Cardiovascular Research (2011) **90**, 344–352
doi:10.1093/cvr/cvq411

Activation of vascular BK channels by docosahexaenoic acid is dependent on cytochrome P450 epoxygenase activity

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Received 24 May 2010; revised 7 December 2010; accepted 22 December 2010; online publish-ahead-of-print 27 December 2010

Time for primary review: 21 days

Aims *n*-3 Polyunsaturated fatty acids (PUFAs) are known to protect the cardiovascular system and improve blood pressure control. These important dietary constituents are converted into bioactive metabolites, but their role in regulation of the cardiovascular system is unclear. In particular, the functions of the cytochrome P450 (CYP) metabolites of *n*-3 PUFAs remain virtually unexplored. In this study, we examined the effects of docosahexaenoic acid (DHA) on the regulation of large-conductance calcium-activated potassium (BK) channel activities in coronary arterial smooth muscle cells.

Methods and results Using whole-cell patch-clamp techniques, we found that DHA is a potent activator of BK currents in rat coronary arterial smooth muscle cells with an EC_{50} of 0.23 ± 0.03 μM. This effect was abolished by pre-incubation with the CYP epoxygenase inhibitor, SKF525A (10 μM). The effects of DHA on the BK channels were reproduced by 16,17-epoxydocosapentaenoic acid (16,17-EpDPE) with an EC_{50} of 19.7 ± 2.8 nM. The physiological role of the CYP metabolites of DHA was confirmed by measuring DHA-mediated vasodilatation in isolated rat coronary arteries. DHA dilated pressurized isolated coronary arteries in a dose-dependent manner, and the DHA effects were abolished after pre-treatment with SKF525A (10 μM) or with iberiotoxin (100 nM). In addition, 16,17-EpDPE directly produced coronary vasodilatation that was iberiotoxin sensitive.

Conclusion These results suggest that DHA-mediated vasodilatation is mediated through CYP epoxygenase metabolites by activation of vascular BK channels.

Keywords Docosahexaenoic acid • Coronary arterial smooth muscle cell • BK channel • Cytochrome P450 epoxygenase • 16,17-Epoxydocosapentaenoic acid

1. Introduction

Consumption of *n*-3 polyunsaturated fatty acids (PUFAs) in the diet or as a supplement is known to be associated with beneficial cardiovascular effects.[1,2] Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the major *n*-3 PUFAs in fatty fish and are the recommended fish oil supplements endorsed by the American Heart Association for patients with coronary heart disease and hypertriglyceridaemia.[3] Although both DHA and EPA bestow protective effects on the cardiovascular system, DHA is more abundant than EPA in the human myocardium,[4] and DHA supplementation may be of greater therapeutic value than EPA because it produces more favourable results on lipid profile,[5] thrombotic risk,[6] and ambulatory blood pressure.[7]

Like *n*-6 PUFAs, *n*-3 PUFAs are converted by multiple enzymatic pathways into bioactive products. In fact, DHA and EPA are competitive substrates for the enzymes of arachidonic acid (AA) metabolism.[8] Eicosapentaenoic acid is metabolized by the cyclo-oxygenase pathway into 3-series prostaglandins and thromboxanes, and by 5-lipoxygenase into 5-series leukotrienes, whereas DHA is metabolized into other autocoids, such as resolvins, docosatienes, and neuroprotectins.[8] More recently, EPA and DHA have been shown to be metabolized by cytochrome P450 (CYP) epoxygenase into fatty epoxides.[9]

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While the CYP products of AA have been extensively studied, very little is known about the function of the CYP products of DHA and EPA. The CYP products of AA metabolism are known as epoxyeicosatrienoic acids (EETs), which are endothelium-derived hyperpolarizing factors that produce potent vasodilatory effects. One of their major targets is the large-conductance Ca^{2+}-activated K^+ (BK) channel.[10] In this study, we tested the hypothesis that the CYP products of DHA metabolism activate vascular BK channels to produce vasodilatation.

2. Methods

2.1 Materials

Chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA), except DHA, 16,17-epoxydocosapentaenoic acid (16,17-EpDPE), and 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE), which were obtained from Cayman Chemical (Ann Arbor, Michigan, USA), and SKF525A from BIOMOL (Plymouth Meeting, Pennsylvania, USA).

2.2 Animals

Sprague–Dawley rats (200–250 g) were obtained from the Harlan Laboratories (Madison, Wisconsin, USA). Handling and care of animals, as well as all animal procedures, were conducted in conformity with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee, Mayo Foundation.

2.3 Isolation of rat coronary arteries and videomicroscopy

Rats were anaesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal), and their hearts were rapidly excised and placed in ice-cold Krebs solution that contained (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.1 (pH 7.4). The secondary and tertiary branches (150–200 μm diameter) of the coronary arteries were carefully dissected and isolated free of myocardium and connective tissue.[11] Isolated small coronary arteries (1–2 mm in length) were mounted in a vessel chamber filled with Krebs solution and secured between two borosilicate glass micropipettes with 10–O ophthalmic suture. The lumen of each vessel was filled with Krebs solution through the micropipettes and maintained at a constant pressure (no flow) of 60 mmHg. Vessels were equilibrated for 60 min in oxygenated (20% O_2, 5% CO_2, balanced with N_2, 37°C) Krebs solution. Vessels were deemed unacceptable for experiments if they demonstrated leaks, failed to produce > 30% constriction to 60 mM KCl or to graded doses of endothelin-1 (ET-1), or failed to produce 80% dilatation with zero Ca^{2+}.

All compounds were added abluminally. During the 60 min equilibration period, some vessels were pre-treated with β-diethyl-aminoethyl-diphenylpropylacetate (SKF525A, 10 μM), *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH, 10 μM), ryanodine (10 μM), diltiazem (10 μM), or iberiotoxin (IBTX, 100 nM). Vessels were constricted with ET-1 ($\sim 10^{-9}$ M) by 50–70% of baseline passive diameter. Concentration–response relationships of DHA (0.1–10 μM) on vasodilatation were measured and comparisons made between those with and without pre-treatment with designated compounds. To assess the direct effects of the CYP epoxygenase products of DHA, concentration–response relationships of 16,17-EpDPE (0.1–50 nM) on vasodilatation were obtained. At the end of each experiment, vessels were maximally dilated with a Ca^{2+}-free solution, and the percentage dilatation in response to DHA and to 16,17-EpDPE were normalized to the maximal diameter.

2.4 Isolation of rat coronary arterial smooth muscle cells

Rat coronary arterial smooth muscle cells (CASMCs) were dissociated enzymatically, as described previously.[12] Freshly isolated rat coronary arteries were incubated with low-Ca^{2+} saline solution (mM: NaCl 145.0, KCl 4.0, $CaCl_2$ 0.05, $MgCl_2$ 1.0, HEPES 10.0, and glucose 10.0, pH 7.4) that contained 0.1% bovine serum albumin for 10 min at 37°C in a shaking water bath followed by digestion in 1.0 ml of low-Ca^{2+} saline solution containing 1.5 mg papain, 1.0 mg dithiothreitol and 0.1% bovine serum albumin at 37°C for 10 min. The vessels were further digested in 1.0 ml low-Ca^{2+} saline solution containing 1.0 mg collagenase, 1.0 mg trypsin inhibitor, 0.25 mg elastase, and 0.1% bovine serum albumin at 37°C for 10 min. The vessels were then washed three times with 1.0 ml aliquots of low-Ca^{2+} saline solution, and gently triturated with a fire-polished glass pipette until the cells were completely dissociated. Experiments were performed within 18 h after cell isolation.

2.5 Whole-cell BK current recordings

Whole-cell BK currents were recorded as reported previously[12] using an Axopatch 200B amplifier (Molecular Devices, Inc., Sunnyvale, California, USA), filtered at 2 kHz, and sampled at 50 kHz. When filled with the pipette solution, the pipette resistance was 0.5–1 MΩ for whole-cell current recordings. The pipette solution contained (mM): KCl 140.0, $MgCl_2$ 0.5, Na_2ATP 5.0, Na_2GTP 0.5, HEPES 1.0, EGTA 1.0, and $CaCl_2$ 0.465 (200 nM free Ca^{2+}); pH adjusted to 7.35 with KOH. The bath solution contained (mM): NaCl 145.0, KCl 5.6, $MgCl_2$ 1.0, $CaCl_2$ 0.5, HEPES 10.0, and glucose 10.0, pH 7.4. Total potassium currents in CASMCs were elicited from a holding potential (HP) of −60 mV with pulses of 100 ms duration to testing potentials (TPs) from −40 to 160 mV in 10 mV increments. All experiments were performed at room temperature (22–24°C).

2.6 Spontaneous transient outward current recordings

Spontaneous transient outward currents (STOCs) were recorded from freshly isolated rat CASMCs at 0 mV for 2 min as whole-cell currents measured in the perforated-patch configuration as previously described.[13] Events of STOC openings were analysed using Clampfit 10.2 software (Molecular Devices, Inc., Sunnyvale, California, USA) before and after applications of 1 μM DHA and 0.1 μM IBTX. The frequency of STOCs was calculated as the total number of events divided by the recording time. The pipette and bath solutions were the same as those for whole-cell BK channel recordings, except the pipette solution contained 50–100 μM nystatin and the pipette tip was back-filled with pipette solution without nystatin to allow gigaseal formation.[13,14]

2.7 Statistical analysis

Data are presented as means ± SEM. Student's unpaired *t*-test was used to compare data between two groups. Student's paired *t*-test was used to compare data before and after treatment. A one-way ANOVA followed by contrast testing was used to compare data from multiple groups. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Effects of DHA on K^+ currents in rat CASMCs

Docosahexaenoic acid is a potent activator of K^+ currents in freshly isolated CASMCs. At baseline, CASMCs exhibited low levels of outward currents. Upon exposure to 1 μM DHA, the currents were enhanced several-fold, and these effects were reversed upon washout (*Figure 1A*). The time course of the DHA effects is shown

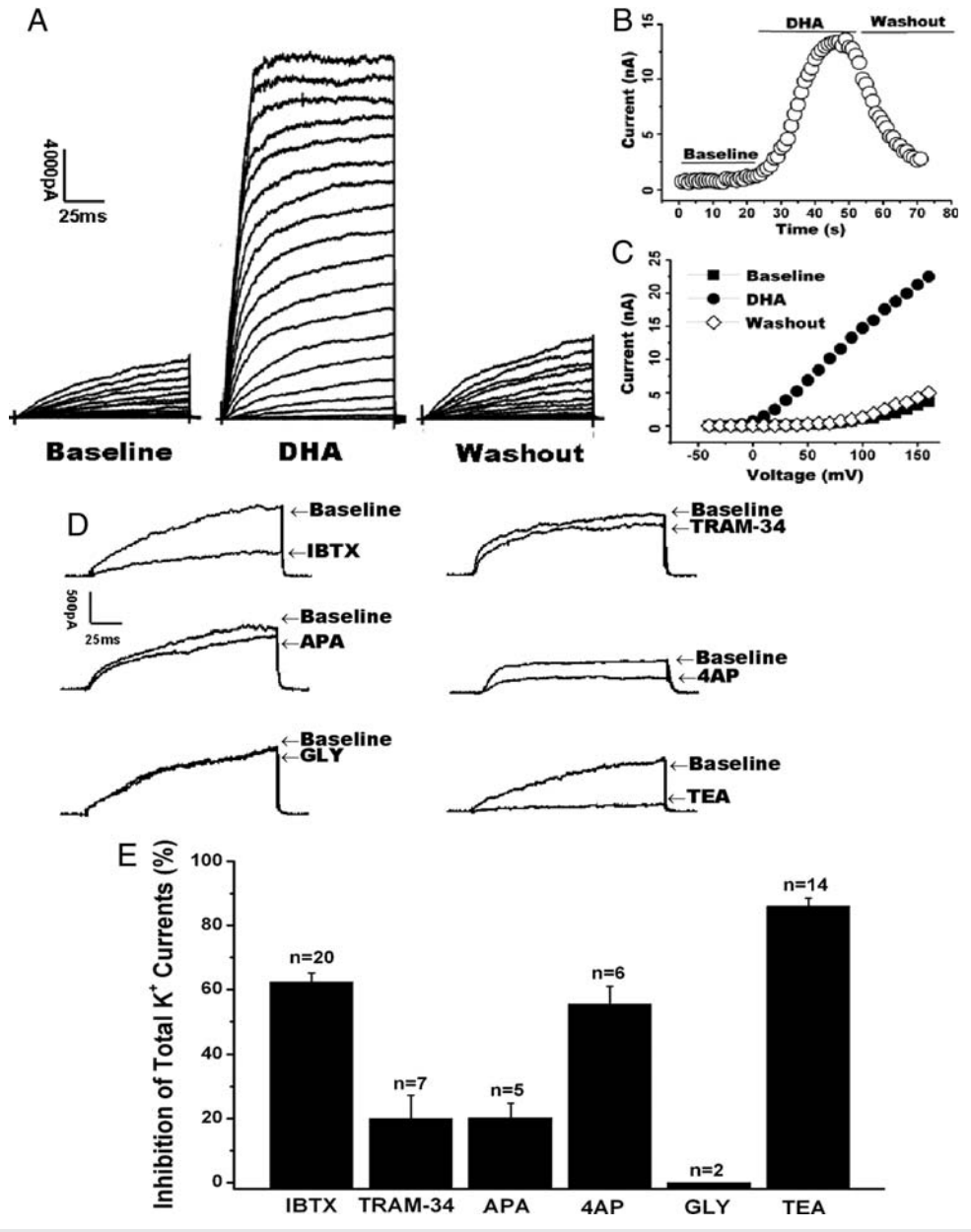

Figure 1 Effects of DHA on total K^+ currents in CASMCs. (*A*) Representative current traces showing outward K^+ currents recorded from freshly isolated rat CASMCs (HP = −60 mV, TP = −40 to 160 mV in 10 mV increments) at baseline, after exposure to 1 μM DHA, and after DHA washout. (*B*) Plot showing the time course of the experiment in *A*. (*C*) *I–V* relationships of total K^+ currents at baseline, with application of 1 μM DHA, and after DHA washout. (*D*) Inhibition of total outward K^+ currents in rat CASMCs by various K^+ channel blockers. Representative current traces showing the inhibition of total K^+ currents in freshly isolated rat CASMCs by IBTX (100 nM), TRAM-34 (200 nM), APA (1 μM), 4AP (5 mM), GLY (10 μM), and TEA (10 mM). (*E*) Group data in bar graphs; *n* = sample size.

in *Figure 1B*. *Figure 1C* shows the current–voltage (*I–V*) relationships of K^+ currents at baseline, with DHA, and after washout.

3.2 BK channels are major targets of DHA activation

To determine the composition of K^+ currents in rat CASMCs, we examined the effects of various ion channel blockers on the K^+ currents in rat CASMCs. These included the BK channel-specific blocker IBTX (100 nM), the intermediate conductance Ca^{2+}-activated K^+ (IK) channel blocker 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34, 200 nM), the small-conductance Ca^{2+}-activated K^+ (SK) channel blocker apamin (APA, 1 μM), the voltage-gated K^+ (Kv) channel blocker 4-aminopyridine (4AP, 5 mM), the ATP-sensitive K^+ channel blocker glyburide (GLY, 10 μM), and the non-specific K^+ channel blocker tetraethylammonium (TEA, 10 mM). The drugs were applied after baseline K^+ current recordings were obtained. Inhibition of total K^+ currents by various K^+ channel blockers was determined by comparing current activities before and after exposure to these drugs and was expressed as the percentage inhibition of baseline

currents. *Figure 1D* shows representative current traces at baseline and after application of specific K^+ channel blockers with the currents elicited from a HP of −60 mV and TP of 100 mV. Group data are summarized in *Figure 1E*. The non-specific K^+ channel blocker TEA inhibited the K^+ currents in rat CASMCs by 86.0 ± 2.5% ($n = 14$). With the specific K^+ channel blockers, the total K^+ currents were inhibited 64.2 ± 2.7% ($n = 20$) by IBTX, 55.5 ± 5.4% ($n = 6$) by 4AP, 20.2 ± 4.5% ($n = 5$) by APA, 20.0 ± 7.3% by TRAM-34 ($n = 7$), and none by GLY ($n = 2$), suggesting that the BK and Kv currents are the major constituents of K^+ currents in rat CASMCs. Since BK currents are known to be major targets of modulation by *n*-6 PUFAs and their metabolites,[15–17] and because we suspect that DHA may have properties similar to *n*-6 PUFAs, we determined the effects of DHA on CASMC BK channel activities.

3.3 DHA is a potent activator of BK currents in rat CASMCs

We measured the effects of DHA on whole-cell BK currents in freshly isolated rat CASMCs with 5 mM 4AP, 1 μM APA, and 200 nM TRAM-34 in the bath solution to eliminate the Kv, SK, and IK currents in these cells, and to allow BK currents to be determined. Docosahexaenoic acid activated the BK currents in a concentration-dependent manner. Representative raw current traces are displayed in *Figure 2A* (left panel, HP = −60 mV, TP = 100 mV). Docosahexaenoic acid had no effects on BK currents at concentrations of 0.01 and 0.03 μM, but increased BK currents by 5 ± 3, 170 ± 69, and 220 ± 73% at 0.1, 0.3, and 1.0 μM DHA, respectively ($n = 7$, *Figure 2A*). Curve fitting of individual concentration–response curves showed that DHA is a potent activator of BK currents with a half-maximal effective concentration (EC_{50}) of 0.23 ± 0.03 μM (*Figure 2C*). The effects of DHA were inhibited by 100 nM IBTX, indicating that BK channels are the major target of the DHA effects (*Figure 2D*).

3.4 Effects of DHA on BK currents in CASMCs are dependent on CYP

To determine the role of CYP on the effects of DHA in BK channel activation, freshly isolated rat CASMCs were pre-treated for 60 min with 10 μM SKF525A, a CYP inhibitor. Unlike other commonly used CYP inhibitors, such as miconazole, we found that SKF525A does not have off-target effects that may interfere with interpretation of results.[18] The effects of DHA on BK channel activation (HP = −60 mV, TP = 100 mV) were virtually abolished after incubation with SKF525A ($n = 6$, $P < 0.05$ vs. control conditions, *Figure 2B* and *C*). SKF525A by itself had no effect on the BK currents in CASMCs (*Figure 2E*). After pre-incubating the rat CASMCs with 10 μM MS-PPOH, another commonly used CYP inhibitor, DHA failed to activate BK currents, similar to the results after SKF525A pre-incubation (*Figure 2F*). These findings suggest that activation of BK channels by DHA in CASMCs is dependent on CYP activity at the concentrations studied.

3.5 16,17-EpDPE is a potent activator of BK currents in CASMCs

We examined the effects of 16,17-EpDPE, a DHA epoxide and a CYP epoxygenase product of DHA, on BK currents in CASMCs and found that applied in the extracellular solution 16,17-EpDPE is a potent activator of IBTX-sensitive BK currents. Exposure to 20 nM 16,17-EpDPE produced a significant increase in BK currents (*Figure 3A* and *B*). The effects of 16,17-EpDPE on the BK channel *I–V* relationships are shown in *Figure 3C* (HP = −60 mV, TP = −40 to 160 mV, $n = 4$). The effects of 16,17-EpDPE were significant at TP ≥ 100 mV ($P < 0.05$) as indicated. The effects of 16,17-EpDPE were IBTX sensitive and not affected by SKF525A pre-incubation (*Figure 3D* and *E*), suggesting that BK channels are the major targets of 16,17-EpDPE activation. In a dose-dependent manner, 16,17-EpDPE potently activated the BK currents with an EC_{50} of 19.7 ± 2.8 nM ($n = 6$; *Figure 4F* and *G*), indicating that this CYP epoxygenase product of DHA metabolism is a potent vascular BK channel activator.

3.6 Mechanisms of BK channel activation by DHA

We further determined the mechanism through which DHA and DHA epoxide activates BK channels. First, we examined whether the effects of 16,17-EpDPE were mediated through mechanisms similar to those of EETs by using the EET antagonist, 14,15-EEZE.[19] After pre-incubation with 14,15-EEZE (500 nM), 16,17-EpDPE (500 nM) was able to activate the BK currents significantly, doubling the current density (*Figure 4A*), suggesting that BK channel activation by EpDPE is different from its *n*-6 PUFA epoxide counterpart. Next, we tried to determine whether DHA would enhance the activity of STOCs. Exposure to 1 μM DHA increased the frequency of STOCs in rat CASMCs 4.6-fold, from 121 ± 47 events/min at baseline to 552 ± 235 events/min ($n = 5$, $P < 0.05$ vs. baseline), and the STOCs were inhibited by 100 nM IBTX (71 ± 41 events/min), indicating the source of these currents to be BK channels (*Figure 4B*). These results suggest that activation of BK currents by DHA may be mediated through an increase in local Ca^{2+} concentration, i.e. Ca^{2+} sparks.

3.7 DHA-mediated dilatation of rat coronary arteries is CYP dependent

To determine the physiological relevance of these findings, we examined the effects of DHA on the dilatation of isolated rat small coronary arteries using videomicroscopy.[11] Docosahexaenoic acid produced dose-dependent vasodilatation of vessels pre-constricted with ET-1, producing dilatations of 0.5 ± 0.5% at 0.1 μM, 8.1 ± 1.8% at 0.5 μM, 22.6 ± 2.8% at 1 μM, 27.3 ± 2.6% at 5 μM, and 29.8 ± 2.9% at 10 μM DHA. Docosahexaenoic acid is a potent vasodilator with an EC_{50} of 0.67 ± 0.07 μM ($n = 7$), but it has only limited efficacy, as its effects plateaued at 30% vasodilatation (*Figure 5A*).

To determine the contribution of the CYP pathway to the effects of DHA-mediated vasodilatation, freshly isolated rat coronary arteries were incubated with 10 μM SKF525A for 60 min before the effects of DHA were measured. Incubation with SKF525A not only eliminated DHA-induced vasodilatation but also produced a 3.5 ± 1.1% vasoconstriction by 10 μM DHA ($n = 4$, P = NS, *Figure 5A*). These results indicate that all the vasodilatory effects of DHA were mediated through CYP metabolites.

To determine the role of BK channels in mediating the DHA-induced vasodilatation, rat coronary arteries were incubated with 100 nM IBTX for 30 min before the effects of DHA were measured. After incubation with IBTX, the effects of DHA were abolished ($n = 4$, *Figure 5A*). These results indicate that the vasodilatory effects of DHA were mediated through activation of BK channels. Pre-

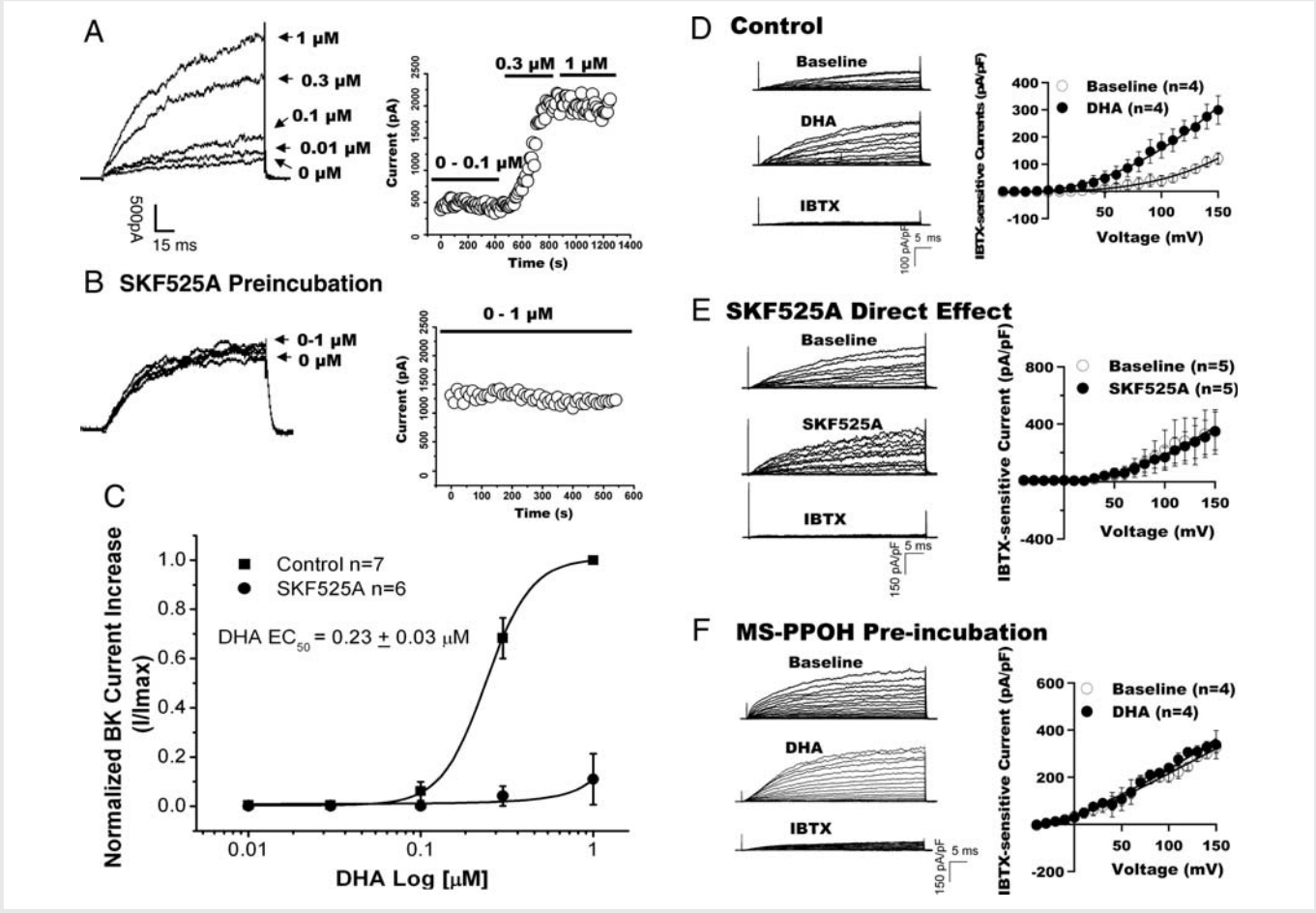

Figure 2 Effects of DHA on BK currents in rat CASMCs and the role of CYP in mediating these effects. (*A*) Representative current traces showing BK currents at baseline and after exposure to 0.01, 0.1, 0.3, and 1.0 μM of DHA (left panel). Time course of changes in BK current density in response to the different concentrations of DHA (right panel). (*B*) After incubation with 10 μM SKF525A for 60 min, CASMCs no longer responded to activation by DHA (0.01–1.0 μM) with experimental protocols similar to that in *A*. (*C*) Concentration–response relationships showing the effects of DHA on BK channel activation with and without treatment with SKF525A. In control conditions, DHA EC_{50} was 0.23 ± 0.03 μM. (*D*) The DHA-activated K^+ currents were IBTX sensitive. Cells were pre-treated with TRAM-34 (200 nM), APA (1 μM), and 4AP (5 mM). Upon exposure to 1 μM DHA, K^+ currents were activated three-fold, and the DHA-activated currents were sensitive to IBTX (100 nM). (*E*) Acute exposure to SKF525A (10 μM) had no effect on the IBTX-sensitive currents in CASMCs. (*F*) After pre-treatment with MS-PPOH (10 μM), DHA could no longer activate BK currents, confirming that the effects of DHA were dependent on the CYP epoxygenase activities.

incubation of the vessels with both SKF525A and IBTX resulted in vasoconstriction in response to DHA (*Figure 5A*). Pre-incubation with another CYP epoxygenase inhibitor, MS-PPOH (10 μM), produced results similar to those with SKF525A (*Figure 5A*). These results confirmed that the vasodilatory effects of DHA are associated with activation of BK channels and that the CYP pathway is critical for DHA-mediated vasodilatation.

To further determine the mechanism through which DHA produces vasodilatation in rat coronary arteries, we examined the effects of ryanodine, which inhibits the Ca^{2+} release channels in the sarcoplasmic reticulum. After pre-incubation with ryanodine (10 μM) for 10 min, DHA could no longer dilate rat coronary arteries (*Figure 5B*). Likewise, after pre-incubation with diltiazem (10 μM) for 30 min, which did not produce maximal vasodilatation, the DHA-mediated vasodilatation was significantly blunted (*Figure 5B*). These results suggest that DHA may exert its effects through modulation of intracellular Ca^{2+} homeostasis in CASMCs.

3.8 16,17-EpDPE is a potent vasodilator

To confirm that the DHA effects on vasodilatation are mediated through its CYP epoxygenase products of metabolism, we measured the effects of 16,17-EpDPE on the reactivity of isolated rat coronary arteries. The 16,17-EpDPE produced potent and dose-dependent vasodilatations of 5.1 ± 1.9% at 0.1 nM, 10.6 ± 1.8% at 0.5 nM, 23.6 ± 1.0% at 1 nM, 29.7 ± 1.1% at 5 nM, 31.5 ± 1.1% at 10 nM, and 32.5 ± 0.8% at 50 nM ($n = 6$). The EC_{50} for 16,17-EpDPE was 0.76 ± 0.07 nM ($n = 6$), reaching a plateau effect of about 30% vasodilatation at concentrations around 3 nM (*Figure 6*).

To determine the role of BK channels in mediating the 16,17-EpDPE-induced vasodilatation, rat coronary arteries were incubated with 100 nM IBTX for 30 min before the effects of 16,17-EpDPE were measured. After incubation with IBTX, the effects of 16,17-EpDPE were abolished ($n = 4$, *Figure 6A*). These results indicate that the vasodilatory effects of 16,17-EpDPE were mediated through activation of BK channels. In addition, pre-

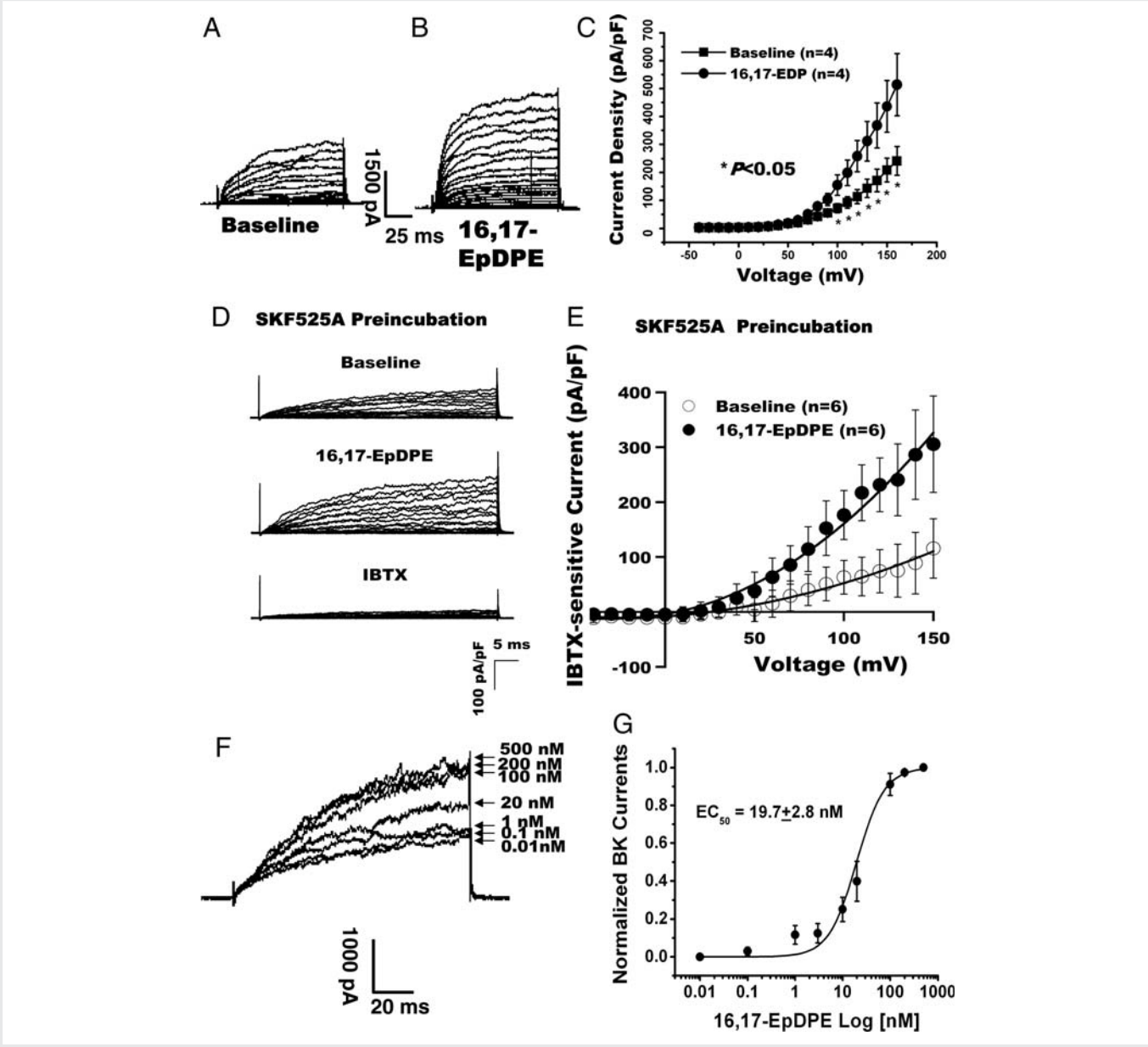

Figure 3 Activation of BK currents by 16,17-EpDPE. Representative current traces showing BK currents recorded from a freshly isolated rat CASMC at baseline (*A*) and after exposure to 20 nM of 16,17-EpDPE (*B*). The *I–V* relationships of group data are shown in (*C*); $^*P < 0.05$. (*D*) Representative BK currents in CASMCs recorded in the presence of SKF525A (10 μM), TRAM-34 (200 nM), APA (1 μM), and 4AP (5 mM) were activated by 16,17-EpDPE (500 nM), and the effects were sensitive to IBTX (100 nM). Group data on the IBTX-sensitive *I–V* relationships are shown in (*E*). (*F*) Representative current traces showing the effects of different concentrations of 16,17-EpDPE (0.01–500 nM) on BK currents (HP = −60 mV, TP = 100 mV). (*G*) Concentration–response relationship showing that16,17-EpDPE is a potent BK channel activator with an EC_{50} of 19.7 ± 2.8 nM.

incubation with ryanodine completely abolished the 16,17-EpDPE-mediated vasodilatation (*Figure 6A*), while pre-incubation with SKF525A (10 μM for 30 min) had no effect (*Figure 6B*). These results are in agreement with the effects of DHA on rat coronary artery vasodilatation and with the effects of DHA and 16,17-EpDPE on BK channel activation. Together, our findings suggest that the CYP epoxygenase metabolites of DHA, namely the DHA epoxides, play an important physiological role in the regulation of coronary arterial tone through modulation of vascular BK channels.

4. Discussion

In this study, we have reported several important findings. First, DHA activates BK channels in rat CASMCs at sub-micromolar levels with an EC_{50} of 0.23 μM. Secondly, DHA-mediated vascular BK channel activation is dependent on the activity of the CYP pathway. Thirdly, the DHA effects on BK channel activation were reproduced by 16,17-EpDPE, a CYP epoxygenase product of DHA metabolism. Fourthly, DHA dilates isolated rat coronary arteries at sub-micromolar levels with an EC_{50} of 0.67 μM, and these effects were

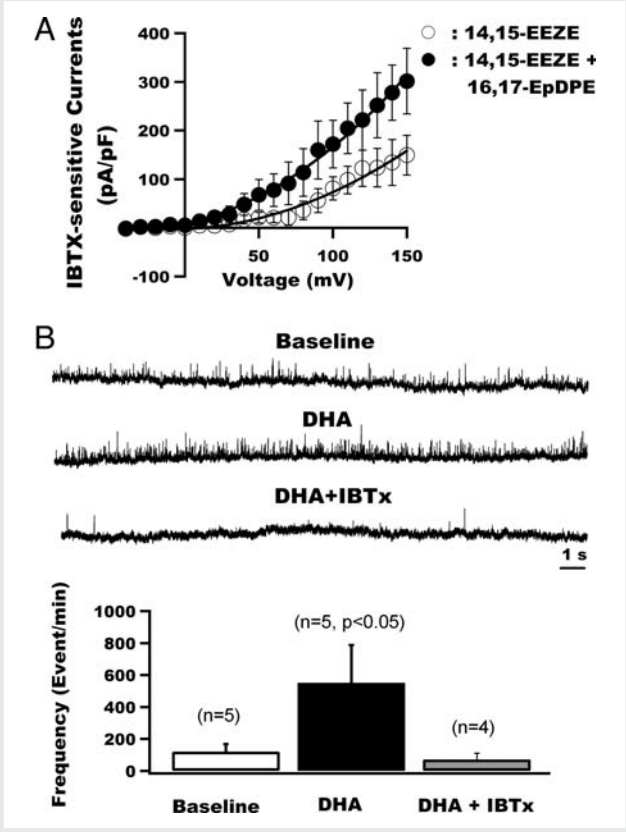

Figure 4 (*A*) 14,15-EEZE did not inhibit BK channel activation by 16,17-EpDPE. Freshly isolated rat CASMCs were treated with 14,15-EEZE (500 nM), an inhibitor of EET. After pre-incubation with 14,15-EEZE, 16,17-EpDPE (500 nM) was able to significantly activate BK currents by two-fold. (*B*) Effects of DHA on STOCs. The STOCs in CASMCs were recorded at 0 mV in whole-cell recordings using the perforated patch technique. Exposure to 1 μM DHA increased the frequency of STOCs by 4.6-fold, and this was inhibited by 100 nM IBTX to below baseline values, suggesting that the source of these currents was from BK channels.

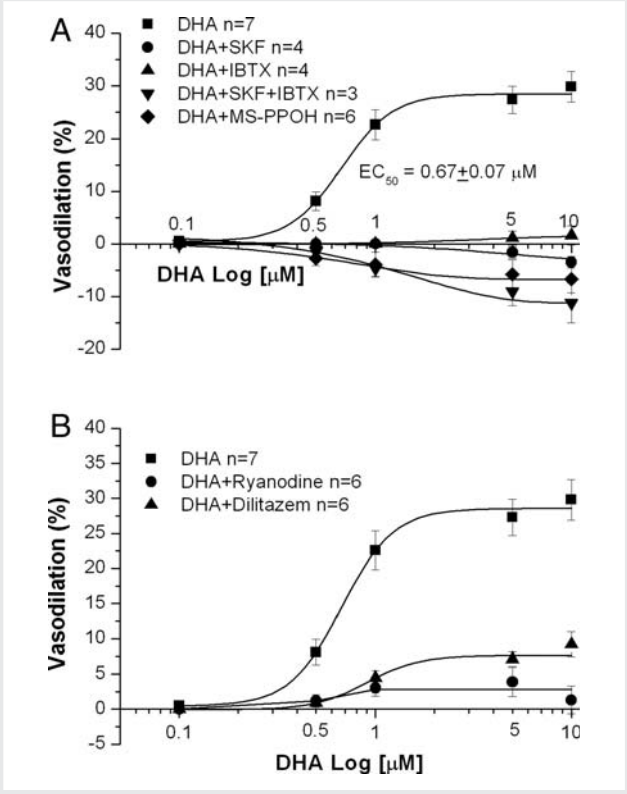

Figure 5 Effects of DHA on dilatation of isolated rat coronary arteries. (*A*) DHA produced potent dose-dependent dilatation of rat coronary arteries with an EC_{50} of 0.67 ± 0.07 μM. However, after incubation with the CYP inhibitor SKF525A (10 μM for 60 min, $n = 4$), MS-PPOH (10 μM for 60 min, $n = 6$), IBTX (100 nM for 30 min, $n = 4$), or with SKF + IBTX ($n = 3$), the vasodilatory effects of DHA were totally abolished ($n = 4$). (*B*) After pre-treatment with ryanodine (10 μM for 10 min, $n = 6$), or with diltiazem (10 μM for 30 min, $n = 6$), the ability of DHA to produce vasodilatation in isolated rat coronary arteries was abolished.

completely dependent on the activities of the CYP pathway. Fifthly, the effects of DHA-mediated vasodilatation were reproduced by 16,17-EpDPE. Sixthly, DHA activates STOCs, and DHA- and 16,17-EpDPE-mediated vasodilatation were abolished by ryanodine. These findings suggest that DHA is an important modulator of vascular function, producing potent vasodilatation through activation of BK channels in vascular smooth muscle cells by its CYP epoxygenase metabolites, which may modulate intracellular Ca^{2+} homeostasis.

A multitude of cellular and physiological mechanisms have been proposed through which *n*-3 PUFAs exert their beneficial effects,[1,2] including reduced platelet aggregation, anti-inflammatory effects, reduced plasma triglycerides, improved autonomic function, direct membrane effects and modulation of ion channel function, and improved vascular endothelial function. Our finding that DHA is a potent activator of the vascular BK channels delineates a basic mechanism through which *n*-3 PUFAs provide cardiovascular protective effects by serving as vasodilators to modulate systemic blood pressure and to improve tissue perfusion.

Docosahexaenoic acid has been shown to inhibit numerous ion channels, such as the cardiac voltage-gated sodium currents, the L-type Ca^{2+} currents, the T-type Ca^{2+} currents, delayed rectifier K^+ currents, and the transient outward K^+ currents.[15,16] It is intriguing that DHA uniformly inhibits the cardiac currents, whereas it activates vascular BK currents. As BK channels are major determinants of vascular tone, activation of BK channels causes hyperpolarization of membrane potentials, which leads to the closing of voltage-gated Ca^{2+} currents and subsequent vasorelaxation.[17] Since human plasma concentrations of DHA are in the micromolar range,[20] our finding that BK currents are activated by sub-micromolar concentrations of DHA suggests that these effects are physiologically relevant and that important vascular beds, including the coronary vasculature, may be significantly regulated by DHA and its metabolites.

Like the *n*-6 PUFAs, *n*-3 PUFAs are metabolized by similar enzymes and pathways.[8] Our findings that DHA-mediated BK channel activation and coronary vasodilatation are CYP dependent are similar to those reported for *n*-6 PUFAs, such as AA. Indeed, the CYP epoxygenase metabolites of AA, EETs, are known endothelium-derived hyperpolarizing factors that activate BK channels.[21,22] Both EETs and 16,17-EpDPE activate vascular BK channels and produce significant coronary vasodilatation in the nanomolar range. However, in contrast to the AA-mediated effects on BK channels and vasodilatation, which

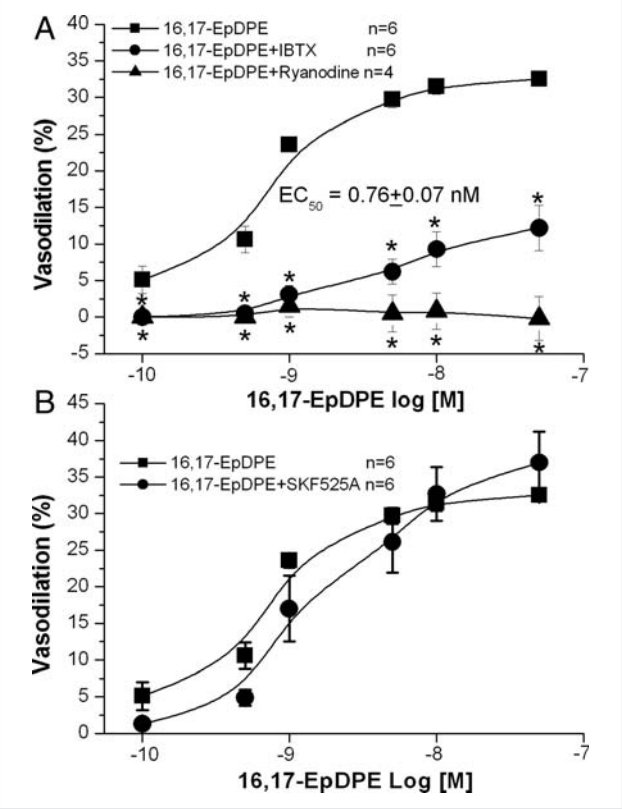

Figure 6 Effects of 16,17-EpDPE on dilatation of isolated rat coronary arteries. (*A*) 16,17-EpDPE produced potent dose-dependent dilatation of rat coronary arteries with an EC_{50} of 0.76 ± 0.07 nM. After incubation with the BK channel inhibitor IBTX (100 nM for 30 min, $n = 6$), or with ryanodine (10 μM for 10 min, $n = 4$), the vasodilatory effects of 16,17-EpDPE were abolished ($n = 6$). *$P < 0.05$ vs. control. (*B*) Pre-incubation with SKF525A (10 μM for 30 min) had no effects on 16,17-EpDPE-mediated vasodilatation.

were only partly reduced by CYP inhibitors,[11] the effects of DHA were totally abolished by the CYP inhibitors, SKF525A and MS-PPOH. The difference may be because AA is metabolized into many bioactive metabolites by multiple enzymatic pathways, including cyclo-oxygenase, lipoxygenase, and the CYP epoxygenase.[23] Inhibition of CYP thus only partly suppresses the production of the AA metabolites. In contrast, DHA is a poor substrate for cyclo-oxygenase and lipoxygenase,[24] and at 1 μM DHA, only CYP epoxygenase produces significant bioactive metabolites. The effects of DHA were not inhibited by 14,15-EEZE, which antagonizes the effects of EET through specific binding to the putative EET receptor.[19,25] Our results indicate that 16,17-EpDPE activates vascular BK channels through mechanisms that are different from its *n*-6 PUFA counterparts.

Very little is known about the DHA-derived epoxides. Epoxygenase metabolites of DHA have been shown to inhibit platelet aggregation with a half-maximal inhibitory concentration of 0.7–1.5 μM.[26] Our results demonstrated that 16,17-EpDPE is a potent vascular BK channel activator. In addition, we showed that the DHA effects were exclusively dependent on CYP activity, indicating that the CYP epoxygenase products of DHA may be important dietary regulators of vascular functions. Recently, among 15 human recombinant CYPs examined, CYP2C9, CYP2C19, and CYP1A2 were found to be the most efficient in DHA epoxidation, equal to or more efficient in epoxidizing DHA than AA.[9] Since the CYP2C enzymes are abundantly expressed in the vasculature,[27] the production of EpDPEs and their role in the regulation of vascular function may depend on DHA availability.

The mechanism through which the CYP metabolites of DHA activate vascular BK channels is not entirely clear. Our findings that DHA promotes the development of STOCs and that ryanodine inhibits the vasodilatory effects of DHA and 16,17-EpDPE suggest that the DHA metabolites may facilitate Ca^{2+} release from the sarcoplasmic reticulum, possibly through regulation of calcium sparks.[28] Indeed, the Ca^{2+} channel, ryanodine receptors, and BK channels operate as a functional unit in the regulation of vascular tone,[13] and our findings (*Figures 4B*, 5*B*, and 6*A*) are consistent with this scheme. However, these properties of DHA metabolites are different from 17,18-epoxyeicosatetranoic acid, the CYP epoxygenase product of EPA, which activates the vascular BK channels independent of intracellular Ca^{2+} concentration or local sarcoplasmic reticulum Ca^{2+} release events.[29] The difference in properties of these metabolites might explain in part the difference in outcomes and effects between DHA and EPA. As accumulating evidence establishes the cardiovascular protective roles of *n*-3 PUFAs, the DHA metabolites bear great significance in mediating these effects. Our present findings have helped demonstrate the role of CYP and EpDPE in mediating the vascular protective effects of DHA. Mechanistic delineation of the cardiovascular function of these important DHA metabolites may provide further insights into the beneficial effects *n*-3 PUFAs.

Conflict of interest: none declared.

Funding

This work was supported by funding from the National Natural Science Foundation of China (81070157 to R.W.), the Basic Medical Institute, Shandong Academy of Medical Sciences (Q.C.), the American Diabetes Association (ADA-JFA-07-39 to T.L.) and the National Institutes of Health (HL080118 and HL074180 to H.L.).

References

1. Breslow JL. n-3 fatty acids and cardiovascular disease. *Am J Clin Nutr* 2006;**83**: 1477S–1482S.
2. Lavie CJ, Milani RV, Mehra MR, Ventura HO. Omega-3 polyunsaturated fatty acids and cardiovascular diseases. *J Am Coll Cardiol* 2009;**54**:585–594.
3. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 2002;**106**:2747–2757.
4. Metcalf RG, James MJ, Gibson RA, Edwards JR, Stubberfield J, Stuklis R *et al.* Effects of fish-oil supplementation on myocardial fatty acids in humans. *Am J Clin Nutr* 2007;**85**: 1222–1228.
5. Mori TA, Burke V, Puddey IB, Watts GF, O'Neal DN, Best JD *et al.* Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 2000;**71**:1085–1094.
6. Woodman RJ, Mori TA, Burke V, Puddey IB, Barden A, Watts GF *et al.* Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis* 2003; **166**:85–93.
7. Mori TA, Bao DQ, Burke V, Puddey IB, Beilin LJ. Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. *Hypertension* 1999;**34**:253–260.
8. Schmitz G, Ecker J. The opposing effects of *n*−3 and *n*−6 fatty acids. *Prog Lipid Res* 2008;**47**:147–155.
9. Fer M, Dreano Y, Lucas D, Corcos L, Salaun JP, Berthou F, *et al.* Metabolism of eicosapentaenoic and docosahexaenoic acids by recombinant human cytochromes P450. *Arch Biochem Biophys* 2008;**471**:116–125.
10. Spector AA. Arachidonic acid cytochrome P450 epoxygenase pathway. *J Lipid Res* 2009;**50**(Suppl):S52–S56.

11. Zhou W, Wang XL, Lamping KG, Lee HC. Inhibition of protein kinase Cβ protects against diabetes-induced impairment in arachidonic acid dilation of small coronary arteries. *J Pharmacol Exp Ther* 2006;**319**:199–207.
12. Lu T, Zhang DM, Wang XL, He T, Wang RX, Chai Q, , *et al*. Regulation of coronary arterial BK channels by caveolae-mediated angiotensin II signaling in diabetes mellitus. *Circ Res* 2010;**106**:1164–1173.
13. Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ, *et al*. Relaxation of arterial smooth muscle by calcium sparks. *Science* 1995;**270**:633–637.
14. Horn R, Marty A. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* 1988;**92**:145–159.
15. London B, Albert C, Anderson ME, Giles WR, Van Wagoner DR, Balk E *et al*. Omega-3 fatty acids and cardiac arrhythmias: prior studies and recommendations for future research: a report from the National Heart, Lung, and Blood Institute and Office Of Dietary Supplements Omega-3 Fatty Acids and their Role in Cardiac Arrhythmogenesis Workshop. *Circulation* 2007;**116**:e320–e335.
16. Xiao YF, Sigg DC, Leaf A. The antiarrhythmic effect of n-3 polyunsaturated fatty acids: modulation of cardiac ion channels as a potential mechanism. *J Membr Biol* 2005;**206**: 141–154.
17. Eichhorn B, Dobrev D. Vascular large conductance calcium-activated potassium channels: functional role and therapeutic potential. *Naunyn Schmiedebergs Arch Pharmacol* 2007;**376**:145–155.
18. Zhou W, Lu T, Spector AA, Lee HC. Inhibition of PGI_2 signaling by miconazole in vascular smooth muscle cells. *Prostaglandins Other Lipid Mediat* 2006;**80**:28–34.
19. Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR *et al*. 14,15-Epoxyeicosa-5(*Z*)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. *Circ Res* 2002;**90**:1028–1036.
20. Harper CR, Edwards MJ, DeFilippis AP, Jacobson TA. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J Nutr* 2006; **136**:83–87.
21. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 1996;**78**: 415–423.
22. Lu T, Katakam PV, VanRollins M, Weintraub NL, Spector AA, Lee HC. Dihydroxyeicosatrienoic acids are potent activators of Ca^{2+}-activated K^+ channels in isolated rat coronary arterial myocytes. *J Physiol* 2001;**534**:651–667.
23. Brash AR. Arachidonic acid as a bioactive molecule. *J Clin Invest* 2001;**107**:1339–1345.
24. Russo GL. Dietary *n*−6 and *n*−3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. *Biochem Pharmacol* 2009;**77**:937–946.
25. Yang W, Tuniki VR, Anjaiah S, Falck JR, Hillard CJ, Campbell WB. Characterization of epoxyeicosatrienoic acid binding site in U937 membranes using a novel radiolabeled agonist, 20-^{125}I-14,15-epoxyeicosa-8(*Z*)-enoic acid. *J Pharmacol Exp Ther* 2008;**324**: 1019–1027.
26. VanRollins M. Epoxygenase metabolites of docosahexaenoic and eicosapentaenoic acids inhibit platelet aggregation at concentrations below those affecting thromboxane synthesis. *J Pharmacol Exp Ther* 1995;**274**:798–804.
27. Fisslthaler B, Hinsch N, Chataigneau T, Popp R, Kiss L, Busse R *et al*. Nifedipine increases cytochrome P4502C expression and endothelium-derived hyperpolarizing factor-mediated responses in coronary arteries. *Hypertension* 2000;**36**:270–275.
28. Jaggar JH, Wellman GC, Heppner TJ, Porter VA, Perez GJ, Gollasch M *et al*. Ca^{2+} channels, ryanodine receptors and Ca^{2+}-activated K^+ channels: a functional unit for regulating arterial tone. *Acta Physiol Scand* 1998;**164**:577–587.
29. Hercule HC, Salanova B, Essin K, Honeck H, Falck JR, Sausbier M *et al*. The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK α channel subunit in rodents. *Exp Physiol* 2007;**92**:1067–1076.