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Metabolism of 12-hydroperoxyeicosatetraenoic acid to vasodilatory trioxilin C_3 by rabbit aorta

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Abstract

Arachidonic acid is metabolized by both the cyclooxygenase and lipoxygenase pathways by rabbit aorta. We investigated the metabolism of 12-hydroperoxyeicosatetraenoic acid by aortic homogenates and microsomes. Rabbit aortic homogenates were incubated in the presence of ^{14}C-arachidonic acid plus 12-lipoxygenase and analyzed by reversed-phase high-pressure liquid chromatography (HPLC). Under these experimental conditions, there was a ^{14}C-metabolite that migrated at 17.6 min. This ^{14}C-metabolite was not observed when aortic homogenates were incubated in the absence of 12-lipoxygenase. Similar results were obtained with aortic microsomes. Further analysis using a different HPLC solvent system resolved the ^{14}C-metabolite into a number of products. Gas chromatography/mass spectrometric (GC-MS) analysis of the major product (labeled peak 3) after conversion to the methyl ester-trimethylsilyl derivative showed two major compounds (compounds A and B) eluting at 13.99 and 14.14 min. The two compounds differed in the intensities of the 213 and 243 m/z ions with 243 being greater than 213 in compound A and the opposite in compound B (relative abundance 213 vs. 243; 100% vs. 43% for compound A and 5% vs. 100% for compound B). Based on the mass spectra, peak 3 contained two metabolites identified as the methyl ester-trimethylsilyl ether derivatives of 8,11,12-trihydroxyeicosatrienoic acid (trioxilin A_3) and 8,9,12-trihydroxyeicosatrienoic acid (trioxilin C_3). Biological activity of the mixture of two trioxilins isolated from aortic homogenates was tested in phenylephrine-precontracted aortas and found to produce concentration-dependent relaxations (maximal relaxation: 20.1 ± 7.6%). Further testing with authentic trioxilin A_3 and C_3 revealed that trioxilin C_3 was the active metabolite (maximal relaxation: 16.6 ± 1.3%). In conclusion, trioxilin C_3 acid was isolated and identified as a novel biologically active arachidonic acid metabolite formed by rabbit aorta when 12-lipoxygenase is supplied exogenously.

Keywords: Lipoxygenase; Arachidonic acid; Rabbit aorta; Vasorelaxation

1. Introduction

Many studies have documented the importance of the vascular endothelium in the regulation of vascular tone. A number of endothelium-derived relaxing factors (EDRFs) have been identified including nitric oxide [1], prostacyclin [2] and the epoxyeicosatrienoic acids [3]. Our previous work described an endothelium-dependent, lipoxygenase metabolite of arachidonic acid that elicited relaxation of rabbit aorta [4]. Our work showed that arachidonic acid is metabolized by 15-lipoxygenase to 15-hydroperoxyeicosatetraenoic acid (15-HPETE) which undergoes an enzymatic rearrangement to either 11-hydroxy-14, 15-epoxyeicosatrienoic acid (11-H-14, 15-EETA), 15-H-11, 12-EETA or both. Hydrolysis of the epoxy group resulted in the formation of 11,12,15- and 11,14,15-trihydroxyeicosatrienoic acid (THETA). The HEETAs and THETAs relaxed the rabbit aorta, and we proposed that these compounds represent new members of the family of EDRFs.

In the late 1970s, it was reported that platelets from a number of different species including humans convert arachidonic acid to a mixture of 8,9,12-THETA and 8,11,12-THETA [5–7]. These products were produced in amounts equal to or greater than thromboxane B_2. These early studies did not determine any biological effects of the metabolites. Further studies by Pace-Asciak et al. [8,9] reported that the rat lung converts the 12-lipoxygenase

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product 12-HPETE to 8-H-11, 12-EETA, called hepoxilin A_3, and 10-H-11, 12-EETA, called hepoxilin B_3. These compounds are metabolized by epoxide hydrolase into the corresponding trioxilins A_3 and B_3, respectively. Hepoxilins are formed in a number of different tissues, including platelets and rat aorta, and possess a variety of different biological actions [10–12]. In previous studies, we found no evidence for the synthesis of hepoxilins or trioxilins from arachidonic acid by rabbit aorta. Therefore, the purpose of this study was to characterize the metabolism of 12-HPETE by rabbit aorta. Our results showed that if an exogenous source of 12-HPETE is provided to the aortic tissue, the production of the trioxilins was apparent. The major products were identified as 8,11,12-THETA or trioxilin A_3 and 8,9,12-THETA or trioxilin C_3. Further studies indicated that trioxilin C_3 relaxed precontracted rabbit aortas.

2. Methods and materials

2.1. Materials

Arachidonic acid, phenylephrine and indomethacin were all from Sigma Chemical, St. Louis, MO. Leukocyte 12-lipoxygenase was from Caymen Chemical, Ann Arbor, MI. [^{14}C(U)]-Arachidonic acid (specific activity 920 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All solvents were HPLC grade and purchased from Burdick and Jackson, Muskegan, MI.

2.2. Tissue preparation and incubation

Six-week-old male New Zealand White (NZW) rabbits were obtained from New Franken Rabbitry (New Franken, WI). The animals were housed in the Medical College of Wisconsin Animal Care Facilities and maintained on a standard rabbit chow diet and given tap water ad libitum. Rabbits were sacrificed (pentobarbital 120 mg/kg, IV), thoracic aorta removed, and cleaned of adhering connective tissue and fat. In some cases, aortas were purchased from Pel-Frez Biologicals (Rogers, AR). The vessels were rinsed in Tris buffer (0.05 M, pH 7.5), and then cut into small pieces. Aortic tissue was homogenized (five passes with a mechanical homogenizer) in Tris buffer (500 mg of tissue/10 ml of buffer). The homogenate was centrifuged at $750 \times g$ for 15 min and the supernatant used. Aliquots (5 mg/ml) of the homogenate were incubated with [^{14}C (U)]-arachidonic acid in Tris–HCl buffer at 37 °C for 20 min in the presence and absence of 12-lipoxygenase. All incubations were carried out in the presence of indomethacin (10^{-5} M). In all studies, parallel control incubations were performed under identical conditions but without aortic homogenate. For some studies, aortic tissue was homogenized in cold 0.25 M sucrose solution using a Potter–Elvehjem tissue grinder with a Teflon pestle. Subcellular fractions were prepared by differential centrifugation [13]. The homogenate was centrifuged at $5000 \times g$ for 20 min to remove cellular debris, mitochondria and nuclei. The supernatant was further centrifuged at $100{,}000 \times g$ for 60 min. The microsomal pellet was resuspended in 1.15% KCl and homogenized. The homogenate was centrifuged at $100{,}000 \times g$ for 60 min. The pelleted microsomes were resuspended in microsome incubation buffer (Tris 50 mM, KCl 150 mM, $MgCl_2$ 10 mM, pH 7.5) and stored at −80 °C. Protein concentrations were determined using the Bradford technique (Bio-Rad) with IgG as the standard. Aliquots of microsomal protein (2 mg/ml) were incubated with [^{14}C (U)]-arachidonic acid and 12-lipoxygenase in HEPES buffer containing indomethacin (10^{-5} M) at 37 °C for 15 min. Parallel studies were performed with boiled microsomes. For analysis of metabolites by gas chromatography/mass spectrometry (GC-MS), aortic microsomes (3 mg/ml) were incubated with [^{14}C (U)]-arachidonic acid (0.05 μCi, 5×10^{-5} M) plus 12-lipoxygenase.

All reactions were stopped by adding ethanol to a final concentration of 15%. The samples were acidified (pH < 3.5) and extracted using ODS extraction columns as previously described [4]. Recovery of HETEs, HEETAs and THETAs averaged 85–90%. The extracted metabolites were evaporated to dryness under a stream of nitrogen and stored at −40 °C until analysis by HPLC.

2.3. Purification of metabolites by HPLC

The biological samples were first resolved by reversed-phase (Nucleosil-C18 column, 5 μ, 4.6×250 mm) HPLC using solvent system I. Solvent A was water and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program was a 40 min linear gradient from 50% solvent B in A to 100% solvent B. Flow rate was 1 ml/min. The column eluate was collected in 0.2 ml fractions by a fraction collector. An aliquot of each fraction was removed, and radioactivity determined by liquid scintillation spectrometry. The fractions corresponding to the trihydroxy metabolites (fractions 27–35) (5–7.5 min) were collected and extracted with cyclohexane/ethyl acetate (50:50). The solvent was removed under a stream of nitrogen, and the extract redissolved in the HPLC mobile phase. This fraction was rechromatographed on reversed-phase HPLC using solvent system II [14]. In solvent system II, solvent A was water containing 0.1% glacial acetic acid and solvent B was acetonitrile. The program consisted of a 5 min isocratic phase with 35% B in A, followed by a 35 min linear gradient to 85% B. Flow rate was 1 ml/min. The column eluate was collected in 0.2 ml aliquots, and radioactivity determined as described above. The fractions containing the trihydroxy metabolites (19.5–20.5 min; fractions 97–103) were collected, extracted with cyclohexane/ethyl acetate (50:50), and further purified by HPLC using a Kromasil-C18 column (5 μ, 4.6×250 mm). Solvent system III consisted of an isocratic separation using 35% B in A with a flow rate of 1 ml/min. Solvent A was water containing

0.05% glacial acetic acid and solvent B was acetonitrile containing 0.05% glacial acetic acid. The column eluate was collected in 0.2 ml fractions and the radioactivity determined as described above.

2.4. Gas chromatography/mass spectrometry

The major peaks were isolated from solvent system III (peaks 1–4) and extracted with cyclohexane/ethyl acetate, evaporated to dryness under nitrogen and derivatized for GC-MS as previously described [14,15]. The sample was dissolved in 120 μl acetonitrile and then esterifed with ethereal diazomethane for 6 min at room temperature. The reaction mixture was evaporated to dryness under nitrogen and the hydroxyl groups were then silylated with 15 μl bis-TMS-trifluoroacetamide for 60 min at 37 °C. GC-MS was performed with a Hewlett Packard 5989A Mass Spectrometer coupled with a 5890 Series 2 Gas Chromatogram. Ionization of the samples was done by electron impact at 65–70 eV or collisionally using methane as the reagent gas. The methyl ester-TMS ether-derivatized metabolites were resolved using a 14 m capillary DB-5 column with a linear gradient from 100 to 300 °C.

2.5. Vascular reactivity

Rabbit aortic homogenates or microsomes were incubated with 12-lipoxygenase and [^{14}C (U)]-arachidonic acid as described above. In some cases, identical control incubations without tissue homogenate (referred to as cell-free) were carried out in parallel. Following incubation and extraction, the trioxilins were purified by sequential reversed-phase HPLC using solvent systems I, II and III. The fractions 167–170 from solvent system III, corresponding to the trioxilins were collected, extracted and tested for biological activity. For these biological activity experiments, thoracic aortas were obtained from 1- to 2-month-old NZW rabbits and placed in Krebs bicarbonate buffer (118 mM NaCl, 4 mM KCl, 3.3 mM $CaCl_2$, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 11 mM glucose) as previously described [4]. The tissue was carefully cleaned of adhering fat and connective tissue and cut into rings (3 mm length) taking care not to damage the endothelium. Aortic rings were suspended in 6 ml tissue baths containing Krebs bicarbonate buffer maintained at 37 °C and continuously bubbled with 95% O_2–5% CO_2. Isometric tension was measured with force displacement transducers (Grass Instruments), AD Instruments ETH-400 amplifiers and recorded on a Macintosh computer using MacLab 8e software. Resting tension was adjusted to its length tension maximum of 2 g, and vessels allowed to equilibrate for 1 h. Contractions were produced by increasing the KCl concentration of the baths to 40 mM. After the vessels reached peak contraction, tissue baths were rinsed, and vessels allowed to return to resting tension. Once the aortic rings had reproducible, stable responses to KCl, the

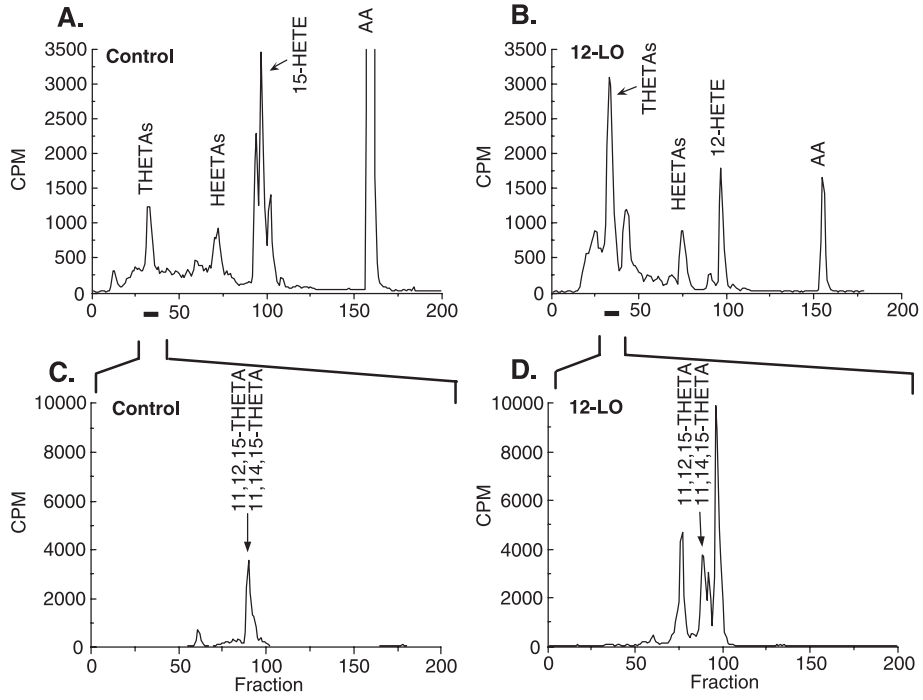

Fig. 1. Effect of 12-lipoxygenase on the metabolism of [^{14}C(U)]-arachidonic acid by homogenates of rabbit aorta. Aortic homogenates were incubated with [^{14}C(U)]-arachidonic acid in the absence (panels A and C) or presence (panels B and D) 12-lipoxygenase for 20 min. The metabolites were separated using reversed-phase HPLC using solvent system I in panels A and B. Metabolites in fractions 27–35 were collected, extracted using cyclohexane/ethyl acetate, and rechromatographed using solvent system II (C and D). Migration times of known standard eicosanoids are shown above the chromatogram and are not meant to identify the corresponding radioactive peaks. CPM = counts/min.

tissue was contracted with phenylephrine (10^{-7} M). The effect of serial dilutions of the trioxilins derived under the various incubation conditions was tested. The samples were suspended in a known volume of ethanol and 10 μl per 6 ml bath was the maximal ethanol concentration administered. In a separate series of experiments, chemically synthesized trioxilin A_3 and C_3 were tested for relaxation responses in phenylephrine-precontracted rabbit aorta [16–18].

3. Results

3.1. Formation of metabolites

Incubation of ^{14}C-arachidonic acid with aortic homogenate in the absence of 12-lipoxygenase is shown in Fig. 1A. Separation of the metabolites by reversed-phase HPLC using solvent system I provided radioactive products that migrated with the THETAs, the HEETAs and the HETEs. If aortic homogenate was incubated with ^{14}C-arachidonic acid plus 12-lipoxygenase, three major radioactive peaks were again observed with solvent system I (Fig. 1B). In the presence of 12-lipoxygenase, the polar peak eluting with the THETAs was increased compared to incubations with only ^{14}C-arachidonic acid. A second less polar peak eluting with the HEETAs was still observed. When 12-lipoxygenase was included in the incubation, a single radioactive peak eluted with 12-HETE. Interestingly, the production of 15-HETE was not detected. This may be due to the rapid consumption of arachidonic acid by 12-lipoxygenase. In incubations without homogenate (cell-free controls), no THETA or HEETA synthesis was detected. Similar amounts of total radioactivity were present in Fig. 1A and B. Because indomethacin was present in all incubations, cyclooxygenase metabolites were not detected. Fractions 27–35 (5–7.5 min) and corresponding to the THETAs, were further analyzed by reversed-phase HPLC using solvent system II (Fig. 1C and D). The results indicated that the pattern of metabolites was different in the aortic homogenates incubated with 12-lipoxygenase compared to ^{14}C-arachidonic acid alone. Homogenate incubated in the presence of ^{14}C-arachidonic acid produced a major peak in fractions 87–93 (Fig. 1C) as previously described [14]. In contrast, radioactive peaks were observed in the homogenates incubated with 12-lipoxygenase and ^{14}C-arachidonic acid (Fig. 1D) eluting in fractions 87–93 and 97–103. If aortic microsomes were incubated with 12-lipoxygenase and ^{14}C-arachidonic acid, the profile of metabolites was similar to aortic homogenate incubations (Fig. 2A). However, since 15-lipoxygenase is a cytosolic protein, only 12-lipoxygenase metabolites were observed in the incubations with aortic microsomes. The early polar peak (fractions 27–35) was collected and analyzed by reversed-phase HPLC using solvent system II. Two major ^{14}C-metabolites were resolved. The major peak comigrated with the major peak seen in the aortic homogenate incubations (Fig. 1D). This peak was collected (19.5–20.5 min; fractions 97–103) and analyzed further by HPLC using solvent system III. In this case, a number of radioactive products were resolved and labeled 1–4 (Fig. 2C). In incubations with boiled aortic microsomes, no THETA or HEETA synthesis was detected.

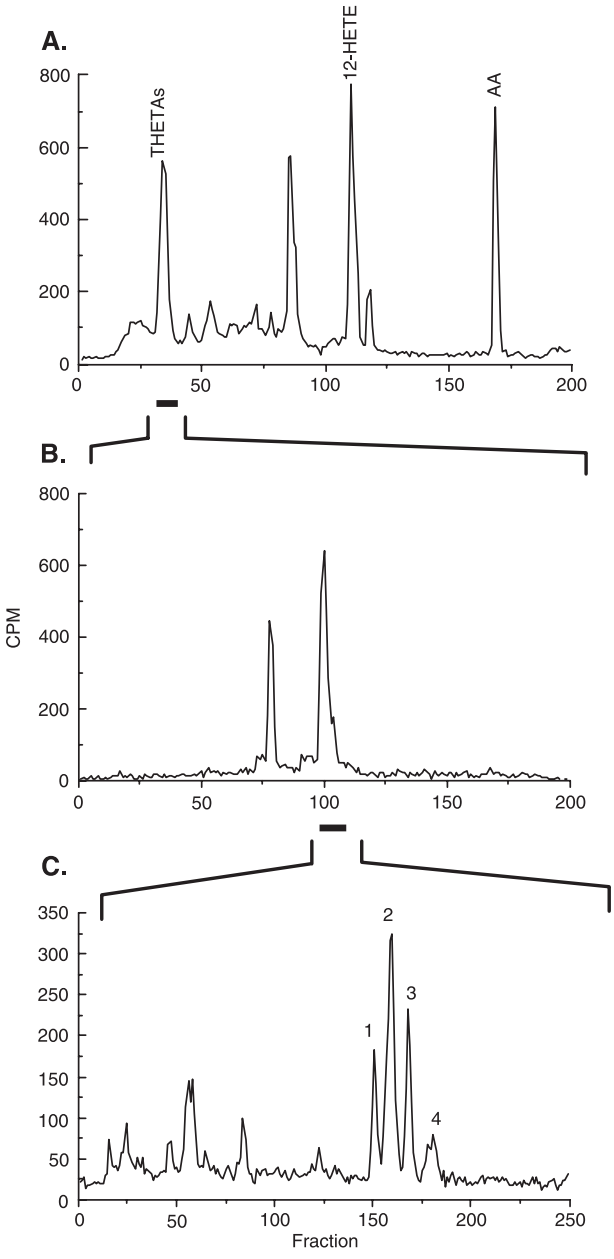

Fig. 2. Synthesis of trioxilins by aortic microsomes. Aortic microsomes were incubated with 12-lipoxygenase and [^{14}C(U)]-arachidonic acid. Metabolites were separated by reversed-phase HPLC using solvent system I (A). Metabolites in fractions 27–35 were collected, extracted using cyclohexane/ethyl acetate, and rechromatographed using solvent system II (B). Metabolites in fractions were collected, extracted using cyclohexane/ethyl acetate, and rechromatographed using solvent system III (C). Migration times of known standard eicosanoids are shown above the chromatogram and are not meant to identify the corresponding radioactive peaks. CPM = counts/min.

3.2. Gas chromatography/mass spectroscopy

Characterization of 12-lipoxygenase products was achieved through GC-MS analysis. Fig. 3 shows the mass spectrum of peak 3 (see Fig. 2C) isolated from the aortic microsomes using solvent system III. The PCI GC-MS analysis of the methyl ester-TMS ether derivative of peak 3 showed major products eluting at 13.99 and 14.14 min (Fig. 3, top panel). The mass spectrum of compound A (Fig. 3B) revealed the presence of major ions at m/z 569 (M^+-15; loss of CH_3), 479 (M^+-90; loss of $(CH_3)_3-SiOH$), 405 (M^+-179; loss of $(CH_3)_3SiOH$ and $(CH_3)_3SiO$), 213 (M^+-371; loss of $((CH_3)_3SiO)-CH-CH-((CH_3)_3SiO)-CH_2-CH=CH-(CH_2)_3-COOCH_3$ and 243 (M^+-341; loss of $CH=CH-CH((CH_3)_3SiO)-CH((CH_3)_3SiO)-(CH_2-CH=CH-(CH_2)_4-CH_3$). The mass spectrum of compound B (Fig. 3C) showed prominent ions at m/z 569 (M^+-15; loss of CH_3), 479 (M^+-90; loss of $(CH_3)_3-SiOH$), 405 (M^+-179; loss of $(CH_3)_3SiOH$ and $(CH_3)_3SiO$), 243 (M^+-341; loss of $((CH_3)_3SiO)-CH=CH-CH-((CH_3)_3SiO)-(CH_2-CH=CH-(CH_2)_4-CH_3$) and 213 ($M^+$-371; loss of $(CH=CH-CH((CH_3)_3SiO)-CH((CH_3)_3SiO)-CH_2-CH=CH-(CH_2)_3-COOCH_3$). The two compounds differed in the intensities of the 213 and 243 m/z ions with 243 being greater than 213 in compound A and the opposite in compound B (relative abundance 213 vs. 243; 100% vs. 43% for compound A and 5% vs. 100% for compound B). Based on these mass spectra, peak 3 isolated from HPLC

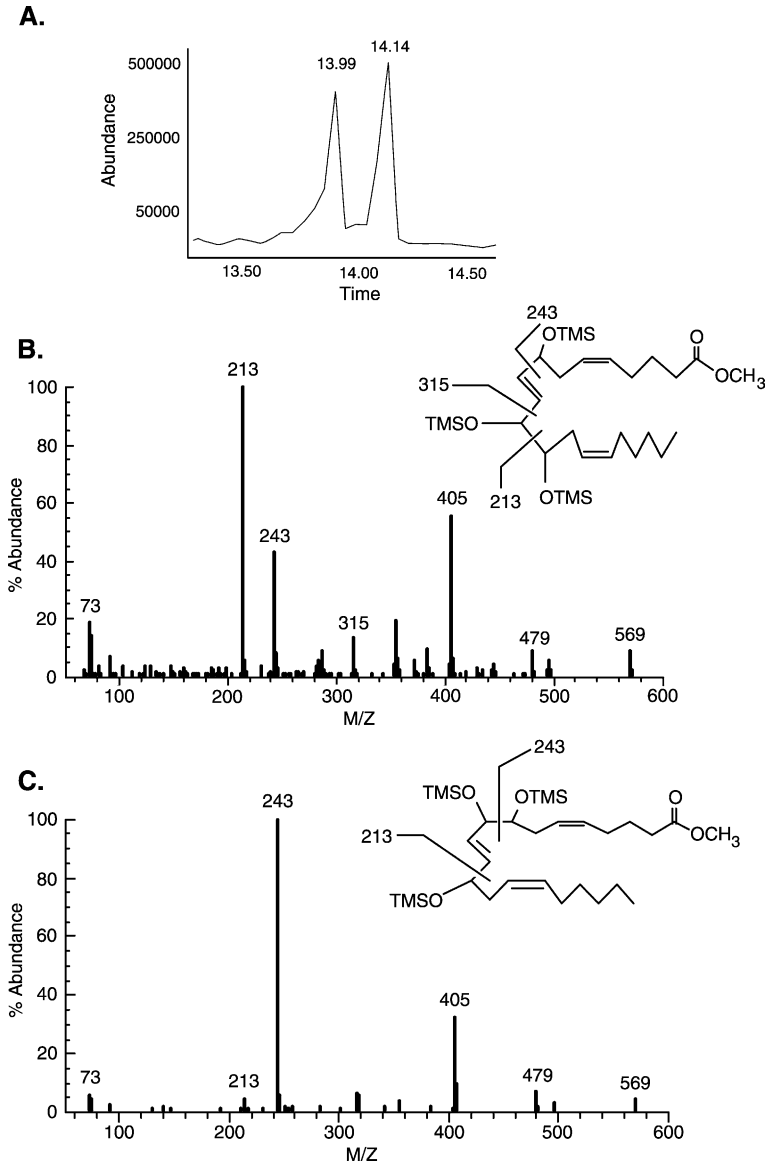

Fig. 3. Identification of trioxilin A_3 and C_3 by GC-MS. Peak 3 (fractions 167–170), of Fig. 2C was derivatized to the methyl ester-TMS ether and analyzed by GC/MS. Panel A is a representative reconstructed ion chromatogram from the gas chromatographic analysis. Panels B and C show the PCI mass spectra for compounds A and B, respectively. The proposed structure of each derivatized compound is given and its prominent ions are indicated. M/Z = mass/charge.

using solvent system III, contains two metabolites, identified as the methyl ester-TMS ether derivatives of 8,11,12-THETA or trioxilin A_3 (peak A) and 8,9,12-THETA or trioxilin C_3 (peak B). The proposed structures for each of the derivatized forms of peak 3 are shown as an inset of Fig. 3B and C.

3.3. Effect of the 12-lipoxygenase products on vascular reactivity

The major peak (fractions 97–103; see Fig. 1B) isolated from aortic homogenates incubated with arachidonic acid and 12-lipoxygenase was tested for activity on isolated rings of rabbit aorta. In precontracted vessels, this peak elicited a concentration-dependent relaxation response (maximal responses: 41.7 ± 18.0%; data not shown). The cell-free incubation with arachidonic acid and 12-lipoxygenase did not relax the rabbit aorta. In a separate study, peaks 1–4 obtained from the aortic microsomes incubated with arachidonic acid and 12-lipoxygenase were collected from the solvent system III, extracted, and serial dilutions tested for biological activity. In precontracted vessels, only peak 3 from the aortic incubations elicited concentration-dependent relaxations (maximal response; 20.7 ± 7.6%; Fig. 4A). The other three peaks were inactive (data not shown). Because peak 3 is a mixture of both trioxilin A_3 and trioxilin C_3, we tested chemically synthesized trioxilin A_3 and C_3 on the precontracted rabbit aorta. Trioxilin A_3 had no effect while trioxilin C_3 elicited a concentration-dependent relaxation (maximal response; 16.6 ± 1.3%) (Fig. 4B). The maximal response to peak 3 did not differ statistically from the maximal response to trioxilin C_3.

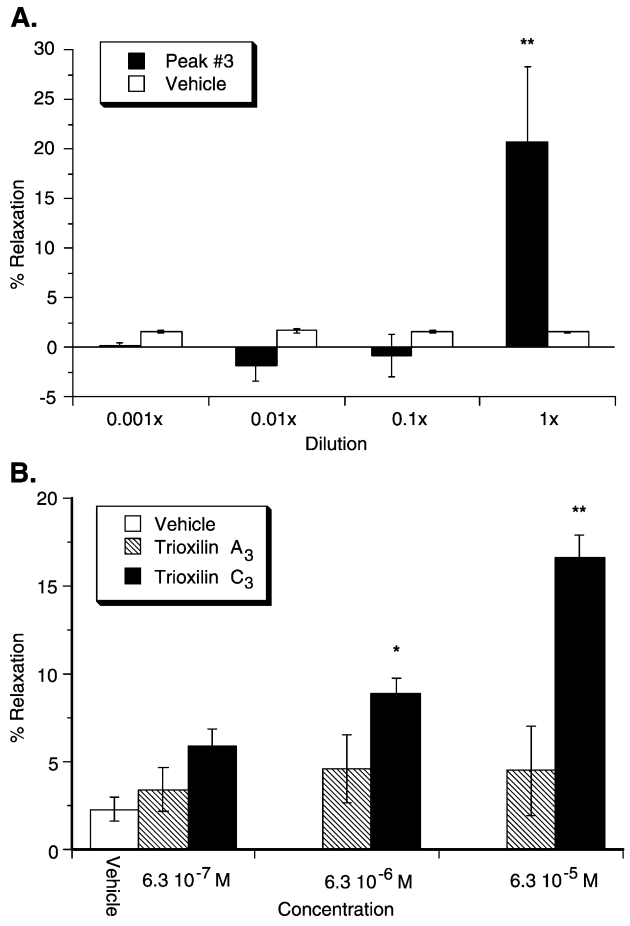

Fig. 4. Effect of peak 3 of Fig. 2C (panel A) and authentic trioxilin A_3 and C_3 (panel B) on precontracted rabbit aorta. Serial dilutions of peak 3 were tested with 1 × representing the stock solution and other concentrations representing 1:10 serial dilutions of the stock. Data are expressed as percent relaxation of the phenylephrine contraction and are shown as the mean ± S.E.M. for $n=6$. $^{**}P<0.001$ treatment vs. vehicle; $^{*}P<0.05$ treatment vs. vehicle.

4. Discussion

Several biologically active products are formed when arachidonic acid is metabolized by 12-lipoxygenase [5–8,10–12,19,20]. The original report by Pace-Asciak et al. [8,9] indicated that hepoxilins were formed through the 12-lipoxygenase pathway via heme-containing proteins that act upon 12(*S*)-HPETE. Further studies showed that this rearrangement occurs in rat vascular tissue and the major products formed are hepoxilin A_3 and hepoxilin B_3 [20]. Hydrolysis of the epoxide groups by an epoxide hydrolase results in the formation of the corresponding trihydroxy metabolites, trioxilin A_3 and B_3. In previous studies, we found no indication of the synthesis of these compounds when rabbit aorta was incubated with arachidonic acid. Rabbit aorta produces a small amount of 12(*S*)-HETE [21]. Instead, our results indicated that in rabbit aortas, arachidonic acid is preferentially metabolized by 15-lipoxygenase to 15-HPETE which undergoes rearrangements to form the HEETAs and THETAs [14]. There are no studies which have shown the expression of 12-lipoxygenase in rabbit vascular tissue and an important aspect of the present study is that when rabbit aortic tissue is provided with an exogenous source of 12-lipoxygenase, 12-HPETE undergoes similar rearrangements as 15-HPETE to form the hepoxilins and trioxilins. Using a variety of HPLC purification steps combined with GC-MS analysis, we report that rabbit aortic tissue produced trioxilin A_3 and trioxilin C_3. The trioxilin synthesis was enzymatic since no production was observed in cell-free, control incubations. There is no indication that aorta produced trioxilin B_3 and this study provides the first evidence that vascular tissue can produce trioxilin C_3.

The enzyme responsible for converting 12-HPETE to the hepoxilins is referred to as a hydroperoxide isomerase. Several heme containing enzymes such as cytochrome P_{450} as well as hemoglobin and hematin, can function as hydroperoxide isomerases and catalyze this rearrangement [22,23]. Our previous studies suggest that rabbit aorta converts 15-HPETE to the HEETAs via a hydroperoxide isomerase [14]. The results of the current study suggest that

the aortic hydroperoxide isomerase may not discriminate between the 15-HPETE and 12-HPETE.

In previous studies, it was shown that the THETAs relaxed precontracted rabbit aorta [14]. The present study tested the effects of the trioxilins on phenylephrine-contracted aorta and found that trioxilin C_3 produced a concentration-dependent relaxation. These results are in contrast to the effects observed with hepoxilin A_3 which produced no direct vasoactive effects but instead sensitized rat aorta to norepinephrine-induced contractions [12]. We did not isolate or test hepoxilin C_3. No studies have shown vasoactive properties associated with either trioxilin A_3 or B_3.

From a physiologic perspective, there is abundant evidence that arachidonic acid undergoes transcellular metabolism or biosynthesis [24–28]. Under these conditions, different cell types may interact and transfer substrate for the biosynthesis of particular compounds. For example, it was shown that platelet-derived arachidonic acid or PGH_2 could be used by endothelial cells to produce prostacyclin [24]. A similar phenomenon occurs when endothelially derived arachidonic acid is used by platelets to produce thromboxane A_2 [29]. Less is known about the transfer of other substrates, such as 12-HPETE. Platelets contain 12-lipoxygenase and synthesize 12-HPETE [30]. While not shown by the current study, it is interesting to speculate that under certain in vivo conditions, the platelet could transfer 12-HPETE to the blood vessel where it can be further metabolized to hepoxilins and trioxilins.

In summary, the present study showed that in the rabbit aorta, arachidonic acid is metabolized by 12-lipoxygenase to 12-HPETE, which undergoes an enzymatic rearrangement to hepoxilins. Hydrolysis of the epoxy group results in the formation of trioxilin A_3 and trioxilin C_3. Trioxilin C_3 relaxes the rabbit aorta.

Acknowledgements

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