Cytochrome P-450 Epoxygenase Metabolites of Docosahexaenoate Potently Dilate Coronary Arterioles by Activating Large-Conductance Calcium-Activated Potassium Channels

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ABSTRACT

Diets enriched in docosahexaenoic acid, a major n-3 fatty acid in fish oil, have hypotensive properties. One mechanism that can lower blood pressure is the direct dilation of arterioles by docosahexaenoic metabolites. Vascular endothelium contains cytochrome P-450 epoxygenases that transform the n-6 fatty acid arachidionate to epoxyeicosatrienoic acids (EETs), potent dilators of coronary arterioles and activators of large-conductance calcium-activated potassium (BKCa) channels. To test whether analogous activations occur for docosahexaenoate, we compared the potency of docosahexaenoate and its five cytochrome P-450 epoxygcnase metabolites, epoxycosapentaenoates (EDPs), in dilating porcine coronary arterioles. Moreover, 13,14-EDP potently activated BKCa channels in myocytes from the porcine coronary arterioles. Moreover, 13,14-EDP potently activated BKCa channels in myocytes from rat coronary small arteries (150–300 \( \mu \)m in diameter); with an EC50 value of 2.2 ± 0.6 pM (\( n = 7 \)), 13,14-EDP was 1000-fold more potent than EETs in activating BKCa channels. We conclude that EDPs potently dilate coronary microvessels and are the most potent fatty epoxides known to activate BKCa channels in coronary smooth muscle cells. Both actions may contribute to the hypotensive effects of dietary fish oils.

Docosahexaenoate and eicosapentaenoate are the major n-3 fatty acids in fish oils. For over 20 years, dietary supplements with fish oils have been considered as indirectly cardioprotective because of their antithrombotic, antiatherosclerotic, and antihypertensive properties. Recent clinical studies suggest that docosahexaenoate may be the active agent in dietary fish oils responsible for lowering systemic blood pressure. In 1989, the daily ingestion of fish oil (5 g of docosahexaenoate and 10 g of eicosapentaenoate) was shown to reduce the blood pressure of patients with essential hypertension (Knapp and FitzGerald, 1989). These antihypertensive effects, which follow eating large amounts of fish oil containing a low docosahexaenoate/eicosapentaenoate ratio, are now widely accepted (Appel et al., 1993; Morris et al., 1993). Recently, the daily ingestion of fish containing small amounts of n-3 fatty acids (3.65 g) with a high docosahexaenoate/eicosapentaenoate ratio was also found to be antihypertensive (Mori et al., 1999). Moreover, daily ingestions of 4.0 g of docosahexaenoate reduced blood pressure in normotensive subjects (Mori et al., 2000). Together, these clinical studies raised the possibility that dietary docosahexaenoate has hypotensive effects.

Epoxyeicosatrienoic acids (EETs) are cytochrome P-450 epoxygenase metabolites of the n-6 fatty acid arachidionate, and they potently dilate coronary arterioles (Oltman et al., 1998; Zhang et al., 2001). Because bradykinin and acetylcholine stimulate the endothelial synthesis and release of EETs...
(Nithipatikom et al., 2000) and because EETs hyperpolarize and relax myocytes in small arteries, EETs have been hypothesized to be endothelial-dependent hyperpolarizing factors (EDHFs) (Campbell et al., 1996; Fisslthaler et al., 1999). Moreover, like EDHF-induced dilations in human vessels (Urakami-Harasawa et al., 1997), EET-induced dilations are more prominent in resistance vessels than in conduit vessels (Oltman et al., 1998). Recently, dietary docosahexaenoate (but not eicosapentaenoate) was found to enhance the blood flow in human arterioles after acetylcholine infusions in the presence of an inhibitor of nitric-oxide synthesis. Thus, dietary docosahexaenoate may enhance the release of an EDHF, perhaps by providing an EDHF precursor (Mori et al., 2000). Yet, unlike eicosapentaenoate (Needleman et al., 1979; Zhang et al., 2001), no docosahexaenoate metabolite has been reported to be a potent dilator of resistance vessels.

Early animal studies suggested that EETs hyperpolarized vascular smooth muscle cells through activation of BKCa channels (Hu and Kim, 1993). Because of a high density in myocyte membranes and large conductance properties, BKCa channels are an important determinant of vascular tone. Moreover, primary and secondary alterations in BKCa channel activities are common in hypertensive rats and mice (Liu et al., 1988). A mixture of six EDP regioisomers was generated by reacting the methyl ester of [1-14C]docosahexaenoic acid with m-chloroperbenzoic acid that converts cis double bonds to trans-epoxides (Chung and Scott, 1974). Individual regioisomers (Fig. 1) were isolated by normal phase-HPLC and collected over ice (VanRollins et al., 1989). One regioisomer, 4,5-EDP, was not processed further because it is chemically unstable due to ready formation of γ-lactones. The other five regioisomers (Fig. 1) were saponified and further isolated by normal phase-HPLC (VanRollins et al., 1989). Based on reversed-phase HPLC (VanRollins et al., 1989), each isomer was >99% free of diole hydrolysis artifacts as well as other regioisomers. Molecular weights, epoxide positions, number of double bonds, and absence of conjugated dienes were established using gas chromatography-mass spectrometry and ultraviolet spectroscopy (VanRollins et al., 1989). In addition, 10 mg of synthetic 13,14-EDP was subjected to acid-catalyzed hydrolysis to yield 13,14-DHDP (Fig. 1) (VanRollins et al., 1989). A 3.0-μg aliquot of the 13,14-DHDP product was derivatized to form a bis(trimethylsilyl) ether, pentfluorobenzylester, and the position of the resulting vicinal diol was confirmed by electron impact, positive- and negative-ion chemical ionization mass spectrometry (VanRollins et al., 1996).

Isolation and Preparation of Porcine Coronary Microvessels. The animal protocols were approved by the University of Iowa Animal Care and Use Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Details for isolating porcine microvessels have been reported (Zhang et al., 2001). In brief, hearts of seven male (118 ± 10 kg) and 12 female pigs (125 ± 4.4 kg) were harvested at a local slaughterhouse and cooled over ice. After being gently flushed with heparinized Krebs’ solution to remove blood, subepicardial arteries were perfused with an India ink solution for visualization purposes. Individual arteries (65–135-μm range and 94 ± 17 (S.D.) μm i.d. ± 1.5 mm in length and ± 16 μg of dry weight) were excised under a dissecting microscope and trimmed of fat and adventitia.

Vasoactivity was tested using a pressurized, no-flow arteriole preparation (Zhang et al., 2001). In brief, each porcine arteriole was cannulated at both ends with glass micropipettes secured by sutures. The micropipettes were connected via tubing containing Krebs’ solution to a reservoir whose height was adjusted to maintain an equilibrium pressure of 100 mmHg.

The present study, we tested whether cytochrome P-450 epoxygenase metabolites of docosahexaenoate (EDPs) dilated coronary arterioles and activated BKCa channels in myocytes from coronary arterioles and small arteries. Dilatory responses in porcine microvessels were examined, because in this species dietary fish oil is known to enhance the endothelial formation of an EDHF (Shimokawa and Vanhoutte, 1989; Naga et al., 1995). To determine a mechanism for EDP-induced dilations, the effects of 13,14-EDP on BKCa channels in inside-out patches from myocytes in porcine coronary arterioles were examined. To compare potencies with EETs, the effects of 13,14-EDP on BKCa channels in inside-out patches from rat coronary myocytes were investigated (Lu et al., 2001). Our findings indicate that cytochrome P-450 metabolites of the n-3 fatty acid docosahexaenoate potently dilate coronary microvessels by activating BKCa channels in vascular smooth muscle cells.

Materials and Methods

Synthesis of EDPs and 13,14-Dihydroxydocosapentaenoic Acid (DHDP). A mixture of six EDP regioisomers was generated by reacting the methyl ester of [1-14C]docosahexaenoic acid with m-chloroperbenzoic acid that converts cis double bonds to trans-epoxides (Chung and Scott, 1974). Individual regioisomers (Fig. 1) were isolated by normal phase-HPLC and collected over ice (VanRollins et al., 1989). One regioisomer, 4,5-EDP, was not processed further because it is chemically unstable due to ready formation of γ-lactones. The other five regioisomers (Fig. 1) were saponified and further isolated by normal phase-HPLC (VanRollins et al., 1989). Based on reversed-phase HPLC (VanRollins et al., 1989), each isomer was >99% free of diole hydrolysis artifacts as well as other regioisomers. Molecular weights, epoxide positions, number of double bonds, and absence of conjugated dienes were established using gas chromatography-mass spectrometry and ultraviolet spectroscopy (VanRollins et al., 1989). In addition, 10 mg of synthetic 13,14-EDP was subjected to acid-catalyzed hydrolysis to yield 13,14-DHDP (Fig. 1) (VanRollins et al., 1989). A 3.0-μg aliquot of the 13,14-DHDP product was derivatized to form a bis(trimethylsilyl) ether, pentfluorobenzylester, and the position of the resulting vicinal diol was confirmed by electron impact, positive- and negative-ion chemical ionization mass spectrometry (VanRollins et al., 1996).

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Fig. 1. Structure of EDP and DHDP metabolites of docosahexaenoate.
intraluminal pressure of 60 mm Hg. Each microvessel was sub-
merged in an organ chamber through which Krebs’ buffer, pre-
warmed to 37°C and aerated with 20% oxygen, 75% nitrogen, and 5% 
CO₂, was continuously circulated. The organ chamber was placed 
on to the stage of an inverted microscope, to which a video camera, 
monitor, and calibrated caliper were mounted. Vessel internal diam-
eters were measured by manually adjusting a video micrometer.

Protocol Testing the Potency of Docosahexaenoate, EDPs, 
and 13,14-DHDP in Dilating Coronary Arterioles. To test arte-
riole viability, 75 mM isometric KCl was applied to a vessel prequili-
brated at the original in situ length for 30 min at 60 mm Hg luminal 
pressure. After 5 min, fresh Krebs’ solution was added to the chap-
mer and the arteriole diameter was allowed to return to the original 
baseline value. To test dilatory potency, the arterioles were first 
constricted to 37 to 61% of the resting diameter using 6.6 ± 3.1 mM 
(S.D.) endothelin-1 (Phoenix Pharmaceutical, Inc., San Francisco, 
CA). Docosahexaenoate (10⁻¹⁰ – 10⁻⁴ M) or EDPs/13,14-DHDP 
(10⁻¹⁰ – 10⁻⁸ M) were added in increasing concentrations directly to 
the organ chambers, and dilation responses were determined every 3 
min. In a few studies, Krebs’ vehicle alone was added to assess the 
contribution of spontaneous dilation with time. Upon completing 
each concentration-dilation study, a single dose of 100 μM sodium 
nitroprusside or 100 μM papaverine (Sigma-Aldrich, St. Louis, MO) 
was applied to test residual dilating capacity. In the rare circum-
stance where a compound produced full-scale dilation, the bath was 
transferred to 80 ml of ice-cold methanol containing 

Data were considered unacceptable if arterioles 1) demonstrated 
visible leaks, 2) failed to constrict or papaverine, or 4) failed to reconstrict 
upon reexposure to endothelin.

Protocol Testing the Stability of Fatty Epoxides in Arte-
riole Baths. Porcine coronary arterioles (n = 2) were isolated, 
pressurized, and preconstricted with potassium and endothelin as 
described above. Each vessel was incubated 37 min (the average 
application time for 12 doses, each dose requiring a 3-min wait for 
measuring vasoactivity) with a mixture of 2.0 μM 13,14-EDP (unla-
beled) and 1.0 μM [1-¹⁴C]11,12-EET (76,000 dpm); the latter tracer 
was synthesized as described previously (VanRlollins et al., 1996) and 
added to assess hydrolysis specificity. After vessel responses to ep-
oxides and sodium nitroprusside were tested, the 20-ml bath solution 
was transferred to 80 ml of ice-cold methanol containing −5 mg of 
N₅CO₃. Each of the vessels studied was considered representative in 
that they responded appropriately to each of the agonists tested.

After storage at −84°C overnight, fractions were flash evaporated 
to near dryness and extracted into ice-cold water-saturated ethyl 
acetate (VanRlollins et al., 1989). After being concentrated under a 
nitrogen stream, metabolites were resolved with guard (5 × 0.46 cm) 
and analytical (25 × 0.46 cm) columns containing 5 μm of Luna 
C18(2) particles (Phenomenex, Torrance, CA) through which 61% 
acetonitrile and 39% water, pH 2.2, flowed at 1 ml/min and 1600 psig 
(VanRlollins et al., 1996). Metabolites in the effluent were monitored 
by absorbance at 194 nm with a photodiode array detector (LC480;
PerkinElmer Instruments, Norwalk, CT) and by on-line liquid scin-
tillation counting (model 500TR fitted with extra lead shielding and 
a 0.5-m1 time-resolved liquid scintillation cell; Packard Instrument 
Company, Inc., Downers Grove, IL). Scintillant cocktail (Ultima-Flo 
M; Packard Instrument Company, Inc.) was introduced into the cell 
at 3.63 ml/min. Similar studies without vessels were performed to 
monitor processing artifacts.

Preparation of Docosahexaenoate, EDP, and 13,14-DHDP 
Solutions. All solutions were prepared on the day of the experiment. 
The Krebs’ solution consisted of 119.7 mM NaCl, 5 mM KCl, 2.5 mM 
CaCl₂, 1.2 mM MgSO₄, 25.5 mM NaHCO₃, 1.2 mM KH₂PO₄, 0.026 
mM Na₂EDTA, and 11 mM glucose, pH 7.4, and was aerated at room 
temperature with 20% O₂, 5% CO₂, and 75% N₂. Upon being synthe-
ized, [1⁻¹³C]EDPs and [1⁻¹⁴C]13,14-DHDP were stored at 5 mM 
concentrations for up to 40 days at −80°C in ethanol. Just before use, 
EDPs and 13,14-DHDP were subjected to serial dilutions with ice-
cold Krebs’ buffer and maintained over ice. The concentrations of the 
stock solutions and initial dilutions were checked daily by liquid 
scintillation counting techniques (Zhang et al., 2001). The final 
concentration of ethanol was <0.01%. In contrast to EDPs and 13,14-
DHDP, sodium docosahexaenoate (100 mg of neat material; NuChek 
Prep, Elysian, MN) was initially suspended in 143 ml of isometric 
NaCl solution and briefly stirred at 40°C until dissolved. Serial 
dilutions were done using a Krebs’ buffer maintained at 25°C. No 
concentration of docosahexaenoate higher than 100 μM could be 
tested for vasoactivity because the high concentrations produced a 
cloudy Krebs’ solution.

Isolation of Smooth Muscle Cells from Porcine Coronary 
Arterioles and Rat Coronary Small Arteries for Patch-Clamp 
Studies. Upon being rapidly excised, five porcine hearts were 
washed and suspended in a 4°C solution containing 145.0 mM NaCl, 
4.0 mM KCl, 0.05 mM CaCl₂, 1.0 mM MgCl₂, 10.0 mM HEPES, and 
10.0 mM glucose, previously adjusted to pH 7.4 with NaOH. Subepi-
cardial microvessels were isolated under a dissecting microscope, 
and their diameters (60–120 μm) were measured using a video 
microscope monitor and micrometer. Single smooth muscle cells 
were isolated as described previously (Ye et al., 2000) but with 
modified digestion conditions to improve cell viability. In brief, the 
microvessels were incubated at 37°C for 35 to 40 min in a Ca²⁺-free 
Tyrode’s solution, pH 7.4, containing 0.18% protease (type XXIV; 
Sigma-Aldrich), 0.15% collagenase (type I A; Sigma-Aldrich), 0.12% 
trypsin inhibitor (type II-S; Sigma-Aldrich), 138.0 mM NaCl, 4.5 mM 
KCl, 0.5 mM MgCl₂, 0.33 mM Na₅HPO₄, 10.0 mM HEPES, and 5.5 mM 
glucose. Digested microvessels were washed by three transfers to 
fresh Krebs’ solutions, pH 7.4, composed of 70.0 mM KOH, 70.0 mM 
KCl, 50.0 mM L-glutamic acid, 20.0 mM taurine, 0.5 mM MgCl₂, 1.0 
M K₂HPO₄, 0.5 mM EGTA, 10.0 mM HEPES, 5.0 mM creatine, 5.0 
mM pyruvic acid, and 5.0 mM Na₅ATP. Single smooth muscle cells 
were isolated by triturating with a fire-polished glass pipette, stored 
at 4°C in the Krebs’ solution, and used within 10 h.

To compare EDP and EET potencies, smooth muscle cells in small 
arteries were isolated from hearts of rats. In brief, male Sprague-
Dawley rats (250–300 g) were anesthetized by inhaling methoxyflu-
orane. Hearts were rapidly excised and placed in a basal, physiolog-
ical saline solution containing 145.0 mM NaCl, 4.0 mM KCl, 0.05 
mM CaCl₂, 1.0 mM MgCl₂, 10.0 mM HEPES, and 10.0 mM glucose, 
and adjusted to pH 7.2 with NaOH. After being isolated under a 
dissection microscope, the secondary and tertiary branches (150–300 
μm i.d.) of the septal coronary arteries were incubated at 37°C for 10 
min in 1.0 ml of basal solution containing 0.1% bovine serum albu-
min. Initially, vessels were enzymatically digested 10 min at 37°C in 
1.0 ml of fresh basal solution containing 1.5 mg of papain (11.9 units 
mg⁻¹; Sigma-Aldrich) and 1.0 mg of dithiothreitol (Roche Applied 
Sciences, Indianapolis, IN). The vessels were further digested 10 min at 
37°C in 1.0 ml of fresh basal solution containing 1.0 mg of collagena-
se (CLS-2, 364 units mg⁻¹; Worthington Biochemicals, Lake-
wood, NJ) and 1.0 mg of trypsin inhibitor (type II-S; Sigma-Aldrich). 
To remove the exogenous enzymes, each vessel was transferred three 
times to 1.0 ml of fresh basal solution, and gently triturated with a 
fire-polished glass pipette until completely dissociated. The resulting 
smooth muscle cell suspension was stored at 4°C and used within 
8 h.

Single BKCa Channel Recording. Unitary membrane currents 
in individual smooth muscle cells were recorded using a patch-clamp 
technique in an inside-out configuration (Hamill et al., 1981). In 
brief, isolated vascular smooth muscle cells were placed in a 1.0-ml 
chamber on the stage of an inverted microscope (CK 40; Olympus 
America Inc., Melville, NY) and were superfused at 1 to 2 ml/min 
using a direct current-powered pump (model 700; Insetch Laborato-
ries, Inc., Plymouth Meeting, PA). Under these conditions, the bath 
solution was entirely replaced within 30 to 60 s. Borosilicate glass
capillaries (Corning 7056; Warner Instrument, Hamden, CT) were used to fabricate patch pipettes. When filled with 140.0 mM KCl, 1.0 mM CaCl$_2$, 1.0 mM MgCl$_2$, 10.0 mM HEPES, and 1.0 mM EGTA, and adjusted to pH 7.4 with KOH, each electrode had a tip resistance between 4 and 10 MΩ and a typical seal resistance greater than 10 GΩ. Single BK$_{Ca}$ channel currents were recorded with an Axopatch 200B integrating amplifier (Axon Instruments, Union City, CA), and the output of the amplifier was filtered through an eight-pole low pass Bessel filter unit (900B/9LSL; Frequency Devices, Haverhill, MA) at 5 kHz and digitized at 20 (pig) or 40 (rat) kHz (12-bit resolution, Digidata 1200; Axon Instruments). Software (pClamp, version 6.05; Axon Instruments) was used to generate the voltage-clamp protocols, and the resulting current recordings were stored in a Pentium-based personal computer (Dimension XPS T450; Dell Computer Corp., Round Rock, TX) for further analysis. A pStat program, implemented in the pClamp software, was later used to calculate the BK$_{Ca}$ channel open probability ($P_o$):

$$P_o = \frac{\sum_{j=1}^{N} t_j}{N} TN,$$

where $P_o$ is the single channel open probability, $T$ is the duration of recording, $t_j$ is the time spent with $j = 1, 2, \ldots, N$ channel openings, and $N$ is the maximal number of simultaneous channel openings observed when $P_o$ was high. An $N$ not more than five was used for all $P_o$ studies. Each dose-response curve was fitted using a Hill equation of the following form: $P_o/P_{o,max} = 1/(1 + (S/EC_{50})^N)$, where $P_{o,max}$ represents the maximal channel activity observed, $S$ represents concentration of the chemical, $EC_{50}$ represents the concentration at half-maximal effect, and $H$ is the Hill coefficient.

For each BK$_{Ca}$ channel study, the membrane potential of the patches was held at +60 mV and the (cytosolic) perfusate was maintained at 21–23°C. The perfusate contained 140.0 mM KCl, 1.0 mM CaCl$_2$, 1.0 mM MgCl$_2$, 10.0 mM HEPES plus 1.0 mM EGTA and was adjusted to pH 7.35 with KOH. Enough CaCl$_2$ was added to provide either 0.2 μM (pig) or 1.0 (rat) μM free Ca$^{2+}$, as calculated using Chelator software (Theo J. M. Schoenmakers, Department of Animal Physiology, University of Nijmagen, Toernooiveld, The Netherlands). After excising each patch, we routinely examined channel sensitivity to Ca$^{2+}$ by removing and replenishing Ca$^{2+}$ in the bath solution. Only patches containing BK$_{Ca}$ channels sensitive to Ca$^{2+}$ were studied further. Individual BK$_{Ca}$ channels were further identified by unitary conductance, voltage sensitivity, and by inhibition with 50 to 100 nMiberiotoxin. Details on BK$_{Ca}$ measurements are available (Lu et al., 2001).

To test BK$_{Ca}$ activation, 13,14-EDP and docosahexaenoate were prepared in absolute ethanol as 2.9 to 5.0 and 50 mM stock solutions, respectively, and stored under nitrogen at −80°C. On the day of the experiment, 13,14-EDP and docosahexaenoate were serially diluted with bath solution and added to the perfusate; the final ethanol concentration was less than 0.0002%.

Statistical Analysis. Data are presented as mean ± S.E.M. values unless otherwise stated. Concentration-dilation curves were subjected to a nonlinear regression program (Prism version 3.0; GraphPad Software, San Diego, CA). From each fitted curve, Hill slope and concentrations that produced maximal dilation and 50% of maximal vasodilation (EC$_{50}$) were determined and evaluated using one-way analysis of variance followed by a Fisher correction (least significant difference) for multiple comparisons. Whether the maximal dilation was less than that induced by sodium nitroprusside was assessed using a one-tailed paired $t$ test. Changes in $P_o$ were evaluated using one-way analysis of variance and the Student’s $t$ test. In all studies, a $p$ value of <0.05 was considered statistically significant.

Results

Each of the five EDP regioisomers diluted porcine arterioles in a concentration-dependent manner (Fig. 2A). The EC$_{50}$ values were 211 ± 172, 4.1 ± 2.1, 2.0 ± 1.4, 20.3 ± 13.3, and 1.6 ± 0.7 pM for 19,20-, 16,17-, 13,14-, 10,11-, and 7,8-EDP, respectively (Table 1). The dilations were thus 50% complete for EDP concentrations ranging from 1.6 to 211 pM. No EDP regiospecificity was detectable in the induced dilations. In contrast to the EDPs, the EC$_{50}$ for the diol 13,14-DHDP was 30 ± 22 nM ($n = 7$). Thus, the conversion of 13,14-EDP to 13,14-DHDP caused a 15,000-fold loss in potency ($p < 0.001$). As an estimate of minimal detectable responses for curves characterized by low Hill slopes (vide infra), the EC$_{20}$ values were 1960 ± 1630, 501 ± 403, 150 ± 132, 552 ± 240, and 199 ± 147 FM for 19,20-, 16,17-, 13,14-, 10,11-, and 7,8-EDP, respectively. Dilated arterioles were detectable at EDP concentrations ranging from 150 FM to 1.96 pM. Thus, EDPs were very potent dilators, with some
TABLE 1
Potency and efficacy of EDP regioisomers in dilating porcine coronary microvessels

Values are geometrical means percentage ± S.E. of the diameter change induced by endothelin. The extent that endothelin had preconstricted porcine arterioles was 42 ± 3, 52 ± 3, 49 ± 3, 39 ± 1, and 48 ± 3% of the resting vessel diameter for 19,20-, 16,17-, 13,14-, 10,11-, and 7,8-EDP, respectively. EC50 represents the concentration producing 50% of maximal vasodilation. The R² of individual curve fittings averaged 0.962 ± 0.016 (S.D., n = 27), and ranged from 0.968 ± 0.021 (n = 6) to 0.990 ± 0.007 (n = 6) in the five studies.

<table>
<thead>
<tr>
<th>EDP Isomer</th>
<th>Log EC50</th>
<th>EDP-Induced Maximum Dilation</th>
<th>Sodium Nitroprusside Dilation</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>19,20-EDP</td>
<td>−10.6 ± 0.5</td>
<td>71 ± 4**</td>
<td>90 ± 4</td>
<td>0.604 ± 0.022</td>
</tr>
<tr>
<td>16,17-EDP</td>
<td>−11.8 ± 0.4</td>
<td>73 ± 4</td>
<td>85 ± 2</td>
<td>0.535 ± 0.070</td>
</tr>
<tr>
<td>13,14-EDP</td>
<td>−12.1 ± 0.3</td>
<td>90 ± 4**</td>
<td>97 ± 2</td>
<td>0.429 ± 0.045</td>
</tr>
<tr>
<td>10,11-EDP</td>
<td>−11.4 ± 0.4</td>
<td>77 ± 4**</td>
<td>89 ± 5</td>
<td>0.432 ± 0.047</td>
</tr>
<tr>
<td>7,8-EDP</td>
<td>−12.0 ± 0.3</td>
<td>75 ± 3*</td>
<td>90 ± 4</td>
<td>0.607 ± 0.094</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01 that was less than sodium nitroprusside-induced dilation.
†p < 0.05 that was greater than all other EDP regioisomers.

EDP regioisomers starting to dilate coronary arterioles at 199 fM, and all EDPs completing 50% of the induced dilations by 211 pM.

Each of the five EDP regioisomers produced slightly less than the maximal dilation induced by 100 μM sodium nitroprusside (Table 1). 13,14-EDP reached 93% of the dilation induced by sodium nitroprusside; however, the other four EDPs produced only 84 ± 2% of the maximal possible dilation. Therefore, at nanomolar concentrations all EDPs induced dilations that were 7 to 16% less than the maximal response produced by sodium nitroprusside (Fig. 2A). Interestingly, the maximal dilations were reached only after increasing the EDP concentration over 6 orders of magnitude. The Hill slope was always ≤0.6 and showed no regiospecificity (Table 1). Nevertheless, together the above-mentioned data indicated that EDPs were both highly potent and efficacious dilators of coronary arterioles.

Docosahexaenoate also dilated porcine subepicardial arterioles in a concentration-dependent manner (Fig. 2B). However, because of solubility problems at greater than 100 μM, the maximal response achieved for docosahexaenoate was only 80 ± 9% and was less than the 102 ± 2% obtained with sodium nitroprusside (p = 0.023). Perhaps more importantly, the apparent EC50 of docosahexaenoate was 8.4 ± 2.6 μM and no dilation was detectable at less than 100 nM. Thus, the parent docosahexaenoate was 100,000 times less potent in dilating coronary arterioles than the EDP metabolites that had an average EC50 of 48 pM.

Upon being added to the arteriole bath, 13,14-EDP underwent little hydrolysis over a 37-min period. Based on integration of absorbance at 194 nm, only 1.6 and 2.8% (n = 2) of 2.0 μM 13,14-EDP were converted to 13,14-DHDP, which eluted at 12.97 min (Fig. 3A, bottom). Based on integration of radioactivity, only 1.1 and 2.8% of [1-14C]11,12-EET were converted to [1-14C]11,12-DHET, which eluted at 13.40 min (Fig. 3A, top). Because the starting concentration for 11,12-EET (1.0 μM) was one-half that of 13,14-EDP (2.0 μM) and because EETs and DHETs have much lower absorbances than EDPs and DHDPs (Fig. 3B, bottom), the small amount of 11,12-DHET generated was not readily detectable at 194 nm.

![Fig. 3](image-url)

**Fig. 3.** A, HPLC chromatogram of lipids in bath of coronary arteriole incubated with 13,14-EDP and 11,12-EET. In brief, 2 μM 13,14-EDP (unlabeled) and 1 μM [1-14C]11,12-EET were incubated 37 min with an arteriole. The bath solution was collected, and lipids extracted and resolved by reversed-phase HPLC. Effluent radioactivity (top) and UV absorption at 194 nm (bottom) were monitored with an on-line flow scintillation and photodiode array detector, respectively. B, HPLC chromatograms of substrates prior to addition to bath. Note that compared with 11,12-EET, which has three double bonds, 13,14-EDP with five double bonds has greater absorption at 194 nm (bottom). Unlabeled arrows depict retention times of [1-14C]11,12-DHET (top) and 13,14-DHDP (bottom) standards. Each panel contains data from a single incubation, but identical chromatograms were obtained from duplicate incubations.
Because of the surprisingly high potency of 13,14-EDP in activating BK_{ca} channels in myocytes from porcine arteries, the effects of 13,14-EDP on BK_{ca} channels in myocytes from rat small coronary arteries were also studied. Although less sensitive to calcium, the BK channels in rat coronary arteries and their responses to EETs/DHETs have been well characterized (Lu et al., 2001). As with porcine arteries, the BK_{ca} channels in rat coronary arteries were activated by 13,14-EDP in a dose-dependent manner. Moreover, increasing the concentration of 13,14-EDP recruited more and more BK_{ca} channels (Fig. 4A). Under these experimental conditions, the ethanol vehicle had a maximum concentration of 0.0002% and did not activate BK_{ca} channels. Channel recruitment by 13,14-EDP was associated with an increased open probability for BK_{ca} channels (Fig. 4B). The open probability increased from 0.062 ± 0.022 (no EDP) to a maximum of 0.34 ± 0.09 (100 pM 13,14-EDP). Open probability climbed 6.04 ± 1.03 fold (p = 0.02) when calculated for each experiment, and 5.5-fold when means were compared. Perhaps more germane for mechanism considerations, the EC_{50} of BK_{ca} channel activation was 6.6 ± 0.6 pM (n = 5), and although statistically different (p = 0.017), was comparable with the 2 pM EC_{50} observed for coronary arteriolar dilation. The Hill coefficient for BK_{ca} channel activation was 1.1. In contrast, the Hill coefficient for arteriolar dilation was only 0.429 (Table 1). Thus, in the same arteriolar vessel, BK_{ca} channel activation and dilations had the same EC_{50} but different Hill coefficients. To our knowledge, this is the first report on eicosanoid potencies and BK_{ca} channel activity in porcine coronary arterioles.

Because of the surprisingly high potency of 13,14-EDP in activating BK_{ca} channels in myocytes from porcine arteries, the effects of 13,14-EDP on BK_{ca} channels in myocytes from rat small coronary arteries were also studied. Although less sensitive to calcium, the BK channels in rat coronary arteries and their responses to EETs/DHETs have been well characterized (Lu et al., 2001). As with porcine arteries, the BK_{ca} channels in rat coronary arteries were activated by 13,14-EDP in a dose-dependent manner. Moreover, increasing the concentration of 13,14-EDP recruited more and more BK_{ca} channels (Fig. 5A). The channel recruitment by 13,14-EDP was associated with an increased probability of opening the BK_{ca} channels (Fig. 5A), which climbed from 0.058 ± 0.026 (no EDP) to a maximum of 0.23 ± 0.05 (100 pM 13,14-EDP). Open probability increased 3.6 ± 0.4-fold (p = 0.02) when calculated for each experiment and 4.0-fold when means were compared. Perhaps more importantly, the EC_{50} of BK_{ca} channel activation was 2.2 ± 0.6 pM (n = 7) and similar (but statistically different, p = 0.001) to

![Fig. 4](image-url) 13,14-EDP activated BK_{ca} channels in myocytes from porcine coronary arterioles. Single BK_{ca} channel currents were recorded on inside-out membrane patches, maintained at +60 mV membrane potential and in the presence of 200 nM cytosolic free Ca^{2+}. A, raw tracings of BK_{ca} channels with 15 pA currents that were exposed to increasing concentrations of 13,14-EDP (0, 0.1 pM, 10 pM, and 1 nM); C, current level observed for closed channels. B, after normalizing for different channel numbers in each patch, the P_{o} of BK_{ca} channels was plotted as a function of increasing concentrations of 13,14-EDP. Data represent mean P_{o}/P_{o,max} Values ± S.E.M. (n = 5). In addition, 13,14-EDP data were fitted to a Hill equation; the EC_{50} value represents the EDP concentration at half-maximal effect, and H represents the Hill coefficient.

![Fig. 5](image-url) 13,14-EDP (A) but not docosahexaenoate (B) activated BK_{ca} channels in myocytes from rat small coronary arteries. Single BK_{ca} channel currents were recorded as described in Fig. 4, but in the presence of 1 μM cytosolic free Ca^{2+}. Top, raw tracings of BK_{ca} channels with 15 pA currents that were exposed to increasing concentrations of 13,14-EDP (A) and docosahexaenoate (B). Bottom, normalized P_{o} of BK_{ca} channels was plotted as a function of increasing concentrations of 13,14-EDP (A) or docosahexaenoate (B). Data represent mean P_{o} values ± S.E.M. (n = 7).
the 6.6 ± 0.6 pM (n = 5) found for porcine arterioles. Because the 1.5 Hill coefficient found for rat myocytes was similar to the 1.1 (p = 0.15) observed for porcine myocytes, there were only minor differences in BKCa channel responsiveness to 13,14-EDP by myocytes from rat small arteries and porcine arterioles. More importantly, 13,14-EDP was a highly potent channel activator in both preparations.

Compared with 13,14-EDP, docosahexaenoate weakly activated BKCa channels (Fig. 5B, top). In fact, up to 10 μM docosahexaenoate (Fig. 5B, bottom) completely failed to activate BKCa channels (n = 7; p = 0.350). In contrast, vessel dilations were apparent at 1 μM docosahexaenoate (Fig. 2B). Thus, docosahexaenoate weakly dilated coronary arterioles at concentrations that had no effect on the BKCa channels in myocytes isolated from small coronary arterioles.

**Discussion**

In the present report, femtomolar-to-picomolar concentrations of EDPs dilated 65 to 135-μm-i.d. porcine coronary arterioles. The EC50 values ranged from 1.6 to 211 pM. As found for coronary arteriolar dilations induced by epoxides of arachidonate (EETs) and eicosapentaenoate (EEQs), EDP-induced dilations were not regioselective. Moreover, the EC50 value for individual EDPs was very similar to that of individual regiosomers derived from eicosapentaenoate and arachidonate (Zhang et al., 2001). Like 11,12-EET, 13,14-EDP underwent little hydrolysis during the time that a dilation was induced (∼0.25%/3 min). Thus, compared with endothelial cells isolated from large arteries (VanRollins et al., 1996), whole arterioles and endothelial cells from microvessels (M. VanRollins, unpublished data) may have little epoxide hydrolase activity. Interestingly, unlike 11,12-EET (Oltman et al., 1998), 13,14-EDP hydrolysis dramatically lowered the potency for arteriolar dilation. Together, these findings indicate that EDPs are some of the most potent dilators of coronary microvessels known and suggest that the induced dilations do not require prior conversions to diols.

In contrast to EDPs, the parent docosahexaenoate was only a weak dilator of coronary microvessels. The EC50 value for docosahexaenoate-induced dilations was 8.4 ± 2.6 μM and was greater than that of arachidonate (0.48 ± 0.19 μM; n = 14) prepared and administered under identical conditions (p = 0.0003). Accordingly, docosahexaenoate was 16- and 160,000-fold less potent than arachidonate and EDPs, respectively, in dilating coronary arterioles. One explanation for the low dilator potency is that the abuminally presented docosahexaenoate must first penetrate the smooth muscle barrier, enter endothelial cells for epoxygenation, and be released as vasodilatory EDPs. If EDP formation mediates the docosahexaenoate-induced dilations, the marked differences in potencies between EDPs and docosahexaenoate suggest that only a small amount of newly formed EDP is released to relax underlying smooth muscle cells and dilate whole arterioles.

13,14-EDP potently activated BKCa channels in coronary myocytes from porcine arterioles and rat small arteries. Unlike other preparations (Li and Campbell, 1997), the porcine and rat inside-out patch preparations are similar in that they require no GTP to demonstrate BKCa channel activation by fatty epoxides and diols (Lu et al., 2001). Moreover, the channels responded similarly to 13,14-EDP, generating comparable EC50 values and maximum channel open probabilities. Yet, the patch preparations differed in that a similar state of activation occurred in the arterioles at 5-fold lower cytosolic calcium concentrations than used for small arteries. Such a difference in calcium sensitivity suggests the presence of BKCa channel isoforms, which may be either vessel- or species-specific. Unfortunately, characterizing and identifying the different isoforms require the analysis of large numbers of channels (Toro et al., 1998). Moreover, quantitating the different proportions of isoforms in coronary conduits and arterioles awaits the advent of specific antibodies. Both goals are beyond the scope of the present investigation. Perhaps more important regarding whether EDPs act as EDHFs, the measured EC50 for 13,14-EDP activation of BKCa channels was 1000-fold less than reported for epoxides and diols derived from the n-6 fatty acid arachidonate when analyzed under identical conditions (Lu et al., 2001; Zhang et al., 2001). Thus, EDPs are the most potent fatty acid epoxides known to activate BKCa channels (Lu et al., 2001; Zhang et al., 2001). In fact, we are unaware of any BKCa channel activator as potent as 13,14-EDP.

In concentrations up to 10 μM, docosahexaenoate did not activate BKCa channels in the smooth muscle cells from coronary small arteries. Moreover, when prepared and administered under identical conditions, arachidonate does not activate BKCa channels (Lu et al., 2001). In light of the comparable EDP potencies for activating the BKCa channels in myocytes from porcine arterioles and rat small arteries, docosahexaenoate was probably a poor activator of arteriolar BKCa channels. Assuming a comparable deficiency in BKCa channel activation in porcine arterioles as found in rat small arteries, there would not be significant BKCa channel activation at the docosahexaenoate doses (EC50 value of ∼8.4 μM) that induce arteriolar dilations. As mentioned above, the weak dilations in whole arterioles may be readily explained by cytochrome P-450 epoxygenases in endothelial cells converting a small amount of docosahexaenoate to EDPs. 13,14-EDP is a highly potent activator of BKCa channels in porcine arterioles, and at least 10,000,000-fold more potent than docosahexaenoate in activating the BKCa channels in small arteries. The inability of either docosahexaenoate or arachidonate to activate BKCa channels may result from the plasmalemmal patches lacking the biosynthetic machinery (cytochrome P-450 epoxygenases) for converting polyunsaturated fatty acids to epoxides. In any case, it is clear that cytochrome P-450 epoxygenases transform docosahexaenoate to very potent coronary vasodilators, BKCa channel activators, and inhibitors of platelet aggregation (VanRollins, 1995). All three actions may contribute to the antithrombotic effects of fish and fish oil diets.

Regarding the mechanism of action for EDP-induced dilations, the EC50 value of 13,14-EDP for activating BKCa channels matched that of arteriolar dilation. Moreover, a tendency to activate channels in the femtomolar range was indicated (Fig. 4B). Both findings suggested that BKCa channel activation is the predominant mechanism for EDP-induced dilations. In contrast, the EC50 values for BKCa channel activation by arachidonate epoxides/diols are orders of magnitude greater than those for dilations (Lu et al., 2001; Zhang et al., 2001), even when the same vessel from the same species is examined (Zou et al., 1996). Such a disparity in EC50 values suggests that activation of potassium channels
other than BKCa channels may mediate the arterial dilations induced by epoxides and diols derived from arachidonic acid (Zhang et al., 2001). Thus, unlike EET vasoactivity, EDP-induced dilations may be mediated exclusively by BKCa channel activation.

However, the molecular mechanisms responsible for EDP-induced dilations are more complex than suggested by simply matching dilation EC50 values with those of BKCa channel activation. Cytoplasmic elements are absent from patches in the inside-out configuration; in such simple plasma membrane preparations, the EDPs seem to act by directly binding to the BKCa channels or their immediate environs. The open probability responses of the coronary channels had Hill slopes of 1.1 (pig) and 1.5 (rat) and indicate that BK channels may possess one or two EDP binding sites. Thus, in the rat small arteries, the EDPs may bind on or close to the a-pores or β-regulatory units of BKCa channels and, by hydrogen bonding with the epoxy ring, induce conformational changes favoring EDP binding to a second site (Lu et al., 2001; Zhang et al., 2001). Future binding studies with cloned BKCa channels and site-directed mutagenesis are needed to specify the precise binding sites.

Surprisingly, arterial dilations by fatty epoxides are characterized by Hill slopes less than 1.0. The Hill slope determined for dilations by 13,14-EDP was 0.429, and the average Hill slope for the five EDP regioisomers was 0.52 ± 0.04 (n = 5). In comparison, the dilation curves of arachidonic acid- and eicosapentaenoic acid-derived epoxides had Hill slopes of 0.32 ± 0.02 (n = 6 enantiomers) and 0.39 ± 0.02 (n = 4), respectively (Zhang et al., 2001). Thus, EDP-dilation curves had Hill slopes that are 33% (p < 0.05) and 62% (p < 0.001) greater than that of arachidonate and eicosapentaenoate epoxides, respectively. Notwithstanding, the Hill slope for arteriolar dilation by 13,14-EDP was only one-third the 1.1 value measured for BKCa channel activation. One possible explanation for the consistently low Hill slopes characterizing arterial dilations is that fatty epoxides interact with G proteins of limited availability in intact vessels, and thus inhibit subsequent EDP binding (Li and Campbell, 1997). Alternatively, the threshold activities of fatty epoxides may result from interactions with several BKCa channel isoforms of varying affinities or with other (non-BKCa) potassium channels. In any case, the fatty epoxide interactions with BKCa channels in whole arterioles seem to involve more than a simple, direct interaction with BKCa channels.

In conclusion, EDPs are cytochrome P-450 epoxygenase metabolites of docosahexaenoic acid, one of the major n-3 fatty acids in fish oils. In the present study, 13,14-EDP activated BKCa channels with an EC50 value of 2.2 to 6.6 pM, which is over 1000 times more potent than observed for epoxides (EETs)/diols (DHETs) derived from arachidonate. To our knowledge, no cyclooxygenase or lipoxygenase metabolite of n-3 fatty acids has been reported to be more active than the corresponding n-6 fatty acid product. As a result of the potent BKCa channel activation by 13,14-EDP, hyperpolarization and relaxation of vascular smooth muscle cells should follow. Consistent with this expectation, 13,14-EDP dilated coronary arterioles with an EC50 value of 2 pM. EDPs are the first docosahexaenoic acid metabolites shown to have potent dilator actions on resistance vessels. Moreover, the EDHF-like actions make it an important goal to determine whether EPDs are synthesized and released from vascular endothelial cells in response to bradykinin and acetylcholine. An endothelial release of EPDs could explain the enhanced endothelium-dependent relaxations and hypotensive effects that accompany dietary supplements of docosahexaenoic acid. In addition, we speculate that an enhanced release of EPDs and EEQs may mediate reductions in myocardial infarctions (Tavani et al., 2001) and ischemia-induced arrhythmias associated with dietary fish oils (Leal, 2001).

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