Albumin modifies the metabolism of hydroxyeicosatetraenoic acids via 12-lipoxygenase in human platelets

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Abstract **12-Lipoxygenase and cyclooxygenase 1 are the dominating enzymes that metabolize arachidonic acid in human platelets. In addition to the conversion of arachidonic acid to 12(S)-hydroxyeicosatetraenoic acid, 12-lipoxygenase can also utilize 5(S)-hydroxyeicosatetraenoic acid and 15(S)-hydroxyeicosatetraenoic acid to form 5(S),12(S)-dihydroxyeicosatetraenoic acid and 14(R),15(S)-dihydroxyeicosatetraenoic acid, respectively. Furthermore, 15(S)-hydroxyeicosatetraenoic acid works as an inhibitor for 12-lipoxygenase. In the present paper we have studied the influence of albumin on the in vitro metabolism of 5- and 15-hydroxyeicosatetraenoic acids, and 5,15-dihydroxyeicosatetraenoic acid by the platelet 12-lipoxygenase. The presence of albumin reduced the formation of 5(S),12(S)- dihydroxyeicosatetraenoic acid from 5(S)-hydroxyeicosatetraenoic acid, however, it had no effect on the 12(S)-hydroxyeicosatetraenoic acid production from endogenous arachidonic acid. In contrast, when 15(S)-hydroxyeicosatetraenoic acid was incubated with activated platelets, the formation of 14(R),15(S)-dihydroxyeicosatetraenoic acid was stimulated by the presence of albumin. Furthermore, albumin reduced the inhibitory action 15(S)-hydroxyeicosatetraenoic acid had on 12(S)-hydroxyeicosatetraenoic acid formation from endogenous arachidonic acid. However, addition of exogenous arachidonic acid (20 μM) to the incubations inverted the effects of albumin on the conversion of 15(S)-hydroxyeicosatetraenoic acid to 14(R),15(S)-dihydroxyeicosatetraenoic acid and the production of 12(S)-hydroxyeicosatetraenoic acid in these incubations. Based on the Scatchard equation, the estimates of the binding constants to albumin were 1.8×10^5 for 15-HETE, 1.4×10^5 for 12-HETE, and 0.9×10^5 for 5-HETE respectively. These results suggest an important role of albumin for the regulation of the availability of substrates for platelet 12-lipoxygenase.**—Dadaian, M., and P. Westlund. **Albumin modifies the metabolism of hydroxyeicosatetraenoic acids via 12-lipoxygenase in human platelets.** ***J. Lipid Res.*** **1999.** 40: **940–947.**

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Within the eicosanoid field, several hypotheses have been evoked concerning transcellular routes for the biosynthesis of some of these substances, e.g., arachidonic acid can be released from one cell and taken up by another cell for synthesis of eicosanoids; one eicosanoid can be taken up by another cell and further used for synthesis of other eicosanoids (1, 2). Within the leukotriene and lipoxin area this has been expressed as the transcellular hypothesis and has been almost a prerequisite for the synthesis of some of these substances, especially for cells lacking one of the enzymes necessary for the synthesis of the compounds (3, 4). The biosynthesis of lipoxins requires the action of two lipoxygenases (LOs) (5). The location of two LOs in the same cell type is not a common requisite, nevertheless exceptions can be found, e.g., the porcine leukocytes carry both a 5- and a 12-LO (6). However, in general, the synthesis of lipoxins requires more than one cell type, at least among the human cells known to be involved in lipoxin biosynthesis (7).

Studies with whole blood, stimulated by calcium ionophore, showed that the main arachidonic acid metabolites released into the serum fraction are 5-HETE, 12-HETE, 12-hydroxy-heptadecatrienoic acid, and LTB_4 together with ω-hydroxy- and ω-carboxy-LTB_4 (8). Human platelets can convert both 5(S)-hydroxyeicosatetraenoic acid (5-HETE) as well as 15(S)-hydroxyeicosatetraenoic acid (15-HETE) to 5(S),12(S)-dihydroxyeicosatetraenoic acid (5,12-DHETE) and 14(R),15(S)-dihydroxyeicosatetraenoic acid (14,15-DHETE), respectively (9, 10). The conversion rate for 15-HETE was shown to be relatively low (10) and 15-

Abbreviations: 5-HETE, 5(S)-hydoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid; 12-HETE, 12(S)-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid; 12-HPETE, 12(S)-hydroperoxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid; 15-HETE, 15(S)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; 5(S),12(S)-DHETE, 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; 14(R), 15(S)-DHETE, 14(R),15(S)-dihydroxy-5,8-*cis*-10,12-*trans*-eicosatetraenoic acid; 14(S),15(S)-DHETE, 14(S),15(S)-dihydroxy-5,8-*cis*-10,12-*trans*-eicosatetraenoic acid; 5,15-DHETE, 5(S),15(S)-dihydroxy-6,13-*trans*-8,11-*cis*-eicosatetraenoic acid; LX, lipoxin; LXB_4, 5(S),14(R),15(S)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 12-LO, 12-lipoxygenase.

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HETE has also been reported to act as an inhibitor of 12-LO in platelets (11).

It has been known for a long time that albumin can carry fatty acids as a transporter from the peripheral tissue to the liver in which the fatty acids are degraded by β-oxidation (12). We have recently shown, both in vitro and in vivo, that physiological concentrations of albumin have a major influence on the synthesis and metabolism of mono- and dihydroxyeicosatetraenoic acids (13, 14). After injection of 12-HETE i.v. into the rabbit, relatively minor compounds were formed in the circulation and the major lipophilic compound at all time points up to 60 min was 12-HETE itself, indicating that the compound was effectively protected, presumably by albumin, from further uptake and metabolism by other cells in the blood (13). Our in vitro data with leukocytes and a combination of leukocytes and platelets also clearly showed that exogenously added arachidonic acid is to some extent available for the synthesis of 12-HETE in the presence of albumin, whereas the metabolism of 12-HETE by 5-LO or cytochrome P450 in the leukocytes is almost negligible (14).

In the present paper we have studied the effect of albumin on the metabolism of 5- and 15-HETE, as well as 5(S),15(S)-dihydroxyeicosatetraenoic acid (5,15-DHETE) in human platelets. To our surprise the effects of albumin on the metabolism of the two HETEs showed a striking difference. The conversion of 5-HETE to 5(S),12(S)-DHETE was reduced, while the conversion of 15-HETE to 14(R),15(S)-DHETE was increased in the presence of a physiological concentration of albumin. Furthermore, albumin also reduced the inhibitory action of 15-HETE on 12-HETE formation from endogenous arachidonic acid. However, when exogenous arachidonic acid was added to the incubations in the presence of albumin, it had the opposite effect and reduced the formation of 14,15-DHETE. The formation of lipoxin B_4 (LXB_4) from 5,15-DHETE by human platelets was generally below 1% of the added substrate. However, addition of exogenous arachidonic acid seemed to stimulate the production and, furthermore, albumin might also interfere with the arachidonic acid effect. Part of these data has been presented in a preliminary form (15).

MATERIALS AND METHODS

Materials

15(S)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-HETE), 5(S)-hydroxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid (5-HETE), 14(S),15(S)-dihydroxy-5,8-*cis*-10,12-*trans*-eicosatetraenoic acid, 14(R),15(S)-dihydroxy-5,8-*cis*-10,12-*trans*-eicosatetraenoic acid, and 5(S),14(R),15(S)-trihydroxy-6-*trans*-8-*cis*-10,12-*trans*-eicosatetraenoic acid (LXB_4) were obtained from Biomol. 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid was obtained from Cayman Chemical Company. [5,6,8,9,11,12,14,15-^{3}H(n)] 15(S)-HETE, specific activity 180 Ci/mmol, and [5,6,8,9,11,12, 14,15-^{3}H(n)]5(S)-HETE, specific activity 200 Ci/mmol, were obtained from DuPont. [5,6,8,9,11,12,14,15-^{3}H(n)]12(S)-HETE, specific activity 194 Ci/mmol, was from NEN™ Life Science Products, Inc. [1-^{14}C]arachidonic acid, specific activity 58 mCi/mmol, was from Amersham and unlabeled arachidonic acid was from Sigma. 12(S)-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid (12-HETE) was prepared by incubation of arachidonic acid with human washed platelets. Trizma, ethylenediaminetetraacetic acid (EDTA), calcium ionophore A23187, and human serum albumin (crystalline, fatty acid free) and aspirin were from Sigma. Dulbecco's phosphate-buffered saline (PBS) was from Gibco. HPLC solvents were obtained from Rathburn, and all other chemicals used were of analytical grade from Merck AG.

Preparation of human platelets

Human platelets were isolated from buffy coat which was diluted with 0.15 M sodium chloride solution containing 15 mM EDTA to give a final concentration of 5 mM EDTA. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min. Then the platelet-rich plasma was further centrifuged at 650 *g* for 20 min and the platelet pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM EDTA and 0.15 mM NaCl. After recentrifugation at 200 *g* and 650 *g* for 20 min, respectively, the platelets in some cases were resuspended in the same Tris-HCl buffer as above, supplemented with aspirin at a concentration of 3 mM, and were incubated for 30 min at room temperature. Before the incubations with the hydroxy acids, the platelets were centrifuged at 650 *g* for 20 min and the platelet pellet was resuspended in PBS, pH 7.4, with a final platelet concentration of 1.2×10^9 platelets/ml unless otherwise stated.

Experimental conditions

The platelets were preincubated for 5 min at 37°C in 0.5–1 ml of PBS in the presence or absence of albumin at a final concentration of 600 μM before the addition of substrate and/or A23187. A23187 and the eicosanoids were all dissolved in ethanol and added to the incubations in a total volume not exceeding 2% of the incubation volume. The final concentrations used for [^{3}H$_8$]5-HETE and [^{3}H$_8$]15-HETE were 10 or 50 μM, 0.1 μCi/1.2×10^9 platelets. The final concentration of 5,15-DHETE was 50 μM, and the final concentration of [1-^{14}C]arachidonic acid, if added, was 20 μM, 0.08 μCi/1.2×10^9 platelets. The incubation time was 60 min with or without stimulation with 3 μM A23187. The reactions were stopped by the addition of 3 volumes of ethanol and the incubations were stored at −20°C until processing.

Purification and analysis

The samples were centrifuged at 900 *g* for 10 min to remove the precipitated cells. In incubations without addition of labeled HETEs, [1-^{14}C]arachidonic acid was added to monitor the recovery of lipophilic compounds. The supernatant was acidified to approximately pH 3.0 with 1 M HCl and the volume was adjusted by addition of acidified water giving a final content of ethanol not exceeding 10%. The samples were extracted using Sep Pak C_{18} (Waters) cartridges eluted with water and HPLC–methanol. After evaporation of the methanol under nitrogen, the material was dissolved in 200 μl of methanol and was further analyzed by reversed phase high performance liquid chromatography (RP-HPLC). Aliquots of the methanol fractions from the incubations containing labeled hydroxy acids were also analyzed by two-dimensional high performance thin-layer chromatography (2D-HPTLC) using silica gel 60, 10 × 10 cm plates from Merck, and the product profile was visualized by autoradiography. The HPTLC plates were first developed in toluene–acetone–acetic acid 80:20:2 (by vol), dried in a desiccator under vacuum for 30 min and brought to normal pressure with nitrogen. The plates were then turned 90° and were developed in a second system, hexane–ethyl acetate–acetic acid 70:30:2 (by vol). After drying, the plates were covered with ^{3}H-Ultrofilm (Leica Instruments) and left for different periods of time depending on the amount of applied radioactivity (16). The radioactive spots were scraped

and transferred into scintillation vials, and the radioactivity was determined using Ready Safe scintillation cocktail (Beckman) and a Tri-Carb 2000CA liquid scintillation analyzer (Packard).

HPLC analyses of the samples were performed using System Gold (Beckman) equipped with a diode array detector module 168 and a Nucleosil C_{18} column (50-5, 250 × 4 mm). The flow rate was 1 ml/min and the samples from the experiments without addition of HETEs were eluted isocratically with methanol–water–acetic acid 80:20:0.01 (by vol) and with methanol–water–acetic acid 75:35:0.01 (by vol) from the incubations with 5- and 15-HETE. The samples from the experiments with addition of 5,15-DHETE were eluted stepwise by the following solutions of methanol–water–acetic acid 65:35:0.01 for 30 min (eluting lipoxins and DHETEs); 80:20:0.01 for 30 min (eluting mono HETEs); and finally with methanol for another 30 min. HETEs and DHETEs were detected at 235 nm and 270 nm and 243 nm, respectively, lipoxins were detected at 302 nm. The quantification of 5-, 12-, and 15-HETE as well as 5,15-DHETE and lipoxin was done by comparison of peak heights with calibration curves established using standard solutions of 5-, 15-HETE, 5,15-DHETE, and lipoxin B_4 in the above-described HPLC systems. The amount of 12-HETE formed was calculated using the calibration curve for 15-HETE, as both compounds have the same ε_{237}. Two-ml fractions were collected from the HPLC, and aliquots were taken from each fraction to determine radioactivity.

Characterization of formed products

The products formed were characterized by co-chromatography with known synthetic and biosynthetic standards and by comparison of the UV spectra with the same standards.

Affinity chromatography

Affinity chromatography was performed using a column (1 × 3 cm) packed with Blue Sepharose CL 6B (Pharmacia) which was equilibrated with 50 mm Tris-HCl, pH 7.0, with 0.1 m KCl. 5-, 15-HETE, or 5,15-DHETE were added to 50 μl of an albumin solution (40 mg/ml) in the same buffer as above or to 50 μl of a buffer solution without albumin and were applied to the column. The concentration of HETEs tested was 10 and 50 μm with an addition of 50,000 cpm of the corresponding radiolabeled HETE. The concentration of 5,15-DHETE was 50 μm. The column was washed with 6 ml of the application buffer without albumin and then eluted with 10 ml of 50 mm Tris-HCl, pH 7.0, with 1.5 m KCl to liberate the albumin from the column. The effluent was monitored at 280 nm (albumin) and 1-ml fractions were collected for radioactivity determinations. The recovery of albumin was estimated by comparison of the absorbance with a standard curve produced under the same conditions. When 5,15-DHETE was applied to the column, the collected fractions (1 ml) were acidified and extracted using Sep Pak C_{18} (Waters) cartridges eluted with water and methanol. The methanol fractions were further analyzed by RP-HPLC under the same conditions as described above (see Purification and analysis) with the mobile phase consisting of methanol–water–acetic acid 65:35:0.01 and the detection at 243 nm.

Equilibrium dialysis

Binding constants of 5(S)-, 12(S)-, and 15(S)-HETE to albumin were determined by equilibrium dialysis. Each hydroxyeicosatetraenoic acid, dissolved in ethanol, was added to 10 ml of PBS, pH 7.4, together with 0.125 μCi of the corresponding labeled compound. The final concentrations of the hydroxyeicosatetraenoic acids were from 1.5×10^{-6} m to 1.5×10^{-5} m, and the amount of the radioactivity was constant. The volume of added ethanol was less than 0.5% of the total volume. The dialysis was performed against 1.5×10^{-5} m solution of human serum albumin (0.5 ml in PBS, pH 7.4) placed in dialysis bags (Union Carbide), which were placed in the vials containing hydroxyeicosatetraenoic acids, for 20 h at 37°C with shaking. After dialysis, aliquots were taken to measure radioactivity inside and outside the dialysis bags. In some experiments the dialysis was performed against PBS without albumin under the same conditions. Calculations of binding constants were performed using the Scatchard equation (17) and Sigma Plot software from Jandel Scientific.

Statistics

The statistical evaluation was performed by Kruskal-Wallis analysis of variances on ranks due to the failure of Normality and Equal Variance tests in some cases. However, the data are presented as mean ± standard error for a better graphic illustration.

RESULTS

Platelets or aspirin-treated platelets were incubated for 60 min with 10 μm or 50 μm [$^3H_8$]5-HETE or [$^3H_8$]15-HETE or 50 μm 5,15-DHETE in the presence or absence of 600 μm albumin. In some of the experiments the platelets were stimulated by the calcium ionophore A23187. The metabolic profile of the respective HETE was analyzed using both 2D-HPTLC and autoradiography as well as HPLC and scintillation counting. Products formed were identified by co-chromatography and by spectral comparison with compounds with known stereochemistry.

Albumin influence on 5-HETE metabolism in human platelets

Typical autoradiograms of the 2D-HPTLC analyses of 5-HETE (50 μm) metabolism in aspirin-treated human platelets during four different conditions are shown in **Fig. 1**. Analysis of the radioactivity in different spots on the HPTLC plates showed that one main metabolite was formed by the platelet 12-LO when no albumin was present in the incubations (panels 3 and 4, Fig. 1). The production of this metabolite was considerably enhanced when the platelets were activated by calcium ionophore (panel 4, Fig. 1). In the presence of physiological concentration of albumin, the formation of this metabolite by both unstimulated and stimulated platelets was markedly reduced (panels 1 and 2, Fig. 1). The same results were obtained during the incubations with the platelets that were not treated by aspirin (data not shown).

HPTLC as well as HPLC of the compound revealed that it was identical to 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid, the major compound formed from 5(S)-HETE by the action of 12-LO in platelets.

The HPLC data from the incubations corroborated the HPTLC data. When unstimulated platelets were incubated with 50 μm of 5-HETE in the absence of albumin, the amount of 5(S),12(S)-DHETE formed was about 0.8 μg/1.2×10^9 platelets, conversion of 8% of added radioactive 5-HETE. Albumin reduced the formation of this compound to 0.09 μg/1.2×10^9 platelets. Ionophore-activated platelets produced about 4 μg/1.2×10^9 platelets of 5(S),12(S)-DHETE. In this case, albumin effectively reduced the formation of this compound to 0.4 μg/1.2×10^9 platelets (**Fig. 2**, panel 1). When the platelets were in-

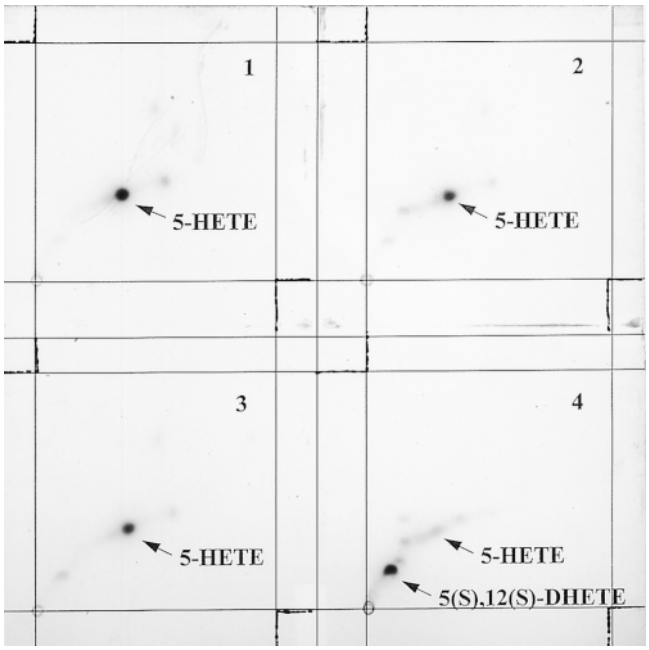

Fig. 1. Profiles of 5-HETE metabolites in aspirin-treated human platelets, analyzed by two-dimensional high performance thin-layer chromatography with subsequent autoradiography. The final concentration of 5-HETE was 50 μm and the added amount of [$^3H_8$]5-HETE was 2.5 × 10^5 dpm. Panel 1: no A23187, albumin (600 μm); panel 2: A23187 (3 μm), albumin (600 μm); panel 3: no A23187, no albumin; panel 4: A23187 (3 μm), no albumin.

cubated with 10 μm 5-HETE, the conversion to 5(S),12(S)-DHETE was about 40%–60% with or without activation and the amount of 5(S),12(S)-DHETE formed was about 1.2 μg/1.2 × 10^9 platelets. The presence of albumin in the incubation medium reduced the production of 5(S),12(S)-DHETE to around 0.12 μg/1.2 × 10^9 platelets. In conclusion, the presence of albumin reduced the production of 5(S),12(S)-DHETE about 90%, thus suggesting that albumin sequesters 5-HETE from the metabolism by 12-lipoxygenase.

The formation of 12-HETE from endogenous arachidonic acid was also followed during the above-described incubations. As expected, the formation of 12-HETE was low without any stimulation of the cells. After ionophore stimulation, 12-HETE was formed in substantial amounts and neither albumin (**Fig. 3**, panel 1) nor 5-HETE (Fig. 2, panel 1) seemed to influence the formation of 12-HETE from endogenous arachidonic acid during the conditions used.

As it appeared that albumin effectively withdrew 5-HETE from the metabolism by 12-LO during long term incuations (1 h) but did not significantly influence the production of 12-HETE from endogenous arachidonic acid, it was of interest to test whether there was any competition between 5-HETE and exogenously added arachidonic acid for albumin binding and thus for the availability of these fatty acids for 12-LO.

Platelets were preincubated for 5 min with or without 600 μm of albumin, and [$^3H_8$]5-HETE (50 μm), [1-^{14}C] arachidonic acid (20 μm), and calcium ionophore (3 μm) were added simultaneously, and the platelet suspension was incubated for 60 min. HPLC analyses of the lipid extracts from these incubations revealed that in the absence of albumin, stimulated platelets produced around 1.6 μg/1.2 × 10^9 platelets of 5(S),12(S)-DHETE, which was half of the amount formed during the incubations without any addition of arachidonic acid (Fig. 2, panel 2). These results suggest that the added arachidonic acid competed with 5-HETE as a substrate for the platelet 12-LO. As would be expected, in the absence of 5-HETE, platelets produced substantially higher amounts of 12-HETE when exogenous arachidonic acid was present in the incubations (Fig. 3, panel 2). However, in the presence of 5-HETE, 12-HETE production was on the same level as results obtained without any addition of exogenous arachidonic acid (Fig. 2, panel 2), although the conversion of the radioactive, exogenous arachidonic acid to 12-HETE was in

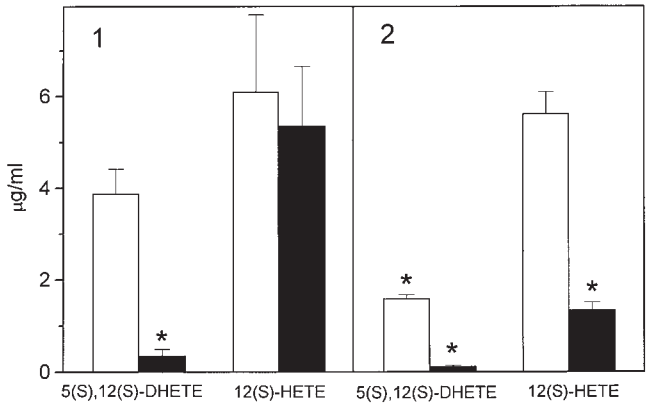

Fig. 2. Effect of albumin on 5,12-DHETE and 12-HETE production by platelets (1.2 × 10^9 platelets/ml) incubated with 5-HETE (50 μm) and stimulated with calcium ionophore A23187. The platelets were incubated in the absence (panel 1) and presence (panel 2) of exogenous arachidonic acid (20 μm). Open bars, without albumin; filled bars, with albumin. The values are mean of four experiments. *$P < 0.05$.

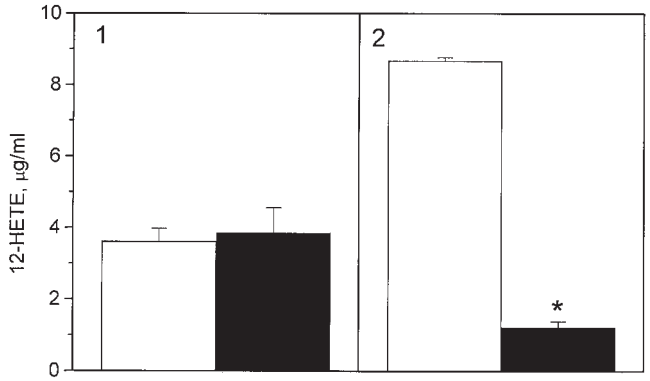

Fig. 3. Effect of albumin on 12-HETE production by platelets stimulated with calcium ionophore A23187. The platelets (1.2 × 10^9 platelets/ml) were incubated in the absence (panel 1) and presence (panel 2) of exogenous arachidonic acid (20 μm). Open bars, without albumin; filled bars, with albumin. The values are mean of four experiments. *$P < 0.05$.

the range of 55%–65%. In this case the presence of albumin decreased the formation of 5(S), 12(S)-DHETE, as well as the conversion of exogenous arachidonic acid to 12-HETE, which was about 11% of the added [1-^{14}C]arachidonic acid (Figs. 2 and 3, panel 2). These results show that albumin sequesters 5-HETE as well as exogenously added arachidonic acid from the metabolism by 12-LO. However, albumin had no effect on the metabolism of endogenous arachidonic acid by the platelet 12-LO during a 1-h incubation.

Albumin influence on the metabolism of 15-HETE by platelets

When 15-HETE was incubated with either untreated or aspirin-treated human platelets with and without albumin, one radioactive spot with an R_f value slightly more polar than that of 15-HETE appeared in all the incubations; however, this product was also formed nonenzymatically during incubation of 15-HETE in buffer alone. The production of another more polar metabolite was enhanced several fold in the presence of albumin and calcium ionophore A23187 as judged by HPTLC (**Fig. 4**, panel 2) and HPLC. The analysis of radioactivity in the spots on HPTLC plates showed that in the presence of albumin and during stimulation of platelets, the formation of the metabolite increased to 18% of the added radioactivity (Fig. 4, panel 2) compared to 2% in the absence of albumin (Fig. 4, panel 4) with/or without stimulation (Fig. 4, panels 1 and 3).

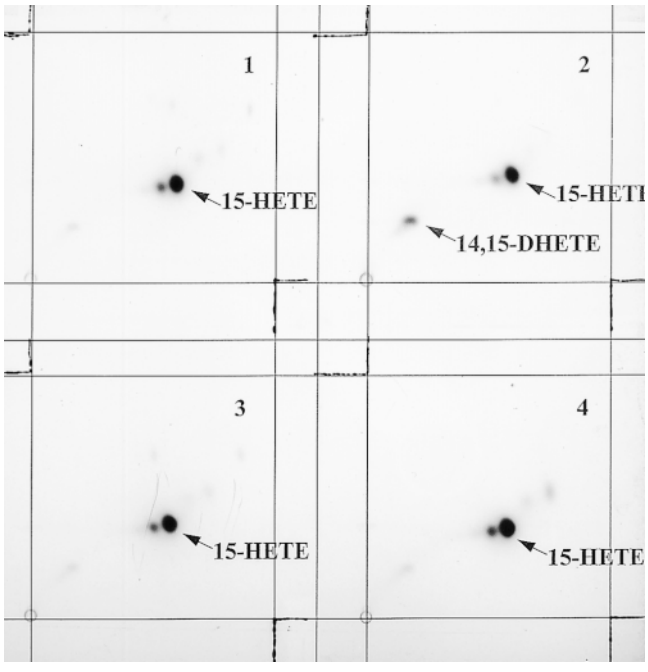

Fig. 4. Profiles of 15-HETE metabolites in aspirin-treated human platelets analyzed by two-dimensional high performance thin-layer chromatography with subsequent autoradiography. The final concentration of 15-HETE was 50 μm and the added amount of [$^3H_8$]15-HETE was 2.5×10^5 dpm. Panel 1: no A23187, albumin (600 μm); panel 2: A23187 (3 μm), albumin (600 μm); panel 3: no A23187, no albumin; panel 4: A23187 (3 μm), no albumin.

The major metabolite of radiolabeled 15-HETE was further characterized by spectral and chromatographic properties in HPLC as free carboxy acid as well as methyl ester. The metabolite contained a conjugated triene structure with absorbance UV maxima at 264, 274, and 284 nm. That indicated, as expected, that the compound had retained its 15-hydroxyl group and had added another oxygen at carbon atom 8 or 14. The UV spectrum, maximum at 274 nm, indicated that the product was a 14,15-DHETE, with the S-configuration retained at the 15 hydroxyl group and the *cis-cis* geometry retained at the double bonds at carbon atoms 5 and 8. Two synthetic isomers were compared with the compound, 14(R),15(S)-dihydroxyeicosatetraenoic acid (14(R),15(S)-DHETE), and 14(S),15(S)-dihydroxyeicosatetraenoic acid (14(S),15(S)-DHETE). The chromatographic properties and UV spectrum of the synthetic isomer 14(R),15(S)-DHETE and of the formed compound were identical.

The presence of albumin clearly stimulated the formation of 14(R),15(S)-DHETE by activated untreated or aspirin-treated platelets. HPLC analysis showed that the formation of this compound in the presence of albumin and calcium ionophore was increased 7-fold (absolute amount 1.83 μg/1.2×10^9 platelets) in comparison to formation in the absence of albumin and/or unstimulated platelets (absolute amount 0.26 μg/1.2×10^9 platelets), **Fig. 5**, panel 1.

When the formation of 12-HETE from endogenous arachidonic acid was recorded during these incubations, the synthesis of 12-HETE was reduced when compared to the incubations without 15-HETE. 15-HETE in two concentrations tested (10 and 50 μm) significantly inhibited the production of 12-HETE from endogenous arachidonic acid in the absence of albumin, which is in agreement with previous findings (11). However, in the presence of albumin, the formation of 12-HETE from endogenous arachidonic acid by activated platelets was enhanced and reached the same level as in the absence of 15-HETE (Fig.

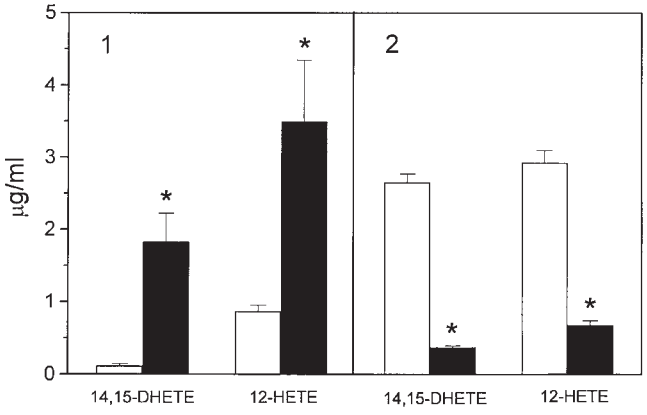

Fig. 5. Effect of albumin on 14,15-DHETE and 12-HETE production by platelets incubated with 15-HETE and stimulated with calcium ionophore A23187. The platelets (1.2×10^9 platelets/ml) were incubated in the absence (panel 1) and presence (panel 2) of exogenous arachidonic acid (20 μm). Open bars, without albumin; filled bars, with albumin. The values are mean of four experiments. *$P < 0.05$.

5, panel 1, compare to Fig. 3). Thus the inhibitory action of 15-HETE on 12-LO was diminished in the presence of albumin and furthermore, the conversion of 15-HETE to 14,15-DHETE by the same enzyme was enhanced in this case.

The addition of exogenous arachidonic acid to the platelets incubated under the same conditions reversed the above-described effects of albumin on the 15-HETE metabolism as well as on the 12-HETE synthesis by 12-LO. When 20 μm [1-^{14}C]arachidonic acid together with 50 μm [$^{3}H_{8}$]15-HETE was incubated without albumin, a substantial amount of 14,15-DHETE (2.6 μg/1.2 × 10^9 platelets, 23% conversion of the added 15-HETE) was formed by activated platelets (Fig. 5, panel 2). Albumin reduced the production of the dihydroxy acid to 0.36 μg/1.2 × 10^9 platelets, 3% of the added radioactivity, as well as the formation of 12-HETE (Fig. 5, panel 2). Only 8% of the added arachidonic acid was converted to 12-HETE in the incubations with albumin, which is in contrast to experiments with endogenous arachidonic acid. About 40% of the added arachidonic acid was converted to 12-HETE without albumin; however, the formation of 12-HETE in these experiments with exogenous arachidonic acid never reached the same level as in the absence of 15-HETE.

Metabolism of 5,15-DHETE in human platelets

The experiments described above dealt with the platelet metabolism of hydroxyeicosatetraenoic acids that differed by the position of the hydroxy group. In the presence of albumin, the metabolism of 5-HETE by platelet 12-LO was reduced, while that of 15-HETE was enhanced by the same enzyme. It was therefore of interest to follow the platelet metabolism of a compound with hydroxyl groups at both carbon atoms 5 and 15 in its structure. To understand whether the position and the number of the hydroxy groups and the stereochemistry of the double bonds played any role in the effect of albumin, human platelets were incubated with 5(S),15(S)-DHETE under the conditions outlined above.

In these experiments, 5(S),15(S)-DHETE was converted to a product with absorbance at 301 nm and eluting as one peak on HPLC. The metabolite contained a conjugated tetraene structure as judged by UV spectroscopy with UV maxima at 286, 301, and 315 nm. According to an early report (18) one of the possible biosynthetic pathways for lipoxin B_4 (LXB_4) is conversion of 5(S), 15(S)-DHETE by reticulocyte lipoxygenase. It has also been shown that leukocyte 12-lipoxygenase can react with 5,15-DHETE resulting in the production of LXB_4 (19). Co-chromatography of the compound formed from 5,15-DHETE with the authentic standard showed that it coeluted with LXB_4.

The conversion of 5,15-DHETE to LXB_4 in platelets without any addition of exogenous arachidonic acid was comparably low, about 0.6% (absolute amount formed was 0.1 μg/1.2 × 10^9 platelets); furthermore, albumin did not interfere with the production of LXB_4 (**Fig. 6**, panel 1). However, addition of 20 μm arachidonic acid somewhat but significantly enhanced the production of this compound (absolute amount 0.17 μg/1.2 × 10^9 platelets)

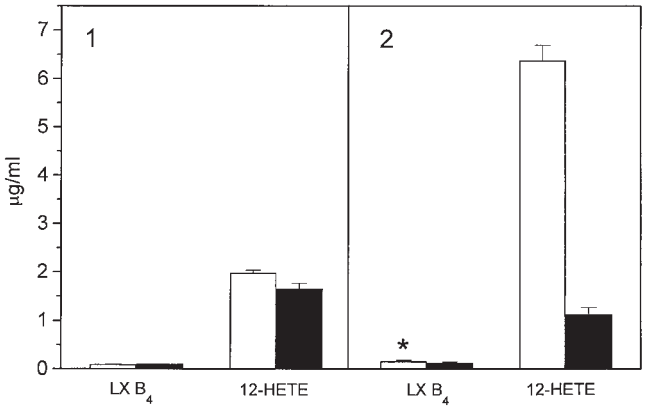

Fig. 6. Effect of albumin on LXB_4 and 12-HETE production by platelets incubated with 5,15-DHETE and stimulated with calcium ionophore A23187. The platelets (1.2 × 10^9 platelets/ml) were incubated in the absence (1) and presence (2) of exogenous arachidonic acid (20 μm). Open bars, without albumin; filled bars, with albumin. The values are mean of four experiments. *$P < 0.05$.

in the absence of albumin. Albumin presence in these incubations reduced its formation slightly but significantly (about 0.1 μg/1.2 × 10^9 platelets), Fig. 6, panel 2. Thus the addition of arachidonic acid to platelets enhanced the production of lipoxin B_4. However, the production of 12-HETE from endogenous as well as exogenous arachidonic acid was not influenced by 5,15-DHETE addition neither in the presence nor in the absence of albumin (Fig. 6, panels 1 and 2).

Albumin binding of hydroxyeicosatetraenoic acid

When 5-HETE, 15-HETE, or 5,15-DHETE were applied separately to a column with Blue Sepharose, none of the fatty acids were retained by the column. However, when they were added together with albumin, more than 85% of the monohydroxy acids and about 50% of 5,15-DHETE were retained by the column and could be eluted by increasing salt concentration, eluting both albumin and the fatty acid together (**Fig. 7**). Using 10 or 50 μm concentra-

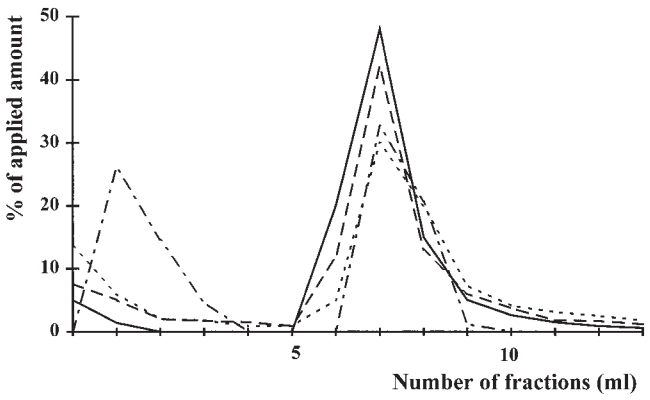

Fig. 7. Percent of the amount of the radioactivity of 5-HETE (····) and 15- HETE (----), and concentration of 5,15-DHETE (·····) applied to a Blue Sepharose column together with albumin (——) in different fractions.

tion of the fatty acid did not influence the binding of the fatty acids to albumin and Blue Sepharose. Equilibrium dialysis was performed to estimate the binding constants of hydroxyeicosatetraenoic acids to albumin. Based on the Scatchard equation, the estimates of the binding constants were 1.8×10^5 for 15-HETE, 1.4×10^5 for 12-HETE, and 0.90×10^5 for 5-HETE respectively.

DISCUSSION

Albumin is a well-known transporter of several types of compounds, e.g., fatty acids and different kinds of drugs and metals, thus interacting with the bioavailability of the compounds. Within the eicosanoid field albumin is known to stabilize thromboxane A_2 and, as a result, to prolong its half-life in aqueous media and also to react with TXA_2 and bind some of it covalently (20). Furthermore, LTA_4, the highly reactive intermediate in the leukotriene biosynthesis, is stabilized by the addition of low concentration of albumin (21). As it has been shown that stimulated neutrophils release almost exclusively LTA_4 versus LTB_4 (22, 23) this albumin effect is essential for the biological impact of LTA_4. However, LTA_4 undergoes a time-dependent disappearance in plasma, probably due to covalent binding to albumin (23).

In the present paper we have studied the influence of albumin on the in vitro metabolism of 5-, 15-HETE, and 5,15-DHETE in human platelets. These hydroxy acids have been reported to interact with the 12-LO in different ways.

The incubations of 5-HETE with human platelets as well as with the partially purified platelet 12-LO showed that 5-HETE can be metabolized by this enzyme with the production of 5(S),12(S)-DHETE (8, 24, 25). Although the rate of the reaction of the purified enzyme with 5-HETE was reported to be 3% of that of arachidonic acid (24), our results showed that during long-term incubations, activated platelets can convert a relatively huge amount of 5-HETE to 5(S),12(S)-DHETE. The concentration of 5-HETE used in this study did not show any competitive inhibition of the 12-HETE synthesis from endogenous arachidonic acid. However, when exogenous arachidonic acid (20 μm) was added to the incubations, we observed some inhibitory effect of 5-HETE on the 12-HETE production. Exogenous arachidonic acid at the same time competed with 5-HETE as a substrate for 12-LO, as the production of 5(S),12(S)-DHETE was reduced by 50% and furthermore, the production of 12-HETE was lower compared to incubations without 5-HETE. Obviously, there are differences in the availability of the endogenous and exogenous substrates for 12-LO, and albumin seems to reduce the availability of exogenous arachidonic acid, as well as 5-HETE as substrates for the 12-LO in platelets. Thus our data demonstrate that albumin binding is important for the availability of exogenous arachidonic acid and 5-HETE for the metabolism of these compounds by the 12-LO in platelets, whereas the endogenous pool of arachidonic acid is less dependent on albumin.

However, in the incubations with 15-HETE, the conversion of 15-HETE to 14,15-DHETE was increased in the presence of albumin which was in contrast to the data found for 5-HETE. Interestingly, the formation of 12-HETE from endogenous arachidonic acid was also increased in the presence of albumin, indicating that albumin had bound some of the 15-HETE and by that reduced its inhibition of the 12-LO.

The data from the Blue Sepharose column clearly showed that both 5- and 15-HETE, as well as 5,15-DHETE, were retained by the column in the presence of albumin. Comparison of the binding constants of 5- and 15-HETE to albumin showed that the binding of albumin to 15-HETE was twice as high. This means that the effect of albumin in terms of sequestering (and by that reducing) the metabolism for 15-HETE should be more pronounced than for 5-HETE. However, we observed the opposite effect, which cannot be explained by the differences in the binding constants for these hydroxy acids.

It should be noted that in the discussed events concerning the studies with the platelets activated by calcium ionophore A23187, the crucial point is the activation of 12-LO. It has been reported that 12-HPETE, the labile lipoxygenase product, stimulates its own production by increasing platelet 12-LO activity (26). Furthermore, it also stimulates the production of lipoxins by platelet 12-lipoxygenase (27). We propose that the formation of the hydroperoxide at C-12 but not at C-14 is the factor that can reactivate the enzyme. As soon as enough 12-HPETE is produced to fully activate 12-LO, it might efficiently metabolize both arachidonic acid and 15-HETE during long time incubations. Thus it seems that the ratio of arachidonic acid to 15-HETE is important in regulating the activity of 12-LO. Our results show that in the absence of albumin, 15-HETE probably competes efficiently with endogenous arachidonic acid for 12-LO, reducing the availability of endogenous arachidonic acid and by that decreasing the production of 12-HPETE. This leads to inhibition of the enzyme activity. However, in the presence of albumin, part of the added 15-HETE is bound to this protein, and more of endogenous arachidonic acid is available for metabolism by 12-LO. The reduced availability of 15-HETE in the presence of albumin allows the production of increased quantities of 12-HPETE, enough to initiate the reaction and activate the enzyme. This can explain the reversibility of the inhibitory effect of 15-HETE on 12-LO in the presence of albumin. However, it seems that activated enzyme can efficiently metabolize 15-HETE in addition to arachidonic acid during long-term incubation.

When exogenous arachidonic acid was added to the incubations together with 15-HETE in the absence of albumin, we observed the formation of substantial amounts of 14,15-DHETE, which confirms our explanation of the key role of 12-HPETE in the activation of 12-LO for metabolism of 15-HETE. In the presence of albumin it effectively sequestered both 15-HETE and exogenous arachidonic acid from the metabolism by platelets, as in the case with 5-HETE.

When 5,15-DHETE was incubated with activated platelets, we observed the formation of LXB_4, although the

conversion to this product was very low. Nevertheless, in agreement with the studies described above, addition of exogenous arachidonic acid somewhat but significantly increased the formation of LXB_4. Albumin did not influence the metabolism of 5,15-DHETE when no arachidonic acid was added to the incubations. However, when exogenous arachidonic acid was present, albumin significantly reduced the formation of LXB_4. This is in agreement with the previous study, which showed that lipoxin synthesis from leukotriene A_4 in human platelets is activated by 12-HPETE (27) and according to our explanation of the effect of albumin on the metabolism of 5- and 15-HETE by platelet 12-LO.

The studies presented here show that albumin interferes with the metabolism of hydroxy- and dihydroxyeicosatetraenoic acids by human platelet 12-lipoxygenase. However, the differences in the metabolism of 5- and 15-HETE that we observed did not relate to some specific albumin effects. It seems that albumin binds and simply sequesters the compounds from platelets. The important point in this case is the availability of the substrates for 12-lipoxygenase and the amount of 12-hydroperoxide produced by the enzyme. Thus, due to its ability to bind fatty acids, albumin can be considered as a regulatory factor for the activity of platelet 12-lipoxygenase.

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