Increased Levels of 12(S)-HETE in Patients With Essential Hypertension

Daniel González-Núñez, Joan Claria, Francisca Rivera, Esteban Poch

Abstract—The platelet-type 12-lipoxygenase (12-LO) catalyzes the transformation of arachidonic acid into 12-hydroperoxyeicosatetraenoic acid [12-(S)HPETE], which is reduced to 12-hydroxyeicosatetraenoic acid [12-(S)HETE]. These metabolites exhibit a variety of biological activities such as mediation of angiotensin II–induced intracellular calcium transients in cultured rat vascular smooth muscle cells. It has recently been reported that platelet 12(S)-HETE production is enhanced in the spontaneously hypertensive rat. The pronounced hypotensive effect of LO inhibition in SHR suggests that LO activity may play a role in this form of hypertension. The aim of this study was to determine the basal and thrombin-induced platelet 12(S)-HETE production and the urinary 12(S)-HETE excretion in essential hypertension. We studied 19 patients with this disease (57±2 years of age) and 9 normotensive control subjects (48±5 years of age) (P=0.074). 12(S)-HETE was measured in Sep-Pack–extracted samples with specific ELISA and high-performance liquid chromatography. The platelet basal level of 12(S)-HETE was significantly higher in patients than in control subjects (3.56±1.22 versus 0.64±0.13 ng/10^6 platelets, P<0.025). In contrast, there were no differences in thrombin-stimulated (1 U/mL) 12(S)-HETE generation: 7.66±2.14 in patients versus 4.87±1.46 in control subjects (P=0.61). Platelet 12-LO protein levels, measured by Western blotting with a polyclonal antibody, were higher in the patients than in the control subjects. The urinary excretion of 12(S)-HETE was higher in patients than in control subjects: 36.8±7.24 versus 17.1±3.14 ng/mg creatinine (P<0.01). These results indicate that 12(S)-HETE levels and 12-LO protein are increased in patients with essential hypertension, suggesting a role for this metabolite in human hypertension. (Hypertension. 2001;37:334-338.)

Key Words: arachidonic acid ■ hypertension, essential ■ lipoxygenase ■ thrombin

Lipoxygenases constitute a family of lipid-peroxidizing enzymes that oxygenate free and esterified polyenic fatty acids to the corresponding hydroperoxide derivatives. The platelet-type 12-lipoxygenase (platelet 12-LO; EC 1.13.11.31) was the first mammalian lipoxygenase discovered and is expressed in platelets, megakaryocytes, and epithelial cells.1 Platelet 12-LO catalyzes the transformation of arachidonic acid into 12-hydroperoxyeicosatetraenoic acid [12-(S)HPETE], which is reduced by different mechanisms to 12-(S)hydroxyeicosatetraenoic acid [12-(S)HETE].1 A dual origin for 12-HETE has been described because it also appears as an R stereoisomer, 12(R)-HETE, which is produced by cytochrome P-450.2

A number of cardiovascular biological activities have been reported for 12(S)-HETE and 12(R)-HETE. Vascular tissue exhibits both lipoxygenase and cytochrome P-450 activities, and both 12(S)-HETE and 12(R)-HETE have been demonstrated to act as vasoconstrictors in small renal arteries.3 In addition, a role for 12(S)-HETE in the development of angiotensin II–dependent hypertension has been described.4 Moreover, 12(S)-HETE also participates in the modulation of angiotensin II–induced aldosterone secretion5 and in angiotensin II–induced intracellular calcium transients in cultured rat vascular smooth muscle cells.6 An enhanced platelet 12(S)-HETE production in the spontaneously hypertensive rat (SHR) has been recently reported.7,8 In addition, the prolonged and dose-dependent hypotensive effect of 12-LO inhibition observed in SHR7 suggests that this enzyme may play a role in this form of hypertension. Recent studies have evaluated the 12(S)-HETE profile in patients with non–insulin-dependent diabetes mellitus,9,10 with conflicting results. On the one hand, one study reported increased urinary excretion of 12 (S)-HETE in diabetic patients as compared with control subjects.9 In this study, there was no influence of the blood pressure level on urinary excretion of 12(S)-HETE. Moreover, a subgroup of nondiabetic patients with essential hypertension did not show increased urinary excretion of 12(S)-HETE. On the other hand, a Japanese study demonstrated that the activity of 12-LO in platelets from diabetic subjects is lower than in control subjects.10 However, platelet production of 12(S)-HETE has not yet been reported in human hypertension.

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In this study, we examined 12(S)-HETE production by platelets and the urinary 12(S)-HETE excretion in patients with essential hypertension and in normotensive control subjects. Moreover, we analyzed the protein expression of 12-LO in the different platelet subcellular localizations in hypertensive and normotensive subjects.

Methods

Study Subjects

Nineteen essential hypertensive outpatients were consecutively recruited from the Nephrology Service of the Hospital Clinic, Barcelona, Spain. The diagnosis of essential hypertension was considered on the basis that no known cause of high blood pressure could be detected after complete clinical, biochemical, and radiological examination. All the patients had ≥3 office blood pressure measurements >140/90 mm Hg after 4 weeks of an unrestricted salt diet and without antihypertensive medication. Informed consent was obtained from all the participants. Patients and control subjects were maintained on a normocaloric diet with a sodium intake of ~100 mmol/d and were asked to collect a 24-hour sample of urine the day before the study. Subjects were studied in the fasting state between 8 and 10 AM. Twenty milliliters of venous blood was sampled without stasis for the measurement of platelet 12(S)-HETE and for the isolation of platelet proteins. Mean arterial blood pressure was calculated as the diastolic pressure plus one-third the pulse pressure. Body mass index (BMI) was calculated as weight (kg)/height (m)². Nine volunteer subjects with no history of hypertension or cardiovascular disease with blood pressure <130/80 mm Hg in 3 separate determinations were selected as normotensive control subjects.

Platelet Preparation

Human platelets were isolated in polypropylene tubes from blood anticoagulated with ACD (in mmol/L: 3.8 Trisodium citrate, 7.5 Trisodium citrate, and 13.6 dextrose) at a ratio of 9:1 (blood:ACD) from 9 healthy donors and 9 patients with essential hypertension who had not taken aspirin (or other medications) for ≥10 days. To obtain platelet-rich plasma, the blood was centrifuged at 200 g for 15 minutes at room temperature. The platelet-rich plasma was carefully collected by aspiration and was then centrifuged at 1200 g for 15 minutes. Plasma was removed, and the platelet pellet was washed twice in Tyrode’s-HEPES buffer and 14 mmol/L EDTA. Isolated platelets were resuspended in 2 mL Tyrode’s-HEPES buffer, and the sample was mixed by inversion. One aliquot of 900 μL (aliquot A) was incubated with 1 U/mL thrombin (Sigma-Aldrich, Germany) for 30 minutes at 37°C, and the second aliquot was incubated without thrombin (aliquot B). After the incubation, 2 volumes of methanol were added to both aliquots and the samples were stored at −20°C until the assay. Finally, an aliquot of 50 μL was used to determine platelet count number in a Neubauer chamber.

12(S)-HETE Production in Platelets

To measure 12(S)-HETE generation from platelets, 50 μL of the supernatants of aliquots A and B were evaporated with N₂, and the residue was resuspended with 250 μL of ice-cold EIA phosphate buffer containing 0.01 mol/L phosphate, 0.9% NaCl, 0.1% MgCl₂, 0.1% BSA, and 0.1% sodium azide at pH 7.4. The levels of 12(S)-HETE in urine and in platelet incubations were measured by ELISA (DRG Diagnostica) after extraction of samples on Sep-Pak C₁₈ cartridges (Waters). The 12-HETE ELISA is specific for 12(S)-HETE, with <2.5% cross-reactivity with 12(R)-HETE, 0.3% with 15-HETE, 0.2% with 5-HETE, 0.1% with PGE₂, and 0.1% with PGL₁. 12(S)-HETE levels in platelet incubations were also determined by reverse-phase (RP) high-performance liquid chromatography (HPLC). Briefly, Sep-Pack–extracted samples from platelet suspensions were injected into an RP-HPLC system that consisted of a Waters integrated system controller (model 600E) equipped with a 996 photodiode array detector and a Millennium HPLC analysis software. For analysis of 12(S)-HETE, a Tracer Kromasil 100 C₁₈ (5 μm, 4.6×250 mm) column eluted with MeOH/H₂O/acetic acid (65:35:0.01; vol/vol/vol) as phase 1 (0 to 20 minutes) and a linear gradient with MeOH/acetic acid (99.9:0.1, vol/vol) as phase 2 (20 to 45 minutes) at a flow rate of 1.0 mL/min was used, and the UV detector was set at 234 nm.

Subcellular Fractionation of Platelets and Detection of 12-LO Protein

Platelets were resuspended in sonication buffer consisting of (in mmol/L): 100 KCl, 15 NaCl, 12 sodium citrate, 2 MgSO₄, 10 glucose, and 25 HEPES, plus 0.2 mmol/L PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A, 0.05 U/mL aprotinin, and 1 mmol/L DTT at pH 7.5. All subsequent steps were carried out at 4°C. The cells were sonicated for 60 seconds and the lysate was subjected to centrifugation at 4000g for 5 minutes. The supernatant was further centrifuged at 100 000g for 75 minutes, and the supernatant was regarded as cytosolic fraction and the pellet as membrane fraction, which was resuspended in 1 mL of Tyrode’s-HEPES buffer with protease inhibitors and 1% of SDS. Protein content of all samples was determined by the Bradford method. Protein (10 μg) of the subcellular fractions was subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane. Preliminary 10% SDS-polyacrylamide gels were run and were stained with Coomassie blue to confirm equality of loading in each lane. The PVDF membranes were incubated with a 1:1000 final dilution of rabbit polyclonal antiserum (Cayman Chemical) against human platelet 12-LO for 60 minutes. The immunocomplexes were detected by the enhanced chemiluminescence method (Amersham). The blots were quantified by densitometry (Non Linear Dynamics Ltd). The band density values were normalized by dividing by the mean value for the control subjects and multiplying by 100%.

Determination of Urinary 12(S)-HETE, Prostaglandins, and Urinary Albumin Excretion

Urinary 12(S)-HETE was determined as detailed above for the platelet incubations. Prostaglandin (PG)E₂ and 6-keto-PGF₁α levels in urine were analyzed by ELISA (Amersham International) after extraction of samples on Sep-Pack C₁₈ cartridges. Urinary albumin excretion rate from 2 separate 24-hour urine collections was measured with an immunonephelometric assay (Boehringer Mannheim). Microalbuminuria was defined as urinary albumin excretion rate between 20 and 200 μg/min.

Statistical Analysis

Data are presented as mean±SEM. Comparisons between means were determined by 2-sided Mann-Whitney U test for unpaired data. Correlation coefficients were calculated by the least-squares method. The 5% probability level was regarded as significant.

Results

The clinical characteristics of the patients with essential hypertension and the normotensive control subjects are shown in the Table. There were no major differences or significant trend in the age and gender distribution among groups. Likewise, urinary sodium excretion was similar in both groups. Urinary albumin excretion was measured in the patients with hypertension, and the mean value was 8.5±2 μg/min (range, 2 to 39).

The in vitro platelet generation of 12(S)-HETE was measured in basal conditions and after stimulation with human thrombin in patients and control subjects. The basal generation of 12(S)-HETE was significantly higher in patients with essential hypertension than in the normotensive individuals: 3.56±1.22 versus 0.64±0.13 ng/10⁶ platelets (P<0.025) (Figure 1A, left). There were no significant differences
between patients with essential hypertension and control subjects in the thrombin-stimulated 12(S)-HETE platelet generation: 7.66±2.14 versus 4.87±1.46 ng/10⁶ platelets (P<0.001) (Figure 1A, right). Figure 2 shows representative chromatograms obtained during HPLC analysis of plasma from a control subject and a patient with essential hypertension.

To investigate the possible mechanism of increased platelet production of 12(S)-HETE in essential hypertension, the amount of 12-LO protein was measured in the platelet cytosolic and membrane fractions by Western blotting in 9 patients and 9 control subjects. Most of the 12-LO protein expression in platelets was observed in the cytosolic fraction rather than in the membrane fraction. Patients with essential hypertension displayed significantly higher levels of 12-LO protein in the cytosolic fraction: 240±64 versus 106±27% (P<0.032) (Figure 1B, a). Although the 12-LO protein level in the membrane fraction was also higher in patients than in control subjects (155±29 versus 87±18%), the difference was not statistically significant (P=0.07) (Figure 1B, b).

Urinary 12(S)-HETE excretion as measured in relation to creatinine excretion was significantly higher in patients with

**Clinical Characteristics of Patients and Control Subjects Studied**

<table>
<thead>
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<th>Clinical Parameter</th>
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<th>Control Subjects (n=9)</th>
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<tr>
<td>Age, y</td>
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<td>3/6</td>
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<td>BMI, kg/m²</td>
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<td>Systolic blood pressure, mm Hg</td>
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<td>&lt;0.001</td>
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<tr>
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<td>88±3</td>
<td>&lt;0.001</td>
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<tr>
<td>Aldosterone, ng/dL</td>
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<tr>
<td>U₉₆ excretion, mEq/d</td>
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<td>138±20</td>
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PRA indicates plasma renin activity; ND, not determined.

**Figure 1.** A, Platelet 12(S)-HETE concentration in subjects with essential hypertension (HT) and normotensive control subjects (CT). Left graph shows basal 12(S)-HETE platelet production; right graph shows thrombin-stimulated 12(S)-HETE platelet production (plat. indicates platelet). B, Representative immunoblot showing platelet 12-lipoxygenase protein abundance in cytosolic fraction (a) and in membrane fraction (b) in 3 control subjects and 3 patients with essential hypertension. 10% SDS-polyacrylamide gels were run and were stained with Coomassie blue to confirm equality of loading in each lane (not shown).

**Figure 2.** Representative RP-HPLC chromatograms of materials from platelets isolated from hypertensive patient (upper panel) or from healthy subject (middle panel). Representative profile of authentic standards of 15-, 12-, and 5-HETE is also shown (bottom panel). Chromatograms were plotted at 234 nm and are representative of 3 experiments. Retention times for 15-, 12-, and 5-HETE standards were 43.69±0.008, 44.39±0.005, and 45.49±0.003 minutes, respectively. Retention times for peaks identified by arrow in upper and middle panels were 44.28±0.02 and 44.26±0.01 minutes, respectively. Inserts: On-line UV spectra of materials eluting beneath peaks identified by arrow.
essential hypertension (n=19) than in the control subjects (n=9): 36.8±7.24 versus 17.1±3.14 ng/mg creatinine (P<0.05) (Figure 3, left). Urinary excretion of PGE\(_2\) and the hydrolysis product of PGI\(_2\) (6-keto-PGF\(_{1α}\)) were also measured in patients and control subjects. There were no significant differences in the levels of PGE\(_2\) excretion between patients and control subjects: 1.56±0.2 versus 1.37±0.16 ng/mg creatinine (P=0.57) (Figure 3, right). In contrast, patients with essential hypertension displayed a significantly reduced PGI\(_2\) excretion as compared with the normotensive control subjects: 0.499±0.07 versus 0.685±0.05 ng/mg creatinine (P<0.05) (Figure 3, center). The 12(S)-HETE/PGI\(_2\) ratio was significantly higher in patients than in control subjects: 76±19 versus 28±7 (P<0.05).

There was no correlation between blood pressure, age, gender, plasma renin activity, or aldosterone and the urinary excretion of 12(S)-HETE, PGI\(_2\), or PGE\(_2\) or the basal and stimulated platelet production of 12(S)-HETE. On the contrary, in the patients with essential hypertension, urinary excretion of PGI\(_2\) was positively correlated with the urinary albumin excretion (r=0.67, P<0.02) and inversely correlated with the BMI (r=-0.74, P<0.05).

### Discussion

In this study, we observed that the production of 12(S)-HETE by platelets from patients with essential hypertension is higher than in normotensive control subjects. The platelet 12-LO protein amount in the cytosolic fraction of platelets from hypertensive patients was higher than in the control subjects. In addition, the patients with essential hypertension excreted higher amounts of 12(S)-HETE and lower amounts of the vasodilatory prostaglandin PGI\(_2\) in the urine than did the normotensive control subjects.

Arachidonic acid is metabolized through the cyclooxygenase, lipooxygenase (LO), and the cytochrome P-450 pathways. The role of cyclooxygenase or cytochrome P-450 metabolites in cardiovascular physiology and hypertension has been intensively studied in the last years.\(^{14}\) In contrast, the possible role of LO metabolites in hypertension has been less explored. Several studies have demonstrated that the administration of the LO inhibitors phenidone and 5,8,11-eicosatrienonic acid reduces blood pressure in several rat models of hypertension such as the SHR and the 2-kidney, 1-clip Goldblatt rats. This response to LO inhibition suggests a role for LO metabolites in the pathogenesis of experimental hypertension.\(^{4,7,8}\) 12(S)-HETE displays vascular actions that are in agreement with their prohypertensive functions.\(^{3}\) In addition, treatment with LO inhibitors attenuates the vasoconstrictor action of angiotensin II in hypertensive rats.\(^{15}\) Evidence of enhanced 12-LO activity in the SHR has been recently provided.\(^{7}\) The basal 12(S)-HETE production by platelets was higher in the SHR than in the control Wistar-Kyoto rats. Moreover, there was a striking correlation between platelet 12(S)-HETE production and systolic blood pressure. Confirming previous results, the administration of a LO inhibitor reduced blood pressure in the SHR, with a concomitant reduction in the platelet production of 12(S)-HETE.\(^{7}\) In addition, increased 12-HETE production has also been demonstrated in the aortas of the SHR.\(^{8}\) However, the mechanisms of the enhanced production of 12(S)-HETE in experimental hypertension have yet not been elucidated.

The role of 12(S)-HETE in human hypertension has not been directly explored. It has been reported that vascular smooth muscle and endothelial cells from patients with diabetes mellitus have increased release of 12- and 15-HETE,\(^{16}\) suggesting a role for this metabolite in this disease. Our finding of a higher basal platelet production and urinary excretion of 12(S)-HETE in a group of patients with essential hypertension is in agreement with the results obtained in rat models of hypertension as detailed above\(^{7,8}\) but is in contrast with previous studies measuring urinary 12(S)-HETE in humans.\(^{9}\) Although the focus of that study was non–insulin-dependent diabetes mellitus, the authors evaluated urinary excretion of 12(S)-HETE in a group of 9 patients with essential hypertension and found no differences as compared with healthy control subjects.\(^{9}\) Increased urinary excretion of 12(S)-HETE may reflect either higher renal production of this metabolite because it can be synthesized in glomeruli and tubuli\(^{17}\) or higher plasma concentration or both. The concomitant decreased urinary excretion of PGI\(_2\) in patients with essential hypertension is striking. It has been demonstrated that HETEs can suppress PGI\(_2\) in vitro as well as inhibit vascular cyclooxygenase.\(^{16}\) In this sense, decreased urinary excretion of PGI\(_2\) in concordance with increased urinary 12(S)-HETE excretion has also been shown in diabetic patients.\(^{9}\) Such high ratios of HETE/prostanoids have been found in the urine of cirrhotic patients\(^{18}\) and in the bronchoalveolar lavage fluid of asthmatic patients after aspirin challenge.\(^{19}\)

In this study, the higher amounts of platelet 12-LO protein in platelet cytosolic fraction from patients with hypertension suggest a possible mechanism underlying the higher platelet 12(S)-HETE production. However, in addition to higher protein expression, other mechanisms may operate because...
the difference in protein expression between patients and control subjects was comparatively less than the 5-fold difference in platelet 12(S)-HETE production observed between the groups. We found a predominant localization of 12-LO protein in the cytosol, and the difference in the amount of this protein between patients and control subjects was more marked in the cytosolic than in the membrane fraction. The intracellular distribution of 12-LO activity varies between different cells, ranging from a predominant cytosolic localization to a preferential localization in membranes. In human platelets, most but not all investigators have observed a predominant (65%) localization of 12-LO activity and protein in the cytosolic fraction. 12(S)-HETE has also been shown to participate in the regulation of platelet function such as aggregation and P-selectin expression. Moreover, a newly developed platelet adhesion inhibitor, OPC-29030, dose-dependently inhibits the production of 12(S)-HETE but not the synthesis of thromboxane B2. This finding may be important because it is known that in essential hypertension, platelets display enhanced secretory and aggregatory responses, reflecting a hyperactivated state that may play a crucial role in the atherosclerotic process relevant in hypertension. Especially, it is interesting that 12-HETE production has been found to be increased in small vessels from ischemic kidneys, thus reflecting renal tissue injury.

In summary, our results indicate that urinary excretion and platelet production of 12(S)-HETE is increased in patients with essential hypertension. The higher levels of 12-LO protein displayed by platelets from patients with hypertension suggest a possible mechanism mediating the increase in 12(S)-HETE. These findings might have clinical implications regarding the platelet disturbances observed in essential hypertension.

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