detergents $C_{10}E_6$ and β-octylglucopyranoside were from Anatrace. BCA protein reagent was from Pierce Biochemical. Complete EDTA free protease inhibitor was from Roche Applied Science. Restriction enzymes were from New England Biolabs, Inc. Ni-NTA was from Qiagen. Methanol and water were HPLC-grade and purchased from Honeywell Burdick and Jackson (Muskegon, MI). Ammonium acetate was AR grade from Mallinckrodt Baker, Inc. (Paris, KY). Ni-NTA was from Qiagen. Trichloroacetic acid (TCA) was purchased from Fischer scientific. Methanol and water were HPLC-grade and purchased from Honeywell Burdick and Jackson (Muskegon, MI). All other materials were purchased from Fisher Scientific.

Mutagenesis, Protein Expression, and Purification. Protocols for expressing and purifying native and S530A huPGHS-2 were similar to those reported previously (Liu et al., 2007; Wada et al., 2007; Yuan et al., 2006; Yuan et al., 2009). A cDNA for huPGHS-2 containing a hexahistidine (His_6) tag at the N-terminus was subcloned into pFastBac plasmid (Invitrogen). S530A mutations of huPGHS-2 was prepared by site-directed mutagenesis, starting with His_6-tagged huPGHS-2 in pFastBac vector and by using the Stratagene QuickChange mutagenesis kit.

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The presence of the mutations was confirmed by DNA sequencing. The primers used to prepare the S530A mutation were as follows: ggAgCACCATTCgCCTTgAAAggACTTATg and CATAAgTCCTTTCAAggCgAATggTgCTCC.

Using the Life Technologies (Grand Island, NY) Bac-to Bac expression system protocol, bacmid DNA was generated and used to transfect Sf21 insect cells. Virus was harvested and used to infect cultures of sf21 cells. After 72-96 h of infection, the cells were collected and used to purify protein.

Sf21 cell pellets were resuspended in Ni-solubilization buffer (20 mM TrisHCl, pH 7.4 and 100 mM NaCl) containing Complete EDTA – free Protease Inhibitor (Roche). The enzyme was solubilized with $C_{10}E_6$ (0.8% vol/vol), and insoluble material was removed by centrifugation. Enzymes were purified from the solubilized supernatant using a combinantion of chromatography on fast-flow Ni-NTA resin (Qiagen) and FLAG-affinity resin (Sigma) essentially as reported previously (Liu et al., 2007; Yuan et al., 2009). Purified protein at a concentration of about 100 μg/ml was concentrated to 0.5-4 mg/ml using a Millipore centrifugal filter with a cutoff molecular weight of 50,000.

Radio-Thin Layer Chromatography Of Prostaglandin Products. Thin layer chromatography was used to separate the products formed from [1-^{14}C]-AA or [1-^{14}C]-EPA by native huPGHS-2 or S530A/S530A huPGHS-2 before and after incubation with 500 μM aspirin for 1 hour at 37 ^{o}C. The reactions were performed in standard assay buffer containing 0.1 M TrisHCl, pH 8.0 containing 1 mM phenol and 5 mM hematin and allowed to proceed for 40 sec at 37^{o} C with or without 12.5 mM diclofenac. The reactions were quenched with stop buffer consisting of ethyl ether/methanol/0.2 M citric acid; 30/4/1. The resultant solutions were centrifuged at 4 °C for 10 min at 1000 x g. An aliquot of the organic layer (100 μl) was subjected

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to thin layer chromatography on a silica gel G plate in ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). After exposing the thin layer plates to x-ray film, the films were scanned and the density of the radioactive bands quantified with the NIH ImageJ program (ver. 1.38x). The product compositions were calculated based on the percentage of the band intensity (e.g. measuring those bands that co-chromatographed with AA, 15-HETE or PGH_2).

Cyclooxygenase Assays. COX assays were performed as detailed previously (Liu et al., 2007). COX reaction mixtures typically contained 3 ml of 0.1 M TrisHCl, pH 8.0, 100 μM arachidonic acid, 1 mM phenol, and 5 μM hematin equilibrated in a glass chamber at 37 °C. Reactions were initiated by adding enzyme to the assay chamber. A Yellow Springs Instruments Model 53 Oxygen monitor was used to monitor O_2 consumption by native or mutant PGHSs with kinetic traces recorded using (DaisyTec) software. The rates reported are maximum rates occurring after a short lag phase. One unit of COX activity is defined as 1 μmol of O_2 consumed/min at 37 °C in the assay mixture. Igor Pro version 6.0 was used for graphing K_m and V_{max}. The errors are reported as standard deviations from multiple kinetic trials.

Quantitation of Aspirin Acetylation. Native huPGHS-2 and S530A huPGHS-2 variants were incubated with 0.5, 1 or 2 mM [1-^{14}C]-acetylsalicylate for different times at either room temperature (ca. 24°) or 37° (Bala et al., 2008). The resulting mixture was treated with cold 10 % TCA and filtered through 1 μM pore-sized filters under vacuum. The filters were washed with 5 ml of cold TCA containing 8 mM unlabeled ASA. After washing, the filters were transferred to scintillation vials and allowed to equilibrate with the scintillation fluid for at least 24 hr. Radioactivity was quantified by liquid scintillation counting.

LC-MS/MS Analysis of EPA and DHA Reaction Products of PGHS-2. Mass spectrometric analysis was performed with a Surveyor HPLC (ThermoFinnigan, San Jose, CA)

interfaced directly to the electrospray ionization source of an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Sample mixtures were separated using a Gemini C6-Phenyl analytical column (150x2.00 mm, 3 micron particles; Phenomenex, Torrance, CA) with a binary gradient at a flow rate of 0.22 ml/min. Solvent A was 10 mM ammonium acetate, pH 8.5 and Solvent B was methanol. A linear gradient from 50% Solvent B to 100% Solvent B over 50 min followed by a hold at 100% B for 10 min was used to elute compounds of interest. The column was maintained at 50^{o} C and the sample compartment was cooled to 4^{o} C. Negative ions were generated in the ESI source using nitrogen as the sheath and auxillary gas under the following conditions: spray voltage, 3.0 kV; tube lens, -52 V; sheath gas, 30 psi; auxilliary gas, 5 units; capillary temperature, 300^{o} C. For identification of the reaction products, the mass spectrometer was set up to collect survey data in full scan mode from *m/z* 150-1000, and four independent MS2 scans based on a mass list of precursor ions for the substrates and predicted monohydroxy reaction products. The normalized collision energy was set to 26%. An additional scan function was added to the method for semi-quantitative analysis. Data were collected using selected ion monitoring of the $[M-H]^-$ ions of the substrates, the products, and the internal standard (d8-15-HETE). Xcalibur software (v 1.4, ThermoFinnigan, San Jose, CA) was used for instrument control and data analysis.

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Results

Properties of Native and S530A huPGHS-2. We used preparations of the native huPGHS-2 homodimer and an S530A/S530A huPGHS-2 homodimer to characterize the interactions of aspirin and diclofenac with huPGHS-2 (Table 1). The V_{MAX} value for S530A/S530A huPGHS-2 was about 35% of that observed with native enzyme, and there was only a small difference in the K_M values with AA between the native and mutant enzymes. The native and mutant enzymes both were stimulated by 11 *cis*-eicosaenoic acid (20:1ω9) and by palmitic acid (Table 1), and so, we infer that the two enzymes are subject to positive allosteric regulation and exhibit half of sites activity with AA (Yuan et al., 2009). It should also be noted that as determined by radio thin layer chromatography with [1-^{14}C]-AA, the products formed by S530A/S530A huPGHS-2 (Supplemental Fig. S-1) and native huPGHS-2 (*vide infra*) contain the same relative amounts of monohydroxy fatty acids (~10%) and PGH_2 (~90%).

Effect of Diclofenac on huPGHS-2. Ser-530 but not Arg-120 is important for inhibition of the COX activity of murine PGHS-2 by diclofenac (Rowlinson et al., 2003). Consistent with this, we found that diclofenac was an ineffective inhibitor of S530A/S530A huPGHS-2 but completely inhibited native huPGHS-2 (Table 1). Diclofenac caused a time-dependent inhibition of native huPGHS-2 (Table 1 and Fig. 1). As shown in Fig. 1A, the activity of enzyme that had been pretreated with diclofenac was not immediately regained—as it would be for a freely reversible inhibitor—when the enzyme was assayed in the presence of a non-inhibitory concentration of diclofenac. This behavior is similar to that observed with indomethacin (Fig. 1A), which has been well-documented to be a time-dependent COX inhibitor. When titrated with diclofenac, maximal inhibition of native huPGHS-2 occurred at a ratio of one diclofenac molecule per molecule of huPGHS-2 dimer (Fig. 1B). Thus, binding of diclofenac to a single

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monomer of huPGHS-2 inhibits the activity of both monomers. Comparison of the results obtained with native huPGHS-2 vs. mutant huPGHS-2 imply that Ser-530 needs to be present in at least a single monomer for time-dependent inhibition by diclofenac to occur (Table 1). Ser530 is not required for inhibition by indomethacin, fluribiprofen or meclofenamate, which are also maximally effective at one inhibitor molecule per dimer (DeWitt et al., 1990; Kulmacz and Lands, 1985; Prusakiewicz et al., 2009; Yuan et al., 2006; Yuan et al., 2009) and mediate an inhibition requiring interaction with Arg-120 (Bhattacharyya et al., 1996; Rieke et al., 1999; Rowlinson et al., 2003; Yuan et al., 2006) but not Ser-530 (Rowlinson et al., 2003). Inhibition by some nsNSAIDs is associated with decreased formation of the Tyr-385 radical involved in catalysis (Rogge et al., 2006; Rogge et al., 2009). It is not known whether this is the case with diclofenac, but Ser-530 does lie across from Tyr-385 in the COX active site channel perhaps with an intervening water molecule (Selinsky et al., 2001).

Aspirin Acetylation of huPGHS-2. Fig. 2A shows a time course for the inhibition of native huPGHS-2 by 0.5 mM aspirin. Aspirin treatment had no detectable effect on the activity of S530A/S530A huPGHS-2. After 150 min at 24^{o}, native huPGHS-2 retained about 40% of its original oxygenase activity as measured by O_2 consumption with an O_2 electrode assay. Native huPGHS-2 was acetylated by aspirin on only one of its monomers; specifically, 0.92 $\pm$ 0.29 moles of acetate were incorporated per mole of native huPGHS-2 dimer (n = 5) during a 150 min incubation with [1-^{14}C]-acetylsalicylate. No additional ^{14}C-acetyl groups were incorporated into native huPGHS-2 during an overnight incubation at 4^{o} nor was there any additional loss of enzyme activity. As anticipated, no ^{14}C-label was incorporated above no enzyme control levels into the S530A/S530A huPGHS-2 homodimer upon incubation with [1-^{14}C]-acetylsalicylate.

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As illustrated in Fig. 2B, diclofenac inhibited the rate of O_2 consumption of aspirin-treated native huPGHS-2 by about two thirds. Interpretation of this result must take into account that following its acetylation by aspirin, huPGHS-2 forms 15*R*-hydroxy-eicosatetraenoic acid (15*R*-HETE) in addition to smaller amounts of PGH_2-derived products (Holtzman et al., 1992; Lecomte et al., 1994; Xiao et al., 1997), and formation of 15-HETE consumes only one oxygen molecule per molecule of AA while formation of PGH_2 involves the incorporation of two oxygen molecules per AA molecule. The experiment depicted in Fig. 2C indicates that 15-HETE is formed by aspirin-treated native huPGHS-2 at significantly greater levels and in higher proportions relative to PGH_2 than by untreated enzyme. Importantly, diclofenac inhibits PGH_2 formation by acetylated huPGHS-2 to a much greater extent than 15-HETE production. Based on studies with S530A PGHS-2, we assume that diclofenac binds to the non-acetylated monomer having an unaltered Ser-530 to block PGH_2 formation but not to the acetylated monomer to block 15-HETE formation. This implies that the acetylated monomer forms 15-HETE and the non-acetylated monomer forms primarily PGH_2.

Oxygenation of EPA by huPGHS-2. EPA and DHA are poorer substrates than AA for huPGHS-2 (Liu et al., 2006; Wada et al., 2007; Yuan et al., 2009). With saturating concentrations of fatty acid substrate (100 μM), the relative COX activities of huPGHS-2 with AA, EPA and DHA were 100%, 45% and 15%, respectively (Table 2). It should be noted that these values are normalized rates of formation of oxygenated fatty acids corrected for differences in rates of oxygen consumption that are intrinsically higher with fatty acids such as AA that are *bis*-oxygenated. Although there is some information in the literature (Serhan et al., 2002), the details concerning the nature and ratios of the products formed from EPA and DHA by highly purified huPGHS-2 have not been reported previously. We found that huPGHS-2 converts 100

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μM EPA to an approximately equal mixture of PGH_3 and mono-hydroxy fatty acids and that treatment of huPGHS-2 with aspirin inhibits the formation of both PGH_3 and mono-hydroxy fatty acid by about 70%. LC-MS/MS analyses revealed that two mono-oxygenated products 11-HEPE (32%) and 14-HEPE (11%) are formed from EPA by native huPGHS-2 (Tables 2 and 3, Fig. 3). The formation of 11-HEPE was confirmed by matching the retention time and MS/MS spectrum with an authentic 11-HEPE standard. No 14-HEPE standard is commercially available. The conclusion that the second monohydroxy acid is 14-HEPE is based on the information summarized in Table 3. The MS/MS spectrum is consistent with the fragments predicted for 14-HEPE, and reduction of the carbon-carbon double bonds of the unknown acid (predicted to be 14-HEPE) yields a product with a 10 Da increase in molecular weight and an MS/MS spectrum expected for 14-hydroxyeicosanoic acid. The only straightforward mechanism for the formation of 14-HEPE would seem to be abstraction of the omega-5 hydrogen from EPA; however, we are unaware of a precedent for this for native PGHSs

Aspirin-acetylated huPGHS-2 converted 100 μM EPA to a mixture of four different hydroxy acids (60% of the products) and PGH_3 (40% of the products) (Fig. 3). A minor product is 18-HEPE, which represents 7% of the total oxygenated products. Oxygenated products were not detected when [1-^{14}C]-EPA (100 μM) was incubated with aspirin-acetylated huPGHS-2 in the presence of diclofenac (12.5 μM) (data not shown). This is unlike what is seen with AA as a substrate (Fig. 2C).

In experiments similar to those reported in Table 2, we analyzed the efficiency of oxygenation of EPA by native huPGHS-2 and aspirin-treated huPGHS-2 at a concentration (5 μM) nearer to that likely to be physiologically relevant (Table 4). We also tested the effect of 25 μM palmitic acid on EPA oxygenation because palmitic acid is the most abundant free fatty acid

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in cells and is a positive allosteric activator of AA oxygenation by PGHS-2 *in vitro* (Table 1; (Yuan et al., 2009)). Palmitic acid had little or no effect on the rate of EPA oxygenation or the pattern of products formed by native huPGHS-2; in the presence of palmitic acid is oxygenated at 25% of the rate of AA. Aspirin-treated huPGHS-2 has only 35% of native huPGHS-2 activity with 5 μM EPA. The product profile is shifted somewhat so that more monohydroxy fatty acids are formed, but the effect of aspirin to increase monohydroxy acid formation from EPA is much less pronounced than what is observed with AA. 18-HEPE is detectable as one of the products of EPA but comprises only 2% of the total products with 5 μM EPA (Table 4).

Oxygenation of DHA by huPGHS-2. The specific activity of highly purified huPGHS-2 with 100 μM DHA is 15% of the specific activity observed with 100 μM AA (Table 2). Following incubation of huPGHS-2 or aspirin-acetylated huPGHS-2 with DHA, the only products visualized following thin-layer chromatography co-chromatographed with authentic 13-hydroxydocosahexaenoic acid (HDHA) and 17-hydroxydocosahexaenoic acid (17-HDHA) standards, and the latter two hydroxydocosahexaenoic acid standards co-chromatographed in the thin layer chromatography system used in our experiments (data not shown). We subsequently analyzed the products by LC-MS/MS (Tables 2 and 4 and Fig. 4). Consistent with what has been reported (Serhan et al., 2002), highly purified huPGHS-2 converts DHA exclusively to 13-hydroxydocosahexaenoic acid (HDHA). The identity of 13-hydroxy-docosahexaenoic acid was confirmed by matching the retention time and MS/MS spectrum with that of a 13-HDHA standard (Fig. 4). Aspirin-acetylated huPGHS-2 has less than one third of the activity of native huPGHS-2 with 100 μM DHA, and forms a mixture of 13-HDHA (~25%) and 17-HDHA (~75%). As was observed using EPA as the substrate, diclofenac (12.5 μM) completely inhibited the oxygenation of DHA (100 μM) by aspirin-acetylated huPGHS-2.

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Experiments were also performed with 5 μM DHA in the presence and absence of palmitic acid (Table 4). The results are qualitatively and quantitatively similar to those seen with 100 μM DHA (Table 2). The relative amounts of 13-HDHA vs. 17-HDHA formed from 5 μM DHA by aspirin-treated huPGHS-2 was unaffected by the presence of 5 μM AA. There was also no appreciable effect of 5 μM DHA on the formation of AA-derived products (data not shown). The latter results are consistent with previous measurements of rates of O_2 consumption when DHA and AA are co-incubated with huPGHS-2 (Yuan et al., 2009).

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Discussion

Earlier studies on the effect of aspirin on PGHS-2 showed that aspirin causes partial inhibition of the enzyme, that the aspirin-treated enzyme forms a new product 15*R*-HETE from AA and that the acetyl group from aspirin is incorporated into the protein at Ser530 (Holtzman et al., 1992; Lecomte et al., 1994). Subsequent work has suggested that 15*R*-HETE is formed from an alternative conformation of AA by aspirin-treated PGHS-2 (Xiao et al., 1997). More recently, studies by Serhan and coworkers on tri-hydroxylated compounds dubbed resolvins have indicated that RvE1 and 17*R*-RvD1 can be derived from 18*R*-HEPE and 17*R*-HDHA that are formed from EPA and DHA, respectively, by aspirin-treated PGHS-2. In this report, we have expanded and refined the results obtained earlier with AA and have also examined the oxygenation of EPA and DHA by purified, aspirin-acetylated PGHS-2.

In the case of PGHS-1 a single monomer of the homodimer is acetylated by aspirin. The result is complete inhibition of the cyclooxygenase activity of the enzyme (Rimon et al., 2010). This is an example of what is becoming recognized as a general mechanism of inhibition of PGHSs by time-dependent inhibitors that include nonspecific NSAIDs that can act on both PGHS-1 and PGHS-2 (Kulmacz and Lands, 1985; Yuan et al., 2006; Yuan et al., 2009) and probably coxibs functioning on PGHS-2 (Walker et al., 2001). These inhibitors act as negative allosteric effectors by binding to one monomer of the enzyme to markedly reduce or even eliminate activity in the partner monomer. In a related manner, fatty acids such as palmitic acid function as positive allosteric effectors of PGHS-2 by binding one monomer of the dimer and promoting the binding and oxygenation of AA in the other monomer (Yuan et al., 2009). In contrast, time-independent, freely reversible inhibitors such as ibuprofen need to bind both

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monomers of PGHSs simultaneously to cause enzyme inhibition and the inhibition is complete (Prusakiewicz et al., 2009).

Our results demonstrate that only a single monomer of PGHS-2 is acetylated by aspirin but that the outcome is somewhat more complex than seen with PGHS-1. The enzyme retains a significantly compromised ability to form PGH_2, and additionally, produces an alternative product 15-HETE. The fact that PGH_2 formation is more than 80% inhibited by diclofenac while the formation of 15-HETE is reduced by only 20% suggests that diclofenac is binding to the non-acetylated monomer of aspirin-treated PGHS-2 and that this monomer be the one that forms PGH_2 while the acetylated monomer forms primarily 15R-HETE. Thus, the effect of aspirin on PGHS-2 is an incomplete allosteric inhibition effect compared to that seen with PGHS-1. Our ability to draw these conclusions depends on the use of diclofenac, which is an unusual anti-inflammatory drug that functions by interacting with Ser530 and not Arg120 (Rowlinson et al., 2003).

Diclofenac completely inhibited the oxygenation of EPA and DHA by aspirin-acetylated huPGHS-2. This suggests that all of the products derived from EPA and DHA are formed by the non-acetylated subunit; however, we cannot exclude the possibility that diclofenac also indirectly interferes with the binding of EPA and DHA to the aspirin-acetylated monomer and inhibits EPA and DHA oxygenation in both monomers. In the context of differential usage of AA, EPA and DHA by the acetylated vs. non-acetylated monomers, the differential effect of palmitic acid may be relevant. Palmitic acid had little or no effect on the pattern of products formed from EPA or DHA by aspirin-acetylated PGHS-2 but had a significant effect on the product profile with AA. With AA and palmitic acid and aspirin-treated huPGHS-2, there was a relative increase in PGH_2

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at the expense of 15-HETE formation. We speculate that palmitic acid preferentially binds the acetylated subunit and interferes with events occurring in that subunit.

Resolvin E1 (RvE1) has potent biological activity in the resolution of inflammation (Serhan et al., 2008). It can be formed in complex tissue systems primed with aspirin pretreatment and exogenous EPA (Arita et al., 2005; Serhan et al., 2000). Moreover, RvE1 can be detected in plasma at a level of up to 1 nM in individuals given a bolus of EPA and treated with aspirin (Arita et al., 2005). A minimal concentration of 1 nM seems to be required for bioactivity (Arita et al., 2005).

The proposed pathway for RvE1 formation involves an initial production of 18*R*-HEPE via aspirin-acetylated PGHS-2 and subsequent further oxygenation. Although 18*R*-HEPE was reportedly formed by microsomes containing recombinant PGHS-2 (Serhan et al., 2000), in our hands, purified recombinant huPGHS-2 did not form this product at saturating concentrations of EPA (100 μM). In agreement with previous studies, 18*R*-HEPE was formed by purified huPGHS-2 following aspirin treatment. However, 18*R*-HEPE was the least abundant of five products formed from EPA, and aspirin-acetylated huPGHS-2 has 35% of the oxygenase activity of native huPGHS-2 with EPA. With concentrations of EPA (5 μM) approaching but likely to be even higher than those available under physiological conditions, 18*R*-HEPE represented only 2% of the products formed from EPA by aspirin-treated, purified huPGHS-2. 18-HEPE formation by aspirin-treated huPGHS-2 from EPA plus palmitic acid occurs at less than 0.25% of the rate of PGH_2 formation from AA plus palmitic acid by native huPGHS-2. In short, while RvE1 is biologically active, we suspect that it could be formed via huPGHS-2 only in quite low abundance and only in highly unusual biological settings.

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17*R*-RvD1 is reportedly formed from DHA, the first step being the conversion of DHA to 17*R*-HDHA (Serhan et al., 2008; Serhan et al., 2002). Again, using the extreme comparison of the relative rates of PGH_2 formation by native huPGHS-2 in the presence of palmitic acid vs. 17-HDHA formation from DHA in the presence of palmitic acid by aspirin-treated huPGHS-2 indicates that 17-HDHA synthesis by aspirin-treated huPGHS-2 occurs maximally at ~5% of the rate of PGH_2 synthesis by native huPGHS-2. Aspirin-acetylated huPGHS-2 showed no obvious preference for AA vs. DHA when these fatty acids were tested together at 5 μM each.

Acknowledgements

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Footnotes

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Figure Legends

Fig. 1. Time-dependent inhibition of native huPGHS-2 by diclofenac and indomethacin. Purified huPGHS-2 (2 μM) was pretreated with (***a,b and c***) no inhibitor for 10 minutes at 37 °C or (***d***) 12 μM diclofenac for 10 minutes at 37 °C or (***e***) 100 μM indomethacin for 5 minutes at 37 °C. No inhibitor was added to the O_2 electrode assay chamber when Sample (***a***) was assayed. Diclofenac (0.16 μM) was included in the assay chamber when sample (***b***) was assayed; sample (***b***) served as the negative control for sample (***d***). Indomethacin (1.3 μM) was included in the assay chamber when sample (***c***) was assayed; sample (***c***) served as the negative control for sample (***e***). Dilution of the aliquot of sample (***d***) yielded a final concentration of 0.16 μM diclofenac in the assay chamber, and no additional diclofenac was added to this assay chamber. Dilution of the aliquot of sample (***e***) yielded a final concentration of 1.3 μM indomethacin in the assay chamber, and no additional indomethacin was added to this assay chamber. Assays were performed at 37 °C in 3 ml of 0.1 M TrisHCl, pH 8.0 containing 100 μM arachidonic acid, 1 mM phenol and 1 μM hematin. (***B***) Purified huPGHS-2 (2.1 μM) was pretreated with the indicated concentrations of diclofenac at 37 ° C for 10 min and then assayed for COX activity with 100 μM AA essentially as described above. Error bars show standard deviations from multiple kinetic trials.

Fig. 2. Inhibition of huPGHS-2 by aspirin. (***A***) Purified native huPGHS-2 homodimer was incubated at 24° C with or without freshly prepared aspirin (500 μM) for the indicated times and then assayed for COX activity using an O_2 electrode. Values are derived from the average of triplicate determinations ± S.E. Similar experiments were performed at least three times with

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different preparations of enzyme and yielded quantitatively similar results. (***B***) Purified native huPGHS-2 was pretreated with 500 μM aspirin for 1 hr at 37° C, which causes maximal inhibition. The sample was then incubated without (E+ASA) or with (E+ASA+Diclo) 12.5 μM diclofenac (Diclo) for 10 min and assayed for COX activity using an O_2 electrode. For the samples pretreated with diclofenac for 10 min, the assay mixture also contained 12.5 μM diclofenac. A control with huPGHS-2 without inhibitor (E) was treated for 1 hr at 37° C with vehicle (in place of aspirin) and then incubated for an additional 10 min without any inhibitor. (***C***) Purified native huPGHS-2 was incubated with or without aspirin (ASA; 500 μM) at 37° C for 1 h, and the oxygenation of [1-^{14}C] AA was assayed by radio thin layer chromatography (TLC) in the presence and absence of 12.5 μM diclofenac (Diclo) as described in Materials and Methods. TLC was used to separate the endoperoxide (PGH_2) and monohydroxy acid products, mainly 15-HETE. To perform the assays [1-^{14}C] AA (100 μM) was mixed with ~ 9 μg of native huPGHS-2 in an assay volume of 0.10 ml, and the reactions were allowed to proceed for 40 sec. The reactions were stopped, and the products were extracted, separated and visualized by autoradiography as shown. The thin layer plates were subsequently scraped and the amount of radioactivity co-chromatographing with AA, PGH_2 and 15-HETE standards was determined by scintillation counting. Numbers obtained from scintillation counting were used to calculate the percentage of total radioactivity found in each product. The experiment was performed three times with consistent results.

Fig. 3. Products formed from EPA by aspirin-acetylated huPGHS-2. EPA (100 μM) was mixed with 9 μg of enzyme, and the reactions continued for 40 sec. The products were extracted

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as described in the legend to Fig. 2C and reconstituted with 50% methanol for LC-MS/MS analysis.

Fig. 4. Products formed from DHA by aspirin-acetylated huPGHS-2. DHA (100 μM) was mixed with 9 μg of enzyme, and the reactions continued for 40 sec. The products were extracted as described in the legend to Fig. 2C and reconstituted with 50% methanol for LC-MS/MS analysis.

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Table 1. Properties of Native huPGHS-2 and S530A huPGHS-2.

Property[a]	**Native huPGHS-2**[b]	**S530A/S530A huPGHS-2**[b]
Specific activity with AA (U/mg protein = μmole O_2/min/mg protein)	40±1	14±1
K_M with AA (μM)	5±1	9±1
Inhibition by diclofenac (%)	100	15±1
Stimulation by 20:1ω9 (%)	132±5	135±7
Stimulation by 16:0 (%)	176±5	155±11

[a]Specific activities were determined using a standard O_2 electrode assay with 100 μM AA as substrate. K_M values were determined using AA concentrations from 1-100 μM. Percentage inhibition by diclofenac was determined in a standard O_2 electrode assay with 100 μM AA following a 10 min preincubation of the enzymes at 24 ^oC with 10 μM diclofenac versus without diclofenac; 10 μM diclofenac was also added to the assay chamber in the case of enzyme pretreated with diclofenac. Activation of activity by 20:1ω9 was measured using 5 μM AA plus 25 μM 20:1ω9 in the assay mixtures. Activation of activity by palmitic acid (16:0) was measured using 5 μM AA plus 25 μM 16:0 in the assay mixtures. The rates reported are maximum rates occurring after a lag phase. Igor Pro version 6.0 was used for graphing rates vs. AA concentrations in obtaining K_M and V_{MAX} values. Errors are reported as standard deviations from at least three kinetic measurements for each substrate or inhibitor concentration.

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Table 2. Comparison of maximal rates of oxygenation and product profiles for AA, EPA and DHA incubated with huPGHS-2 and aspirin-acetylated huPGHS-2.

Fatty Acid Substrate	huPGHS-2		Aspirin-acetylated huPGHS-2	
	[a]Rate of FA oxygenation (μM FA/min/mg)	[b]Products (%)	[a]Rate of FA oxygenation (μM FA/min/mg)	[b]Products (%)
AA	22±0.2	PGH_2 (91%) HETEs (9%)	12±0.5	PGH_2 (53%) 15-HETE (47%)
EPA	10.8±0.5	PGH_3 (50%) 11-HEPE (32%) 14-HEPE (18%)	3.1±0.2	PGH_3 (40%) 11-HEPE (21%) 14-HEPE (16%) 15-HEPE (16%) 18-HEPE (7%)
DHA	3.2	13-HDHA (100%)	0.9	13-HDHA (25%) 17-HDHA (75%)

[a]Rates were determined by measuring O_2 consumption using an O_2 electrode as described in Materials and Methods. Values for rates of O_2 consumption were converted to values for rates of FA consumption based on the percentages of monooxygenated and *bis*-oxygenated (endoperoxide) products. The latter percentages were determined by radioactive thin layer chromatography and autoradiography with [1-^{14}C]-AA or [1-^{14}C]-EPA as substrates or by thin layer chromatography and visualizing the products by staining in the case of DHA; only

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monooxygenated cyclooxygenase products of DHA were observed. Errors are standard deviations from at least three kinetic measurements for each substrate.

[b]Values for AA- and EPA-derived products were determined by measuring the percentage of radioactivity from [1-^{14}C]-AA and [1-^{14}C]-EPA, respectively, that cochromatographed with monohydroxy acid (15-HETE) and PGH_2 upon radio thin layer chromatography and using the ratio of hydroxy acids as determined by LC-MS/MS. Values for DHA were determined by LC-MS/MS directly because only monooxygenated cyclooxygenase products were observed by thin-layer chromatography.

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Table 3. Summary of Evidence That the Peak with a Retention Time of 20.75 min in the HPLC Separation of Products Derived from Incubation of 5,8,11,14,17-Eicosapentaenoic acid with huPGHS-2 is 14-HEPE.

UV, MS, and MS/MS Data for Peak at 20.75 min		
Data	**Result**	**Conclusion**
$[M-H]^-$ = *m/z* 317	Acidic functional group	MW = 318
Fragment ion at *m/z* 299	Loss of H_2O	Monohydroxy
Fragment ion at *m/z* 273	Loss of CO_2	Carboxylic Acid
UV absorbance at 230 nm	UV peak at 20.75 min	Conjugated diene
Intense fragment ion at *m/z* 207	Probable α-hydroxy-β-ene rearrangement	14-Hydroxyl group
MS/MS Data after Reduction of Peak at 20.75 min with Adam's Catalyst		
Data	**Result**	**Conclusion**
$[M-H]^-$ = *m/z* 327	Increase of 10 mass units	5 double bonds
Retention time increase of >10 min	More hydrophobic	Saturated chain
Fragment ion at *m/z* 309	Loss of H_2O	Monohydroxy acid

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Fragment ion at *m/z* 283	Loss of CO_2	Carboxylic Acid
Fragment ion at m/z 325	Loss of H_2	Formation of double bond
Fragment ion at *m/z* 211	Probable α-hydroxy-β-ene rearrangement	14-Hydroxy; C11 – C12 double bond

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Table 4. Comparison of rates of oxygenation and product profiles for 5 μM AA, EPA and DHA with native huPGHS-2 and aspirin-acetylated huPGHS-2.

Fatty Acids	huPGHS-2		ASA-huPGHS-2	
	[a]Rate (μM FA/min/mg)	[b]Products (%)	[a]Rate (μM FA/min/mg)	[b]Products (%)
AA	9.3±0.51	15-HETE 6.8 11-HETE 9.2 PGH_2 84	5.9±0.43	15-HETE 52 11-HETE 9.0 PGH_2 39
AA + 16:0	16±1.0	15-HETE 7.3 11-HETE 9.6 PGH_2 83	6.4±0.51	15-HETE 27 11-HETE 4.1 PGH_2 69
EPA	4.1 ±0.03	14-HEPE 15 15-HEPE 1.9 11-HEPE 22 PGH_3 61	1.5±0.07	18-HEPE 2.2 14-HEPE 16 15-HEPE 16 11-HEPE 18 PGH_3 47
EPA + 16:0	4.3±0.14	14-HEPE 14 15-HEPE 3.3 11- HEPE 18 PGH_3 65	1.5±0.18	18-HEPE 2.0 14-HEPE 12 15-HEPE 23 11-HEPE 18 PGH3 45
DHA	2.0±0.08	13-HDHA 100	1.1±0.23	17-HDHA 78 13-HDHA 22
DHA + 16:0	2.0±0.11	13-HDHA 100	1.1±0.04	17-HDHA 73 13-HDHA 27

[a]Rates were determined as described in the legend to Table 2 but using 5 μM fatty acid substrate and 25 μM palmitic acid (16:0).

[b]Values were determined as described in the legend to Table 2 using 5 μM fatty acid substrate and 25 μM palmitic acid (16:0).

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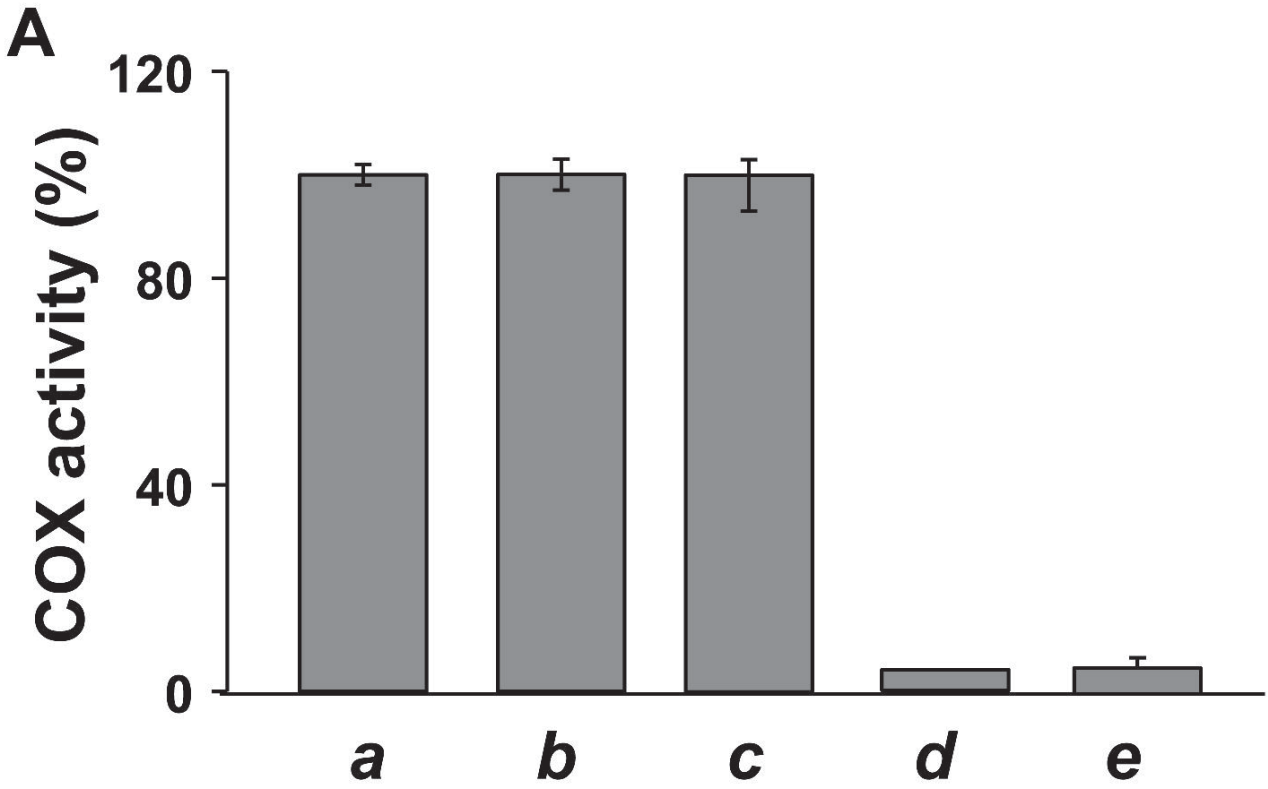

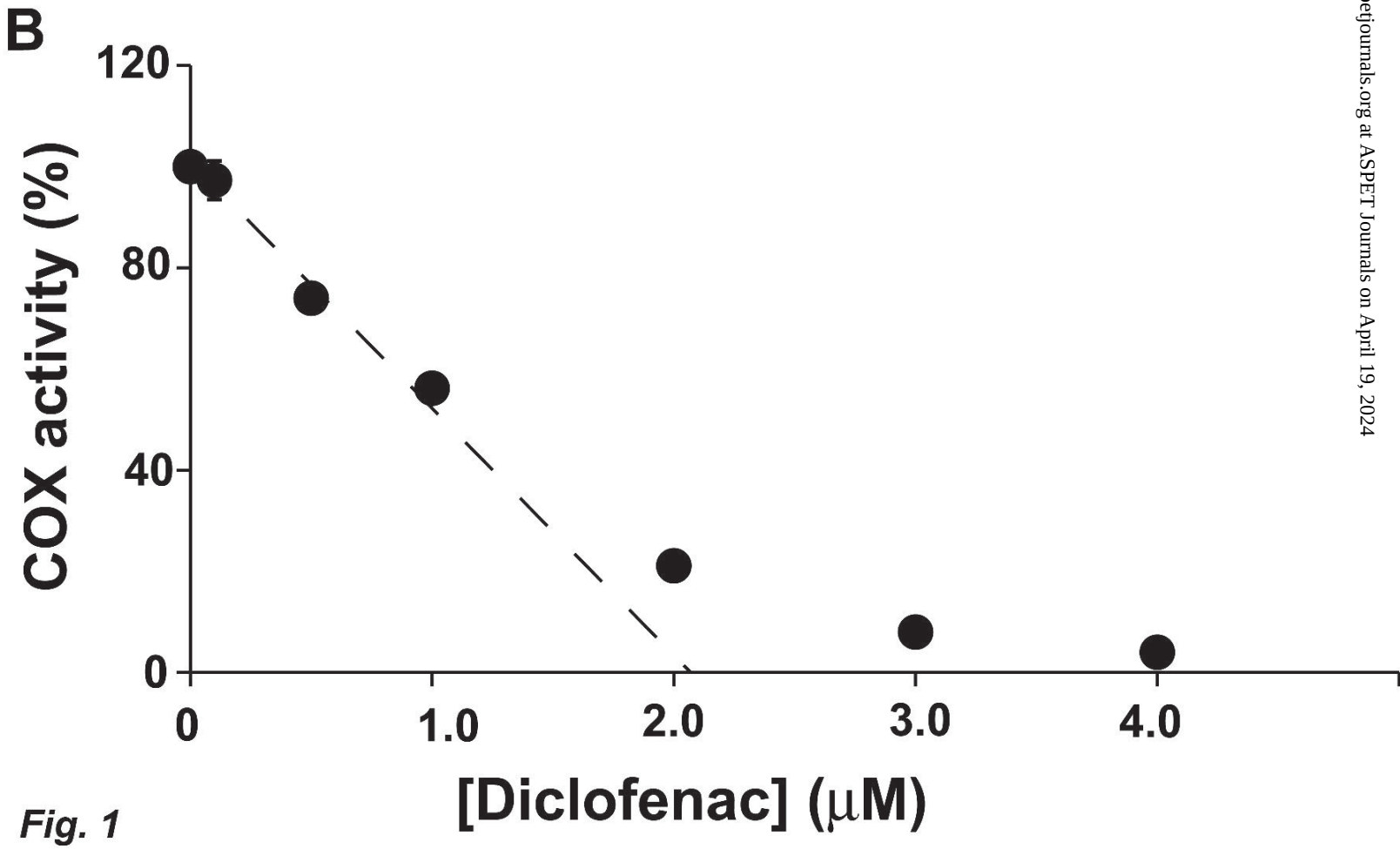

Fig. 1

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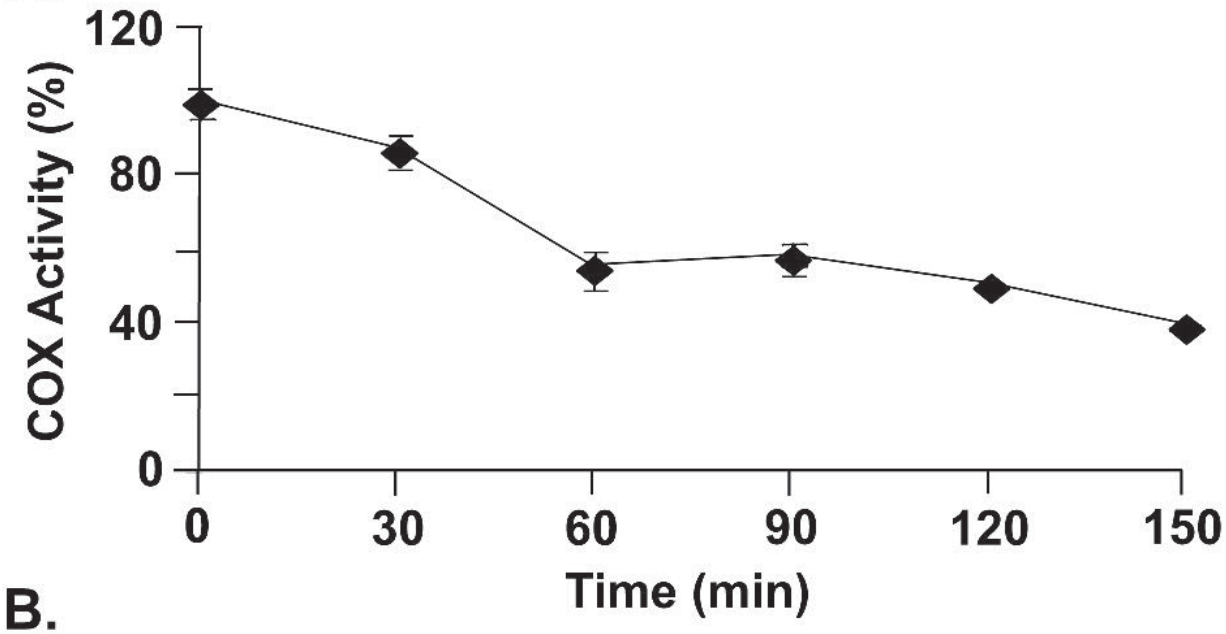

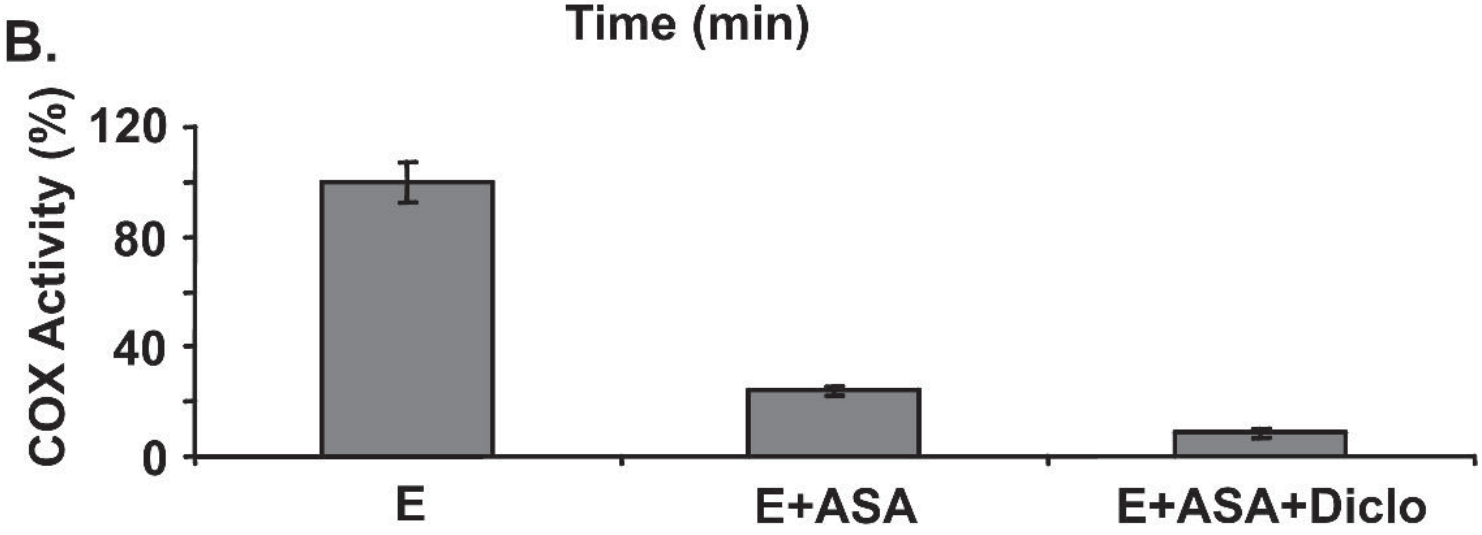

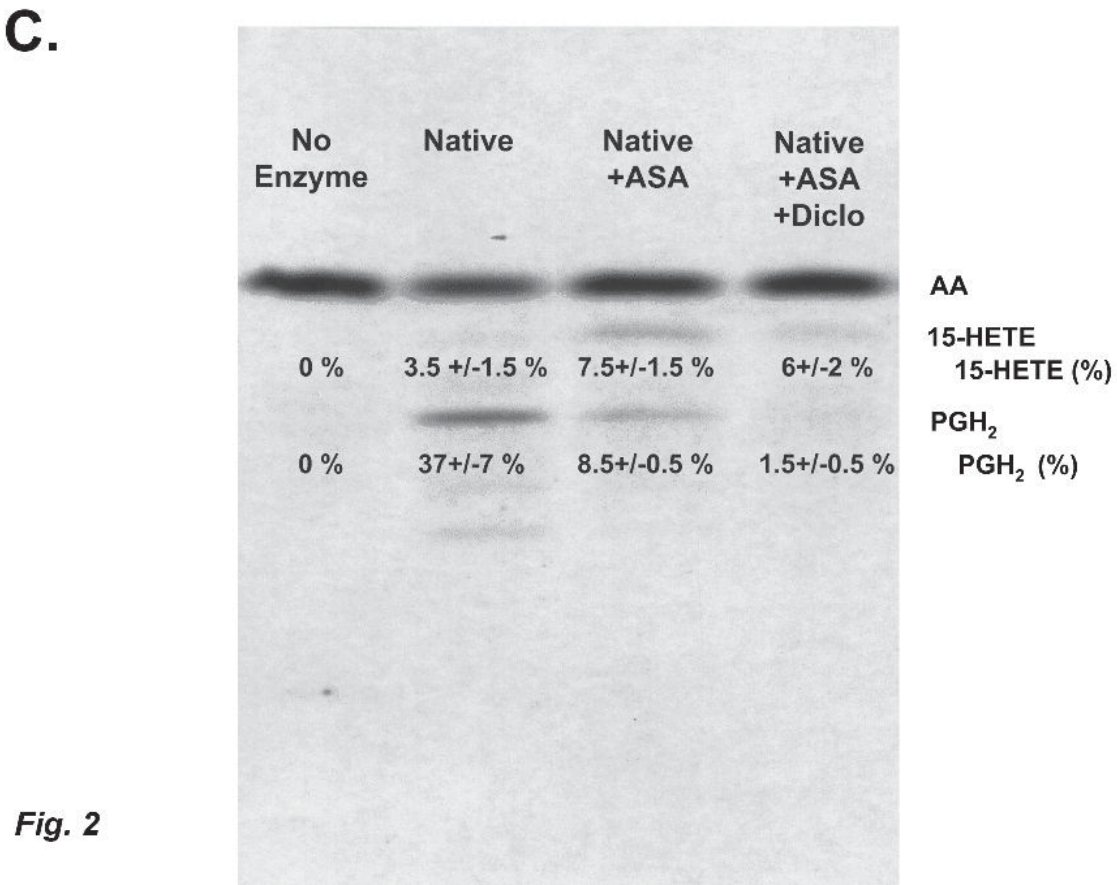

Fig. 2

Fig. 2

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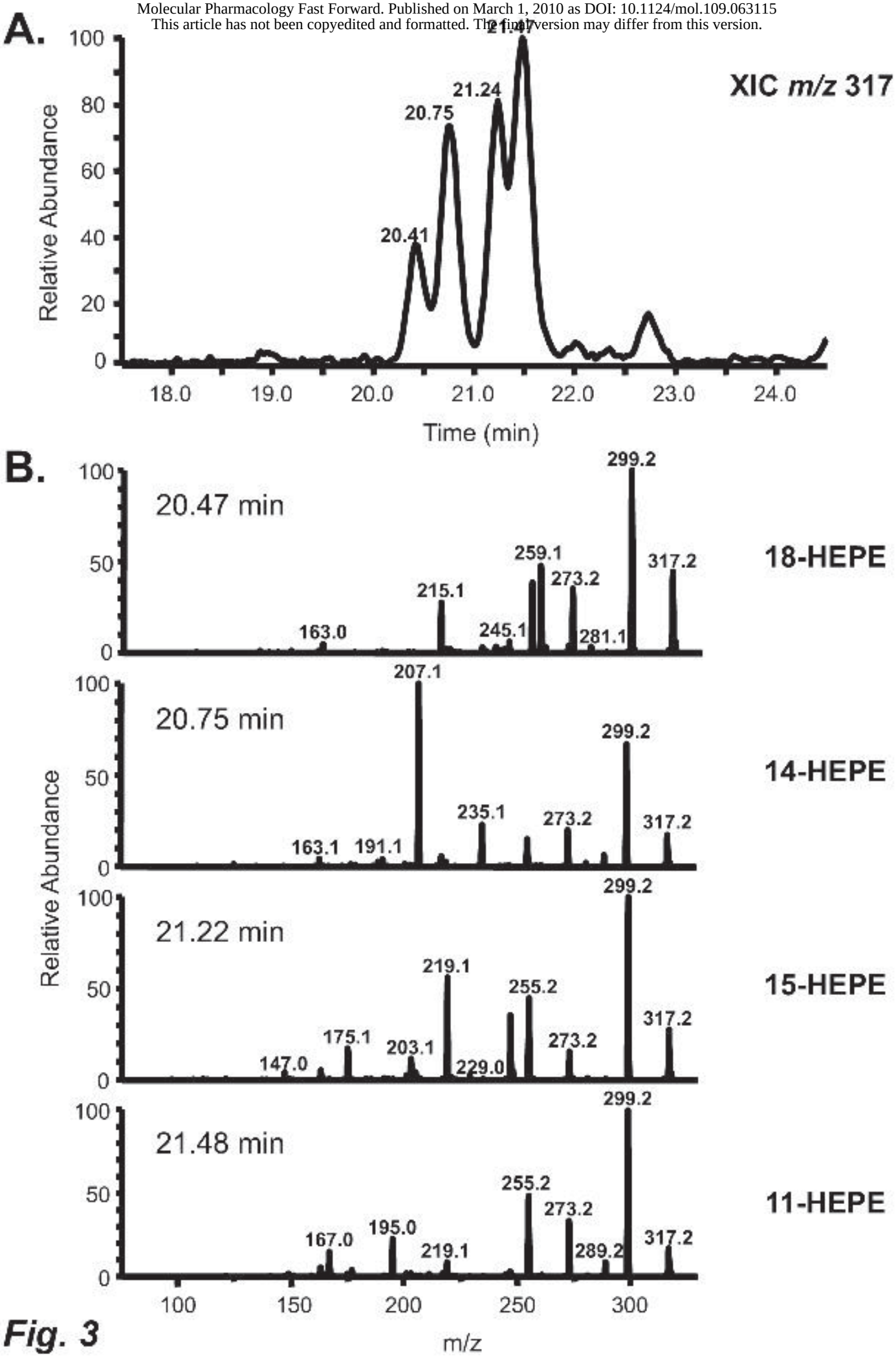

Fig. 3

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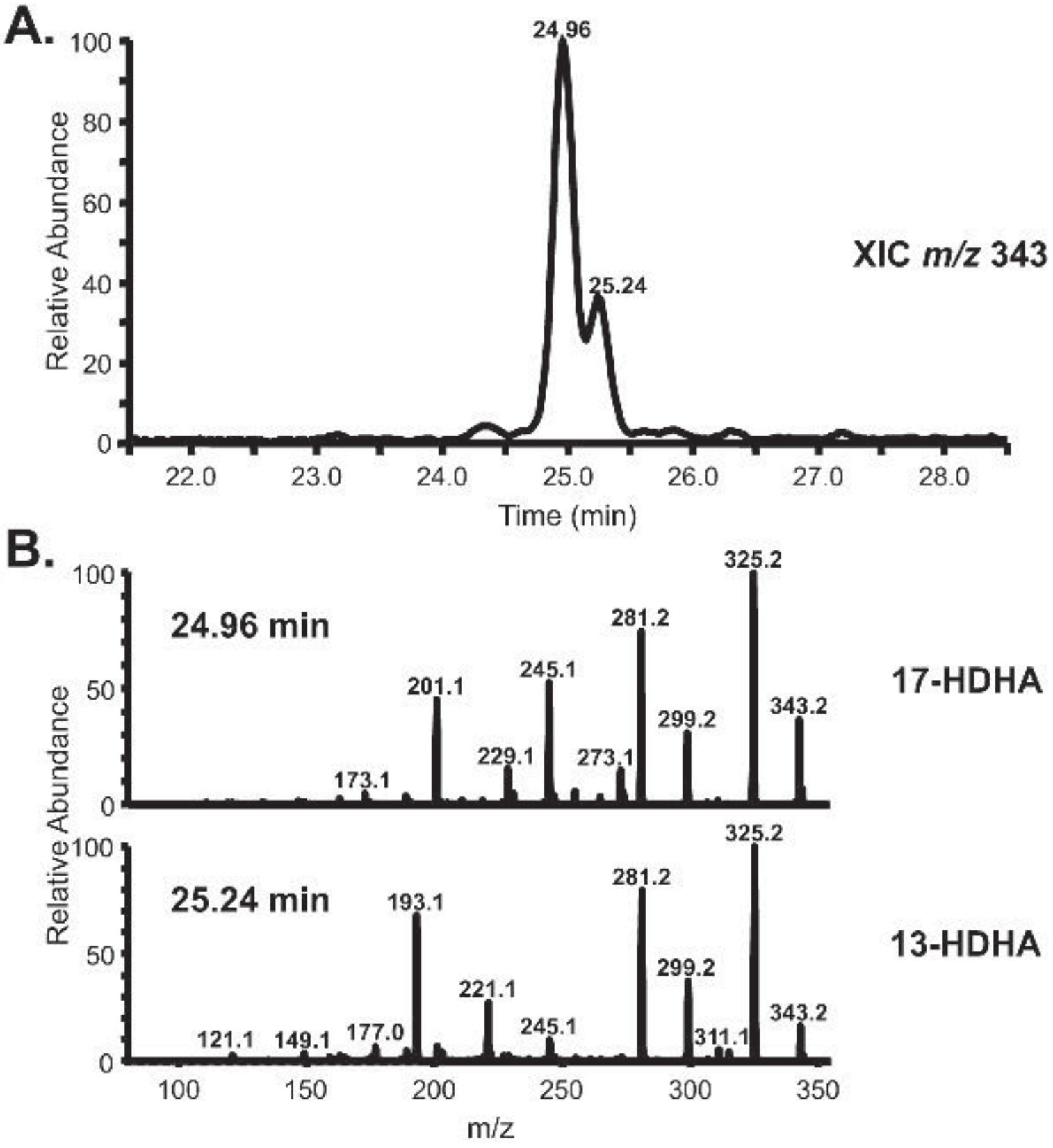

Fig. 4