Cytochrome P450-Dependent Eicosapentaenoic Acid Metabolites Are Novel BK Channel Activators

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Abstract—P450-dependent arachidonic acid (AA) metabolites regulate arterial tone by modulating calcium-activated (BK) potassium channels in vascular smooth muscle cells (VSMC). Because eicosapentaenoic acid (EPA) has been reported to improve vascular function, we tested the hypothesis that P450-dependent epoxidation of EPA produces alternative vasoactive compounds. We synthesized the 5 regioisomeric epoxyeicosatetraenoic acids (EETeTr) and examined them for effects on K⁺ currents in rat cerebral artery VSMCs with the patch-clamp technique. 11(R),12(S)-epoxyeicosatrienoic acid (50 nmol/L) was used for comparison and stimulated K⁺ currents 6-fold at +60 mV. However, 17(R),18(S)-EETeTr elicited a more than 14-fold increase. 17(S),18(R)-EET and the remaining four regioisomers were inactive. The effect of 17(R),18(S)-EETeTr was blocked by tetraethylammonium but not by 4-aminopyridine. VSMCs expressed P450s 4A1 and 4A3. Recombinant P450 4A1 hydroxylated EPA at C-19 and C-20 and epoxygenated the 17,18-double bond, yielding the R, S- and S, R-enantiomers in a ratio of 64:36. We conclude that 17(R),18(S)-EETeTr represents a novel, potent activator of BK potassium channels. Furthermore, this metabolite can be directly produced in VSMCs. We suggest that 17(R),18(S)-EETeTr may function as an important hyperpolarizing factor, particularly with EPA-rich diets.

Key Words: vascular smooth muscle cells ■ endothelium-derived factors ■ potassium channels ■ cytochrome P450

Dietary fish oil or purified (n-3) long-chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) exert a wide range of beneficial effects on vascular function.¹ ² Endothelium-dependent relaxation is enhanced and the vasoconstrictor response to angiotensin II and noradrenaline is reduced because of mechanisms that are incompletely understood.³–⁶ Possibly, EPA and other (n-3) PUFA compete with arachidonic acid (AA) for enzymatic conversion by P450 enzymes. This competition may lead to a reduced formation of vasoactive AA metabolites while alternative metabolites originating from EPA are increased. The P450-dependent AA metabolites result from epoxidation and hydroxylation and include the epoxyeicosatrienoic acids (EET) 5,6-, 8,9-, 11,12 to 14,15-EET, and the ω(ω-1)-hydroxyeicosatrienoic acids (20- and 19-HETE).⁷ EETs are produced in the endothelium by the P450 subfamilies 2C and 2J,⁸,⁹ EETs activate large-conductance, calcium-activated (BK) K⁺ channels in vascular smooth muscle cells (VSMC) and are considered as leading candidates for the endothelium-derived hyperpolarizing factor (EDHF).⁸,¹⁰,¹¹ 20-HETE is produced by P450 4A enzymes in VSMC and acts as endogenous vasoconstrictor that inhibits BK channels. 20-HETE is important for the autoregulation of renal and cerebral blood flow.¹²–¹⁵ How AA metabolite production is influenced by EPA competition for P450 enzymes, and whether or not P450-dependent EPA metabolites modulate BK channels, is unknown. We synthesized the five possible regioisomeric epoxyeicosatrienoic acids: 5,6-, 8,9-, 11,12-, 14,15- and 17,18-EETeTr, as well as the EPA hydroxylation products 20- and 19-OH-EPA. We then performed patch-clamp studies to gain insight into the potential vasoactivity of the metabolites and compared them to another putative EDHF, 11,12-EET.

Methods

Preparation of Metabolites

AA, EPA, and 11,12-EET were from Cayman Chemical, [1-¹⁴C]AA (56.0 mCi/mmol) from Amersharm Pharmacia Biotech, and [1-¹⁴C]EPA (55.6 mCi/mmol) from NEN, respectively. The high-performance liquid chromatography (HPLC) system used was a LC-10Avp (Shimadzu) equipped with a radioactivity monitor (LB 509, Berthold). The columns applied were as follows: Nucleosil 100-5C18HD (250×4 mm) from Machery-Nagel for reverse-phase (RP-) and normal-phase (NP-) HPLC, respectively; Chiralcel OD and OB (250×4.6 mm) from
Electrophysiological Recording

Single VSMC were isolated from rat cerebral arteries of adult male Sprague-Dawley rats (250 to 300 g, Tierzucht Schoenwalde, Germany) and K⁺ channel currents were recorded in the perforated patch configuration with amphoterin B as described previously.19 Currents were recorded from holding potential of −40 mV during linear voltage ramps at 0.53 V/s from −80 mV to +80 mV or 500-ms step pulses to different potentials (holding potential −80 mV). The bath solution contained in mmol/L: 6 KCl, 134 NaCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose (pH 7.4 with NaOH). The patch pipette was filled with a solution containing in mmol/L: 30 KCl, 110 potassium aspartate, 10 NaCl, 1 MgCl₂, 0.05 EGTA, and 10 HEPES (pH 7.2 with KOH). Experiments were performed at approximately 22°C.

Oxidation of EPA by Recombinant P450 Enzymes

The recombinant baculoviruses used for expression of P450s 4A1, 4A2, and 4A3 were constructed as described21 and co-expression with human NADPH-P450 reductase (CPR) was achieved by coinfection of Spodoptera frugiperda (Sf9) cells.22 Microsomes containing the recombinant P450 systems (10 pmol P450 4A1 or 50 pmol P450 4A2 or 50 pmol P450 4A3) were preconditioned for 5 minutes at 37°C with [1-14C]EPA (4.50 nmol, 5.55x10⁵ disintegrations per minute) in 0.1 mol/L potassium phosphate buffer pH 7.2 (total volume 0.5 mL). Reactions were started by addition of NADPH (1 mmol/L final concentration) and carried out for 30 minutes at 37°C. After extraction with ethylacetate, the reaction products were analyzed by RP-HPLC using solvent system 1. 17,18-EETeTr produced by P450 4A1/CPR microsomes was confirmed by hydrolysis (the synthesized racemic 17,18-EETeTr) and 17,18-EETeTr enantiomers were produced according to published procedures.17,18 19- and 20-OH-EPA were generated enzymatically with NADPH-dependent oxygenation of [1-14C]EPA (2 µCi/mmol) with rat liver microsomes18 and separated unresolved by RP-HPLC using solvent system 1 at Rp=14.2 minutes 19- and 20-OH-EPA were resolved by subsequent NP-HPLC using a linear gradient ranging from hexane:2-propanol:acetic acid (99:1:0.1, vol/vol/vol) to hexane:2-propanol:acetic acid (98.3:1:0.1, vol/vol/vol) over 40 minutes at a flow rate of 1 mL/min at 20.8 and 29.2 minutes, respectively.

Statistical Analysis

All values are given as mean and standard error of the mean (SEM). Paired and unpaired Student’s t tests or nonparametric Wilcoxon tests were used as appropriate. P<0.05 was considered statistically significant. The term “n” represents the number of cells tested.

Results

Stereo and Regiospecific Effects of EPA Metabolites on BK Channel Currents

As shown in Figure 1, the K⁺ current showed outward rectification with relatively large noise at membrane potentials positive to 60 mV. The cells exhibited a voltage-
OH-EPA (100 nmol/L), and 20-HETE showed no significant effects.

Oxidation of EPA by Recombinant P450 Isoforms of the 4A Subfamily

An RT-PCR approach was used to examine which of the four known rat P450 4A isoforms are expressed in rat cerebral artery VSMC, as shown in Figure 3. We used primer pairs specific for the amplification of cDNA fragments originating from the mRNAs of P450 4A1, 4A2, 4A3, and 4A8.20 The RT-PCR products were clearly formed with the primer pairs for P450 4A1 and 4A3 (Figure 3A). The fragments had the expected size (351 and 316 bp, respectively), and fragments of identical size were also obtained in control experiments using rat liver mRNA as template. mRNAs for P450 4A2 and 4A8 were not detectable in VSMC but were clearly present in rat liver (Figures 3A and B).

The 3 rat P450 4A isoforms involved in AA metabolism21, P450s 4A1, 4A2, and 4A3, were co-expressed with NADPH-P450 reductase (CPR) in S9 insect cells. As shown in Figure 4, all 3 microsomal P450 isoforms were able to metabolize EPA. Hydroxylation products migrated with a retention time of 14.2-minute in RP-HPLC (Figure 4A). As analyzed in detail for P450 4A1, this product peak was further resolved by NP-HPLC and consisted of 19- and 20-OH EPA (Figure 4B). In addition, each of the P450 isoforms was able to epoxidize EPA as indicated by the presence of products with 19.6-minute retention times (only with P450 4A1) and between 21 and 22 minutes (with P450s 4A2 and 4A3) in RP-HPLC. The metabolite produced by P450 4A1 migrated with chemically synthesized 17,18-EETeTr, whereas those produced by P450s 4A2 and 4A3 may represent an unresolved mixture of other regioisomeric EPA epoxides. To determine the stereoselectivity of P450 4A1 in EPA epoxidation, the product with a retention time at 19.6 minutes was collected from RP-HPLC and converted into the methyl ester with diazomethane. Subsequent analysis by chiral-phase HPLC gave peaks with retention times identical to the chemically and enzymatically synthesized 17,18-EETeTr enantiomers (Figure 4C). As calculated from the peak areas, the R, S enantiomer represented 64%, and the S, R enantiomer 36% of the total epoxidation product. Taken together, P450 4A1 hydroxylated EPA to 19- and 20-OH-EPA and...
catalyzed the epoxygenation of the 17,18 double bond with a stereoselectivity in favor of 17(R),18(S)-EETeTr formation. The total activities of EPA oxidation were indistinguishable from the values reached with AA as substrate (data not shown) and reached 0.9 to 1.0 nmol/min/nmol P450 at a substrate concentration of 10 μmol/L. The ratio of EPA hydroxylase and epoxygenase activities was about 4:1.

Discussion

We synthesized all 5 regioisomeric epoxides of EPA to test their relative effect in activating VSMC BK channels. We demonstrated that 17(R),18(S)-EETeTr stimulated the K⁺ outward current. 17(R),18(S)-EETeTr shares this property with 11,12-EET, the compound that has been proposed as EDHF in a number of vascular beds. In direct comparison, the epoxy-derivative of EPA, 17(R),18(S)-EETeTr, even exceeded the effect of the epoxy-derivative of AA, 11(S),12(S)-EET (15-fold versus 6-fold stimulation). The effect of both metabolites was completely abolished by TEA, but remained unaffected in the presence of 4-AP, showing the characteristics typical for BK channel activation. These findings suggest that 17(R),18(S)-EETeTr may be a novel hyperpolarizing factor in the vessel wall.

A high degree of regio- and stereoselectivity was observed among the various epoxy-derivatives of EPA. Only the 17,18-regioisomer significantly stimulated the K⁺ current and the 17(R),18(S)-, but not the 17(S),18(R)-enantiomer, was effective. Stereospecificity was also found with 11,12-EET, the reference compound. Addition of the R, S-, but not the S, R-enantiomer, elicited BK channel activation, consistent with previous results in rat renal arteriolar VSMC. Recently, all regioisomeric EETeTrs were found to be almost equipotent in producing dilation of coronary microvessels. However, the EETeTrs were not analyzed in terms of BK channel activation. Our results suggested strict structural requirements for EPA metabolites in BK channel activation, which would also imply regio- and stereospecificity in vasodilation. A clear regio- and stereospecificity in terms of BK channel activation and vasodilation in favor of 11(R),12(S)-EET exists in rat renal arterioles. Less specificity has been described in several other vascular beds, including the coronary microvessels. The issue is unsolved; however, the findings suggest important differences in the mechanisms of how various blood vessels respond to EETs and also to EETeTrs.

The signal transduction pathways triggered by 11,12-EET that finally lead to BK channel activation and vasodilation may indeed differ by involving either guanine nucleotide binding proteins and ADP-ribosylation or cAMP/protein kinase A-dependent steps.

Our study is the first to address the question of which P450 isoforms expressed in the vasculature are able to catalyze EPA epoxygenation. Our results show that rat cerebral artery VSMC express P450 4A1 and 4A3. mRNAs for P450 4A2 and 4A8 were not detectable in these cells, whereas the expression of all four P450 4A genes has been reported for rat cerebral microvessels. We found that all rat P450 4A isoforms known to metabolize AA also accept EPA as an efficient substrate. P450 4A1 showed the highest activity and produced 20- and 19-OH-EPA as the major metabolites. In addition, an epoxygenase product was detected that comigrates in RP-HPLC with chemically synthesized 17,18-EETeTr. Chiral-phase HPLC confirmed the identity of the metabolite and revealed a ratio of the enantiomers typical for BK channel activation and vasodilation in favor of the biologically active form. These results indicate that 17(R),18(S)-EETeTr can be directly produced in VSMC.

The involvement of P450 4A1 in the formation of this metabolite may have important implications for the regula-
tion of vascular tone because P450 4A1 was previously reported to be a major isoform generating 20-HETE in the renal vasculature. An additional potential source for 17(R),18(S)-EETeTr are endothelial P450 isoforms of the 2C and 2J subfamilies that otherwise produce EETs from AA. In support of this view, we found that the human P450s 2C8 and 2J2 are able to epoxygenate EPA and to produce 17,18-EETeTr as a major metabolite (unpublished data). However, which of the various rat P450 2C and 2J isoforms share this ability and are actually expressed in the endothelium of cerebral arteries remains to be determined. Taken together, EPA accumulation in the vasculature may shift the profile of P450-dependent metabolites both in VSMC and in the endothelium in such a way that vasodilator mechanisms are enhanced and constrictor responses are reduced. Our results support the hypothesis that competition between AA and EPA for conversion by P450 enzymes and the resulting formation of alternative metabolites such as 17(R),18(S)-EETeTr may contribute to vascular effects attributed to diets rich in EPA.

Acknowledgments

This study was supported by grants to W.H.S. and M.G. from the Deutsche Forschungsgemeinschaft. The authors wish to thank Ramona Zummach and Christel André for excellent technical assistance. We are grateful to Dr Matthias Löhn for initial advice in patch-clamp recordings and Dr Rolf D. Schmid (University Stuttgart) for a sample of bacterial P450BM-3.

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Hypertension, 2002;39:609-613
doi: 10.1161/hy0202.103293

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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