Docosahexanoic acid-induced Coronary Arterial Dilation: Actions of
17S-hydroxy docosahexanoic acid on K+ Channel Activity

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Abbreviations: DHA, docosahexanoic acid; 17S-HDHA, 17S-hydroxy DHA; PUFA, polyunsaturated fatty acid; EC, endothelial cell; SMC, smooth muscle cell; BKCa, large conductance Ca²⁺-activated K⁺; CDC, cinnamyl-3, 4-dihydroxyl-cyanocinnamate.

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ABSTRACT

Despite extensive studies, the mechanisms mediating the cardiovascular actions of omega (ω)-3 polyunsaturated fatty acids has not yet been fully understood. The present study determined the possible actions of an endothelium-derived lipoxygenase product of docosahexanoic acid (DHA), 17S-hydroxy DHA (17S-HDHA) in bovine coronary arteries. HPLC analysis demonstrated that bovine coronary arterial endothelial cells can metabolize DHA via lipoxygenases, and one of the major products was confirmed to be 17S-HDHA by LC/MS/MS. In isolated perfused small bovine coronary arteries, 17S-HDHA (10⁻⁹-10⁻⁵ M) caused a concentration-dependent dilation with a maximum dilator response of 87.8 ± 2.5%, which is much more potent than the dilator response of its precursor, DHA. Moreover, 17S-HDHA-induced vasodilatations was significantly blocked by iberiotoxin, a large conductance Ca²⁺-activated K⁺ (BKCa) channel blocker, but not altered by an ATP-sensitive K⁺ channel blocker, glibenclamide. In patch clamp whole-cell recording, 17S-HDHA markedly increased K⁺ currents in coronary arterial smooth muscle cells. In the inside-out mode, but not in the cell-attached mode, 17S-HDHA dramatically increased the BKCa channel activity which was substantially blocked by iberiotoxin. Collectively, our findings indicate that 17S-HDHA, an endothelium-derived DHA product via lipoxygenase, activates BKCa channels in coronary arterial smooth muscle cells leading to coronary vasodilation, which may represent an important mechanism mediating the beneficial actions of DHA in coronary circulation.
INTRODUCTION

Numerous epidemiological studies, clinical trials, and animal experiments have demonstrated that fish oils, primarily omega (ω)-3 polyunsaturated fatty acids (PUFAs), protect against several types of cardiovascular diseases such as myocardial infarction, arrhythmia, atherosclerosis, stroke, or hypertension (Rapp, 1991; McLennan P, 1996; Nageswari et al., 1999; Kang and Leaf, 2000; Abeywardena and Head, 2001; De Caterina and Zampolli, 2001; Jeerakathil and Wolf, 2001; Leaf et al., 2003; Holub and Holub, 2004; Harrison and Abhyankar, 2005). Two well-known ω-3 PUFAs present in fish oil are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Connor et al., 1993). Recent studies have indicated that DHA may be a major active component in fish oil conferring cardiovascular protection (Horrocks and Yeo, 1999; Nordoy et al., 2001; Hirafuji et al., 2003). In animal experiments, DHA was found more effective than EPA in retarding the development of hypertension in spontaneously hypertensive rats and in inhibiting thromboxane-like vasoconstrictor responses in the aorta from these rats (McLennan et al., 1996). However, it remains poorly understood how DHA exerts its beneficial action on the cardiovascular system, despite that several possible mechanisms have been suggested such as reduction of plasma triglycerides, inhibition of platelet function, enhancement of cardiac excitability, and anti-inflammation (McLennan et al., 1996; Salem et al., 2001; Simopoulos, 2002).

More recently, DHA has been found to be metabolized via cyclooxygenase, lipoxygenase, and P450 metabolic pathways, which generates a series of 17R or 17S mono-, di-, and
tri-hydroxy DHA and various epoxides (Hong et al., 2003). Some of these DHA products possess potent bioactivity, in particular, being active as anti-inflammatory and immune-regulatory compounds (Hong et al., 2003). Inflammation or microinflammation plays important roles in the development of atherosclerosis, ischemic reperfusion injury and cardiac or vascular remodeling. In this regard, the anti-inflammatory or immune-regulatory effects of DHA and its products have been suggested to contribute to the beneficial actions of ω-3 PUFAs or fish oil on the cardiovascular system (Simopoulos, 2002; Holub and Holub, 2004). However, many classical anti-inflammatory drugs such as commonly used indole and arylpropionic acid derivatives do not have similar cardiovascular protective actions to that observed in DHA treatments. This suggests that some other mechanisms are involved in the action of DHA or ω-3 PUFAs on the cardiovascular system in addition to their anti-inflammatory effects. In this regard, previous studies demonstrated that ω-3 PUFA diet enhanced endothelium-dependent vasodilator response in coronary arteries (Shimokawa and Vanhoutte, 1989; Fleischhauer et al., 1993). Therefore, DHA may exert its beneficial action through an endothelium-dependent mechanism in coronary circulation.

The present study hypothesized that 17S-HDHA, a lipoxygenase product, mediates the endothelium-dependent vasodilator action of DHA in small coronary arteries. To test this hypothesis, we first separated and analyzed the lipoxygenase metabolites of DHA produced in coronary arteries and endothelial cells (ECs). Then, we tested the ability and potency of 17S-HDHA to produce vasodilator response in isolated perfused coronary arteries. We further
determined whether vasodilator response to 17S-HDHA is associated with activation of K\(^+\) channels by using patch clamp technique. Our data indicate that 17S-HDHA is a much more potent vasodilator than DHA, and the vasodilator action of 17S-HDHA is associated with activation of large conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{Ca}\)) channels in coronary arterial smooth muscle cells (SMCs).
METHODS

Video Microscopy of Arterial Reactivity. Isolated pressurized small coronary artery preparation was used to study the vasomotor response to DHA and its metabolites as we described previously (Geiger et al., 2000). Briefly, the internal diameter (ID) of these arteries was measured with a microscopic video recording system composed of a stereomicroscope (Leica MZ8), a charge-coupled device camera (KP-MI AU, Hitachi), a video monitor (VM-1220U, Hitachi), a video measuring apparatus (VIA-170, Boeckeler Instrument), and a video printer (UP890 MD, Sony). The arterial images were also recorded continuously with a videocassette recorder (M-674, Toshiba). Before testing any compounds, the cannulated artery was equilibrated for 1–1.5 hours and then precontracted to 40-60% of their resting diameter with a thromboxane A2 analogue, U46619 (2-20 nM). Once steady-state contraction was obtained, cumulative concentration response to 17S-HDHA (10⁻⁹-10⁻⁵ M, kindly provided by Serhan CS, Boston, MA) or DHA (10⁻⁹-10⁻⁵ M, Cayman Chemical, Ann Arbor, MI) were determined by measuring changes in the ID. The vasodilator response was expressed as percent relaxation relative to U46619-induced pre-contraction as calculated in the following formula: percent relaxation = (ID_{dilator}-ID_{U46619})/(ID_{basal}-ID_{U46619}), where ID_{basal} means the arterial ID under resting condition; ID_{U46619} indicates the ID of U46619-precontracted arteries; ID_{dilator} means the arterial ID obtained during treatment of U46619-pre-contracted arteries with compounds tested. Between pharmacological interventions, the arteries were washed three times with PSS and allowed to equilibrate in drug-free PSS for 20-30 min. The arteries were treated with
corresponding inhibitors for 20 min and then concentration-response curves to 17S-HDHA were re-determined. To prepare endothelium-denuded small coronary arteries, the rings of arteries were passed through its lumen by a segment of hair. The endothelium was removed by gently rotating the hair (Ge et al., 2003).

**Extraction and Analysis of DHA Metabolites.** DHA metabolites were analyzed from the reaction mixtures of arterial or cell homogenates with DHA. The homogenates of small coronary arteries were prepared as we reported previously (Geiger et al., 2000). The reactions were performed by incubating 200 μg protein of homogenates prepared from the arteries, ECs or SMCs, with DHA (100 μM, 15 min at 37°C) with a final volume of 200 μL. The incubation was stopped upon addition of 2 ml methanol which was pre-chilled to -40°C and placed at -40°C for 30 min. Then, the reaction mixtures were mixed with an internal standard of 15S-HETE (1 ng/μl), centrifuged at 3000 rpm for 15 min at 4°C, and then acidified to pH 3.5 with HCl. The extraction manifold was set up using a vacuum apparatus and Extract-Clean/RC500 mg C18 Cartridges (Alltech) (one cartridge per sample) and washed with methanol and distilled H2O. The samples ran through the cartridges followed by washing of distilled H2O and hexane. The fraction was then collected following addition of 8 mL methyl formate through the respective cartridges. The samples containing lipid extracts were dried and re-dissolved in methanol and ready for analysis. The identity of DHA metabolite in the extracts was confirmed by using high performance liquid chromatography coupled with a photodiode-array detector and a tandem mass spectrometer (LC-PDA-MS-MS, ThermoFinnigan, San Jose, CA).
In HPLC analysis, C$^{14}$-labeled DHA (1 µCi) was used for each reaction. The extracts obtained as described above were dissolved in acetonitrile and run on a reversed-phase high performance liquid chromatographic system on a nucleosil C$_{18}$ column (5 micrometer, 4.6 x 250 mm, Phenomenex). The solvent program consisted of solvent A that was distilled water and solvent B that consisted of acetonitrile containing 1% glacial acetic acid. A linear gradient from 50% solvent B to 100% solvent B in solvent A over 40 minutes was used at a flow rate of 1 mL/min. The discharge was collected in 0.2-mL fractions and mixed with scintillation fluid, and then the radioactivity was measured by liquid scintillation spectrometry to obtain a profile of radioactive metabolites.

**Western Blot Analysis.** Forty µg of proteins (homogenates) prepared from coronary arteries, ECs and SMCs were boiled, separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked by 5% non-fat dry milk solution and incubated with polyclonal antibody against 15-lipoxygenase (kindly provided by Campbell WB, Milwaukee, WI) for overnight at 4°C. After washing 3 times with TBS-T, the blots were incubated for 1 h with 1:4000 horseradish peroxidase-labeled goat anti-rabbit IgG and developed using enhanced chemiluminescence detection system (ECL, Amersham, IL).

**Patch clamp recording.** K$^+$ currents were recorded using the patch-clamp technique according to the procedures described previously (Hamill et al., 1981; Gebremedhin et al., 1992; Li and Campbell, 1997). Freshly isolated coronary arterial SMCs were prepared for patch-clamp studies as we described previously (Li and Campbell, 1997). The bath solutions used for whole-cell
recording mode contained (in mM) NaCl, 140; KCl, 4; CaCl$_2$, 2; MgCl$_2$, 1; HEPES, 10; glucose, 10 (pH 7.4), and the pipette solution contained (in mM) L-glutamic acid, 145; MgCl$_2$, 1; HEPES, 10; EGTA (K$^+$), 1; Na$_2$ATP, 1; NaGTP, 0.5; and ionized Ca$^{2+}$, 100 nM (pH 7.2). The bath solutions used for single channel recordings in the cell-attached mode contained (in mM) KCl, 145; MgCl$_2$, 1; EGTA (K$^+$), 1; HEPES, 10; and ionized Ca$^{2+}$, 100 nM (pH 7.4). The pipette solution contained (in mM): KCl, 145; CaCl$_2$, 1.8; MgCl$_2$, 1; and HEPES, 5 (pH 7.4). The bath solutions used in the inside-out recording mode contained (in mM) KCl, 145; MgCl$_2$, 1; HEPES, 5; and ionized Ca$^{2+}$, 100 nM (pH 7.2); and the pipette solution contained (in mM) KCl, 1.8; MgCl$_2$, 1; and HEPES, 5 (pH 7.4). All the solutions were stored at 4°C until used.

Patch pipettes were made from borosilicate glass capillary tubing that were pulled with a two-stage micropipette puller (PC-87, Sutter) and heat-polished with a microforge (MF-90, Narishige). The pipettes had tip resistances of 2 to 5 MΩ for whole cell recordings when filled with pipette solution. SMCs were placed in a 1-mL perfusion chamber mounted on the stage of a Nikon inverted microscope. A high resistance seal (5 to 10 GΩ) was formed between the pipette tip and the cell membrane by applying a light suction. The activity of K$^+$ channels in the membrane spanning the pipette tip was recorded. These measurements represented the cell-attached mode. To establish a whole-cell recording configuration, a large pulse of suction was used to rupture the membrane within the pipette, creating a direct continuity between the pipette solution and cytoplasmic contents of the cell. Inside-out membrane patches were excised by lifting the pipette membrane complex to the air/solution interface. Channel currents were
recorded using voltage-clamping mode of a List EPC-7 patch-clamp amplifier (List Biological Laboratories, Inc). For a whole-cell pulse protocol, the patched cell was stimulated by voltage pulses from -60 to +60 mV in steps of 10 mV. Cell capacitance (Cm) was estimated in each cell by integrating capacitative currents generated by 10-mV hyperpolarizing pulses after electronic cancellation of the pipette-patch capacitance (pF). Peak K⁺ current amplitudes were expressed in picoamperes per picofarad (pA/pF) to normalize for differences in cell membrane area between isolated bovine coronary arterial SMCs. Here we kept cells with capacitance as 17.2 ± 0.7 pF. For the cell-attached or inside-out recording configurations, 3-min recordings were obtained at a membrane potential of -40 mV before or after treatments. Single channel conductance (pS) was calculated as amplitude (pA) divided by holding potential (mV). Data were digitized, acquired and stored using a Micron Pentium III computer and Axon pClamp software (8.1).

The effects of 17S-HDHA on the BKCa channel activity were first determined with whole-cell recording mode. After a whole-cell patch was established, control recordings were obtained, and then the bath solution was changed to contain 17S-HDHA and a second pulse recording at each concentration was obtained. To further determine the effects of these 17S-HDHA-induced activation of the K⁺ channels, patch-clamp recordings were performed in the cell-attached and inside-out patch mode. A 3-min control recording was obtained at a membrane potential of -40 mV. The bath solution was then exchanged as described above in recording channel activity by whole-cell patch mode, and then a second successive 3-min recording was obtained at each
concentration. In another group of cells, iberiotoxin (100 nM) was added into the pipette solution and the same recording protocol was repeated.

Average channel activity (NPo) in patches was determined from the following equation (1):

$$NPo = \left( \sum_{j=1}^{N} t_{jj} \right) / T$$  \hspace{1cm} (1)

where N is the maximal number of channels observed in conditions producing high levels of Po, T is the duration of the recording, and t\(_j\) is the time with j=1,2 ... N channels opening.

**Statistics.** Data are presented as mean ± S.E.M.; n represents the number of independent experiments. The significant differences in mean values between and within multiple groups were examined using an analysis of variance for repeated measures followed by a Duncan's multiple range test. \(P < 0.05\) was considered statistically significant.
RESULTS

Metabolism of DHA in Coronary Arteries, ECs and SMCs. We first determined whether small coronary arteries produce hydroxy metabolites of DHA via lipoxygenase pathway. In this regard, bovine coronary arterial homogenate were incubated with $^{14}$C-DHA in the presence and absence of the lipoxygenase inhibitor, cinnamyl-3, 4-dihydroxyl-cyanocinnamate (CDC) (10 μM, both 15- and 5-lipoxygenase are inhibited), and the DHA metabolites in the reaction mixture were extracted followed by separation and analysis by HPLC. Fig. 1A presents typical HPLC chromatograms depicting the hydroxy metabolites of $^{14}$C-DHA with a radioactive peak at 2.5 minutes. Inhibition of lipoxygenase by CDC markedly reduced the peak of DHA metabolites. As summarized in Fig. 1B, the conversion of DHA into hydroxy metabolites was significantly blocked by CDC but not by cyclooxygenase inhibitor, indomethacin (100 μM). Similar to indomethacin, P450 monooxygnase inhibitors, miconazole (10 μM) or 17-octadecynoic acid (1 μM) had no effect on such metabolism of DHA (data not shown). In addition, the hydroxy metabolites of DHA were also detected in homogenates from coronary arterial ECs and SMCs (Fig. 1C). The conversion rate of DHA into hydroxy metabolites was 3 folds higher in ECs than in SMCs (Fig. 1C). 15-lipoxygenase has been shown to hydrolyze the 17S position of DHA to form 17S-HDHA (Hong et al., 2003). As indicated by Western blot analysis, 15-lipoxygenase, has higher abundance in ECs than in SMCs (Fig. 1D), suggesting that the high conversion rate of DHA into hydroxy metabolites in ECs is associated with the higher abundance of 15-lipoxygenase in these cells.
LC/MS Spectrometric Analysis of DHA Metabolites. Fig. 2A shows a typical LC chromatogram of 17S-HDHA obtained from LC/MS analysis, which demonstrates that 17S-HDHA had the largest peak at a retention time of 21 minute. Fig. 2B presents a MS spectrum and structure of 17S-HDHA. The identity of 17S-HDHA was confirmed by ions at m/z of 201 (245-CO2), 229 (273-CO2), 245, 255 (273-H2O), 273, 281 (M-H-H2O-CO2), 299 (M-H-CO2), 325 (M-H-H2O), and 343, which was then further confirmed by MS/MS analysis. Our HPLC and LC/MS analyses further identified that 17S-HDHA is the primary form of hydroxy metabolites, however, other 17S series hydroxy metabolites of DHA including di- and tri-HDHA are also detected (data not shown). 5-lipoxygenase metabolizes 17S-HDHA into di- and tri-HDHA (Hong et al., 2003). Thus, our data indicate that endogenous 15-lipoxygenase and 5-lipoxygenase in bovine coronary arteries are able to metabolize DHA into 17S series HDHA.

Vasodilator Responses to 17S-HDHA and DHA in Small Coronary Arteries. We next test whether DHA or 17S-HDHA causes coronary vasodilation. As shown in Fig. 3A, in isolated precontracted small bovine coronary arteries, DHA induced vasodilator response at relatively high concentration (10⁻⁵ M) with a maximal dilation of 56.9 ± 6.2%. Compared to its precursor DHA, 17S-HDHA induced much stronger vasodilator response with a maximal dilation of 87.8 ± 2.5% at a concentration of 10⁻⁵ M. Moreover, 17S-HDHA at 10⁻⁷ M already induced obvious dilator response (42.9 ± 4.9%), whereas DHA had only trivial dilator response (11.9 ± 2.5%) at the same dose. To quantitate the potency of dilator responses for 17S-HDHA and DHA, we calculated the concentration required to induce 50% dilation (D₅₀) and found that the 17S-HDHA
(D_{50} = 2.0 \times 10^{-7} \text{ M}) is 31-fold more potent than DHA (D_{50} = 6.3 \times 10^{-6} \text{ M}). As shown in Fig. 3B, inhibition of lipoxygenase by CDC significantly reduced DHA-induced vasodilation. Further, removal of the endothelium abolished DHA-induced vasodilation in these coronary arteries.

**Involvement of K^+ Channels in 17S-HDHA-induced Vasodilation.** We further examined whether 17S-HDHA-induced vasodilation is associated with K^+ channel activation. In this regard, we first investigated the effect of the combination of several K^+ channel inhibitors on 17-HDHA-induced coronary vasodilation. It was found that treatment of bovine coronary arteries with a cocktail of inhibitors including iberiotoxin (100 nM), 4-aminopyridine (5 mM) and glibenclamide (100 µM) markedly attenuated 17S-HDHA-induced vasodilation with maximum dilation of 24.7 ± 3.4% at 10^{-5} \text{ M} (n = 4, data not shown). Then, different K^+ channel blockers were used separately to determine the specific subtypes of K^+ channels that are involved in the vasodilator response of 17S-HDHA. As shown in Fig. 4A, the BK_{Ca} channel inhibitor, iberiotoxin (100 nM) significantly attenuated 17S-HDHA-induced maximal vasodilation by 46%. 4-aminopyridine (5 mM), an inhibitor of voltage-gated K^+ channels, also significantly attenuated 17S-HDHA-induced maximal vasodilation by 50% (Fig. 4B). However, the ATP-sensitive K^+ channel blocker, glibenclamide (100 µM) had no effect (Fig. 4C).

**Effects of 17S-HDHA on K^+ Channel Activity Recorded by a Whole Cell Patch Clamp Configuration.** To further demonstrate the involvement of BK_{Ca} channels in 17S-HDHA-induced vasodilation, patch clamp experiments were performed to examine whether this DHA metabolite alters BK_{Ca} channel currents. Bovine coronary smooth muscle cells were
freshly dissociated and transferred to the patch-clamp chamber with a bath solution for whole cell current recording. Fig. 5A shows the recordings of outward K⁺ channel current from SMCs by whole cell configuration. In this cell, 17S-HDHA (10⁻⁶ M) increased K⁺ current by 130%. Outward K⁺ channel currents (pA) were normalized to the averaged cell capacitance (17.2 ± 0.7 pF). Summarized current density-voltage relationship is presented in Fig. 5B. In contrast, DHA at the same concentration did not increase the K⁺ current recorded by whole cell configuration. The currents at different ramp voltage were very similar in bovine arterial SMCs with and without administration of 10⁻⁶ M DHA (Fig. 6A). As summarized in Fig. 6B, administration of DHA (10⁻⁶ M) had no effect on the current density–voltage relationship in these cells.

**Effects of 17S-HDHA on Single BKCa Channel Activity in Cell-attached Patches.** To further examine the mechanism of 17S-HDHA-induced activation of BKCa channels, we performed single channel recordings in the cell-attached mode in SMCs. Fig. 7A shows representative recordings of single BKCa channels in cell-attached mode under control condition and treated with 10⁻⁶ M 17S-HDHA. The BKCa channel conductance was 250.7 pS and opening amplitude was 10 pA with clamped voltage at -40 mV. 17S-HDHA (10⁻⁶ M) slightly increased the channel open probability (NPo) of these BKCa channels. Fig. 7B summarizes the effect of 17S-HDHA on the NPo of BKCa channels. Under control condition, the NPo of this K⁺ channel was 0.0067 ± 0.0029. 17S-HDHA (10⁻⁸-10⁻⁶ M) caused a slight, but not significant increase in the NPo of BKCa channels.

**Effects of 17S-HDHA on Single BKCa Channel Activity in Inside-out Patches.** Fig. 8A shows
representative recordings of BK$_{\text{Ca}}$ channels in an inside-out patch of SMCs in the absence of cytosolic ATP and GTP. Administration of 17S-HDHA (10$^{-7}$ M) into the bath solution, the cytosolic face of the patch, markedly increased BK$_{\text{Ca}}$ channel openings. As summarized in Fig. 8B, 17S-HDHA (10$^{-9}$-10$^{-7}$ M) significantly increased the NPo of these K$^+$ channels in a concentration-dependent manner. This increase in the NPo of BK$_{\text{Ca}}$ channels by 17S-HDHA (10$^{-7}$ M) was almost completely eliminated by iberiotoxin (100 nM) in the patch pipette.
DISCUSSION

The present study reported that 17S-HDHA, a lipoxygenase product of DHA, is produced in coronary arteries and that 17S-HDHA possesses much more potent vasodilator action than DHA in isolated, perfused and pressurized small coronary arteries. The potent vasodilator effect of this DHA metabolite is associated with activation of BKCa channel in coronary arterial SMCs. These findings support the view that DHA may exert its cardioprotective effects through the regulation of vasomotor responses via its metabolites in coronary circulation.

In previous studies, exogenous 15-lipoxygenase was reported to hydrolyze the 17S position of DHA to form 17R- or 17S-HDHA (mono hydroxy), which can be further converted by 5-lipoxygenase into 17S series hydroxy DHA including di- or tri-hydroxy DHA (Hong et al., 2003). By HPLC and LC/MS/MS analysis, we found that DHA in coronary arterial homogenates was mainly converted into 17S series hydroxy DHA including 17S-HDHA (mono hydroxy), di- and tri-hydroxy HDHA and that 17S-HDHA is the major form of this series hydroxy DHA produced by coronary arteries using DHA as substrate. It was also demonstrated that the metabolism of DHA was blocked by lipoxygenases inhibitor CDC, but not by cyclooxygenase inhibitor indomethacin, suggesting that 15- and 5-lipoxygenase synergistically convert DHA into its hydroxy metabolites in small bovine coronary arteries. Consistently, we showed that inhibition of lipoxygenases by CDC markedly attenuated DHA-induced dilation in small bovine coronary arteries, suggesting the dilator action of DHA is primarily mediated through its hydroxy
metabolites. The expression level of 15-lipoxygenase and the conversion rate of DHA into 17S-HDHA are much higher in ECs than in SMCs. We further demonstrated that endothelial removal abolished DHA-induced dilation in small bovine coronary arteries. Therefore, these data indicate that DHA is mainly metabolized into 17S-HDHA in the endothelium rather than smooth muscle of coronary arteries.

To our knowledge, the present study for the first time demonstrated that 17S-HDHA possesses much more potent vasodilator effect than its precursor, DHA, in small bovine coronary arteries. In this regard, several previous studies tested the potency of DHA in dilating porcine or rat coronary arterioles, human coronary arteries or rat aortic rings (Engler and Engler, 2000; Ye et al., 2002; Wu et al., 2007). However, these authors found that DHA significantly induced dilation only when high concentration of DHA was used (typically greater than $10^{-5}$ M). We found here that 17S-HDHA induced potent dilation in small bovine coronary arteries at a low concentration of $10^{-7}$ M, whereas DHA only weakly dilated these arteries at the same concentration. The precise mechanism responsible for this differential vasodilator action between 17S-HDHA and DHA is unknown. The chemical structure of DHA shares similarity with arachidonic acid (AA), which are metabolized into potent vasodilators 14, 15-EET and 14, 15-DHET by cytochrome P-450 in coronary arteries (Campbell et al., 2002). Because these AA metabolites are structurally related with 17S-HDHA, it is plausible that they share some common structural elements required for their vasodilator activity. Falck JR et al. have suggested that the 8, 9-double bond and 14, 15-epoxide/14, 15-dihydroxy group are essential elements for the
vasodilator activity of 14, 15-EET/14, 15-DHETs. The 8, 9-double bond may be involved in p-p bonding with aromatic amino acids of potential receptors (Gauthier et al., 2002; Falck et al., 2003), while the oxygen atom(s) of the epoxide or di-hydroxy group may act as acceptor(s) for hydrogen bonding with receptors of 14, 15-EET/14, 15-DHETs. Based on these findings, we hypothesized that 10, 11-double bond and 16, 17-dihydroxy group are essential structural requirements for the potent vasodilator action of the hydroxy metabolites of DHA. Hydroxylation at carbon 17 of DHA molecule may increase hydrogen bonding with the potential receptor and thereby enhance the vasodilator activity. This may explain why 17S-HDHA exerts much more potent vasodilator effect than DHA.

Regulation of smooth muscle membrane potential through alteration in $K^+$ channel activity is a major mechanism of vasodilation and vasoconstriction under both physiological and pathophysiological conditions (Yokoshiki et al., 1998; Toro et al., 2002; Jackson, 2005). Activation of $K^+$ channels results in hyperpolarization, closure of voltage-dependent $Ca^{2+}$ channels, and vasodilation, whereas inhibition of $K^+$ channels has the opposite effects. Several different types of $K^+$ channels including $BK_{Ca}$ channel, $K_v$ channel and $K_{ATP}$ channel are present in bovine coronary SMCs and involved in the regulation of vascular tone or vasomotor responses of coronary arteries (Yokoshiki et al., 1998; Toro et al., 2002; Jackson, 2005). In the present study, we found that both the $BK_{Ca}$ blocker, iberiotoxin, and the $K_v$ channel blocker, 4-aminopyridine, significantly attenuated 17S-HDHA-induced vasodilation, whereas the $K_{ATP}$
channel blocker, glibenclamide, had no effect. These data suggest that activation of BK_{Ca} and Kv channels may contribute to 17S-HDHA-induced vasodilation in coronary arteries.

To further determine the electrophysiological mechanism mediating actions of 17S-HDHA, we performed patch clamp experiments with a focus on the BK_{Ca} channel in the membrane of coronary arterial SMCs. In whole cell recording mode, 17S-HDHA (10^{-6} \text{ M}) markedly increased K^{+} currents in these SMCs. In contrast, DHA (10^{-6} \text{ M}) had no effect on the K^{+} currents, suggesting that DHA at such dose does not activate BK_{Ca} channels in bovine coronary SMCs. It seems that these results are inconsistent with a recent study demonstrating that DHA activates BK_{Ca} channels in rat coronary arterial SMCs (Lai et al., 2009). In rat SMCs, however, the increase in BK_{Ca} currents was only observed when high concentrations of DHA (2.0 \times 10^{-5} \text{ M} \text{ to } 8.0 \times 10^{-5} \text{ M}) were used (Lai et al., 2009). It is possible that 17S-HDHA and high concentration of DHA may activate BK_{Ca} channels via distinct mechanisms. 17S-HDHA-induced activation of BK_{Ca} channels appears to be specific to the structure of 17S-HDHA as shown in EET studies (Campbell et al., 2002; Gauthier et al., 2002; Falck et al., 2003). 17S-HDHA may directly interact with and change the conformation of BK_{Ca} channels leading to increased channel activity. As reported by Thid et al. (Thid et al., 2007), nevertheless, DHA at high concentrations (greater than 6.0 \times 10^{-5} \text{ M}) dramatically changed the viscoelastic properties of the phosphocholine-supported lipid bilayers. Thus, DHA at high concentrations are more likely to exert its effects as a non-specific lipid compound that modifies biophysical properties of membranes, which results in alteration of BK_{Ca} channel activities.
Previous studies demonstrated that the direct action of $G_{\alpha}$ on the $BK_{Ca}$ channels mediates the effects of 11, 12-EET, an important endothelium-derived hyperpolarization factor (Brown and Birnbaumer, 1990; McDonald et al., 1994; Li and Campbell, 1997). This membrane-delimited action of $G_{\alpha}$ is a ubiquitous mechanism for regulating $K^+$ and $Ca^{2+}$ channels, and requires the presence of GTP (Brown and Birnbaumer, 1990; McDonald et al., 1994). However, our data suggest that a direct activation of G-proteins is not involved in 17S-HDHA-induced $BK_{Ca}$ channel activation. First, in the cell-attached mode, 17S-HDHA had no significant effects on $BK_{Ca}$ channel activity, suggesting that 17S-HDHA does not activate $BK_{Ca}$ channel through intracellular coupling mechanisms. This result suggests a membrane-delimited action of 17S-HDHA on $BK_{Ca}$ channel activity. In excised inside-out patches, 17S-HDHA markedly increased $BK_{Ca}$ channel activity when it was added to the cytoplasmic side of membranes even in the absence of GTP or ATP. This suggests that activation of $BK_{Ca}$ channels by 17S-HDHA does not require any intracellular soluble components, including GTP and ATP. These data rule out the possibility of G-protein coupled mechanism in mediation of 17S-HDHA action. It seems that a membrane-delimited, but G-protein-independent mechanism mediates 17S-HDHA-induced activation of $BK_{Ca}$ channels.

It should be noted that 17S-HDHA also induced a small residual dilation in the presence of a combination of $K^+$ channel inhibitors (i.e. iberiotoxin, 4-aminopyridine and glibenclamide) in bovine small coronary arteries with maximum dilation of $24.7 \pm 3.4\%$ at $10^{-5}$ M (Figure 3B).
This residual dilator response may be attributed to the dilator effects of 17S-HDHA metabolites. It has been shown that 17S-HDHA can be further converted by 5-lipoxygenase into 17S series hydroxy DHA including di- or tri-hydroxy DHA (Hong et al., 2003). It is possible that these 17S-HDHA metabolites (converted by lipoxygenases in the intact endothelium) dilate coronary arteries via K⁺ channel-independent mechanisms. Indeed, we found that 17S tri-hydroxy DHA is also a potent vasodilator in bovine small coronary arteries with a maximal dilation of 59.1 ± 5.5% at 10⁻⁷ M (unpublished data). However, the mechanisms responsible for the dilator effect of 17S tri-hydroxy DHA are unknown and need to be further investigated in future studies.

In summary, the present study demonstrated that 17S-HDHA is a novel and potent vasodilator in coronary arteries. 17S-HDHA activates BKCa channels in SMCs from these arteries via a membrane-delimited mechanism by direct action of 17S-HDHA on channel proteins or their regulatory proteins. Since 17S-HDHA possesses more potent vasodilator effect than DHA itself, this DHA metabolite may serve as an endothelium-derived hyperpolarization factor to regulate vascular tone in coronary circulation and to at least partially mediate the beneficial actions of DHA or ω-3 PUFAs in prevention and treatments of cardiovascular diseases, in particular coronary arterial diseases.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: X. Li, P. L. Li, Zhang

Conducted experiments: X. Li, Hong, Zhang

Performed data analysis: X. Li, Hong, P. L. Li, Zhang

Wrote or contributed to the writing of the manuscript: X. Li, P. L. Li, Zhang
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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1  Metabolism of DHA in coronary arteries. $^{14}$C-DHA was incubated with homogenates of bovine coronary arteries for 15 min at 37°C in the presence or absence of indicated inhibitors. Then DHA and its metabolites in the reaction mixtures were extracted and applied for HPLC analysis. A, typical HPLC chromatograms depicting the metabolism of $^{14}$C-DHA with (Control, left panel) or without addition of lipoxygenase inhibitor, CDC (10 μM, right panel) in coronary arterial tissue homogenates. Metabolites of $^{14}$C-DHA were eluted at peak of 2.5 minutes and $^{14}$C-DHA was eluted at a peak of 33 minutes. B, summarized data showing radioactivity of $^{14}$C-DHA metabolites in the reaction mixtures in the absence or presence of cyclooxygenase inhibitor, indomethacin (Indo, 100 μM) or CDC (10 μM) (n = 4). C, summarized data showing the conversion rates of DHA to its metabolites in homogenates of coronary arterial tissue (Control), endothelial cells (EC) and smooth muscle cells (SMC) (n = 5). D, representative Western blot gel document showing 15-lipoxygenase (15-LO) expression in homogenates of coronary arterial tissue (Control) and their cells (SMC and EC). * $P < 0.05$, significant difference compared with the values from control samples.

Fig. 2  LC/MS analysis of DHA metabolites. (A) Typical LC chromatogram of 17S-HDHA from LC/MS analysis showing that 17S-HDHA had a peak at a retention time of 21 minutes. (B) Typical MS spectrum of lipidomic MS/MS analysis of DHA metabolites isolated
from bovine coronary arteries. Inset: chemical structure of 17S-HDHA as reported previously (Hong et al., 2003). 17S-HDHA was identified on the base of the diagnostic MS-MS ions at m/z of 201 (245-CO$_2$), 229 (273-CO$_2$), 245, 255 (273-H$_2$O), 273, 281 (M-H-H$_2$O-CO$_2$), 299 (M-H-CO$_2$), 325 (M-H-H$_2$O), and 343 (M-H).

Fig. 3 A, DHA and 17S-HDHA induce vasodilation of bovine small coronary arteries. * $P < 0.05$, significant difference compared with the values from the arteries treated with DHA (n = 6). B, effects of CDC (10 μM) and endothelium removal (EC denuded) on DHA-induced vasodilation in bovine small coronary arteries. # $P < 0.05$, significant difference compared with the values from the endothelium intact arteries treated with DHA (n = 6).

Fig. 4 Effects of BK$_{Ca}$ channel blocker, iberiotoxin (IBTX, 100 nM) (A), Kv channel blocker, 4-aminopyridine (5 mM) (B) and K$_{ATP}$ channel blocker, Glibenclamide (100 μM) (C) on 17S-HDHA-induced vasodilation in bovine small coronary arteries (n ≥ 4). * $P < 0.05$, significant difference compared with the values from the control arteries that are treated with 17S-HDHA only.

Fig. 5 Effect of 17S-HDHA on whole cell K$^+$ channel currents in bovine coronary SMCs. A, representative recordings of outward K$^+$ currents in one SMC under control conditions, or treated with 10$^{-6}$ M 17S-HDHA. B, summarized K$^+$ current densities activated by voltage
showing that 17S-HDHA (10^{-6} \text{ M}) induced increases in K^{+} currents. * P < 0.05, significant difference compared with the values from vehicle-treated bovine coronary SMCs.

**Fig. 6** Effect of DHA on whole cell K^{+} channel currents in bovine coronary SMCs. A, representative recordings of outward K^{+} currents in one bovine coronary SMC under control conditions, or treated with 10^{-6} \text{ M} DHA. B, summarized K^{+} current densities activated by voltage showing that DHA (10^{-6} \text{ M}) did not increase K^{+} currents (n = 6).

**Fig. 7** Effects of 17S-HDHA on single BK_{Ca} channel activity in the cell-attached mode. A, representative recordings of the BK_{Ca} channels in bovine coronary SMCs under control conditions or treated with 17S-HDHA (10^{-6} \text{ M}) at voltage of -40 \text{ mV} (o: open; c: close). B, summarized data showing the effects of 17S-HDHA (10^{-8}-10^{-6} \text{ M}) on NPo of BK_{Ca} channels (n = 6).

**Fig. 8** Effects of 17S-HDHA on single BK_{Ca} channel activity in inside-out patches. A, representative recordings of the BK_{Ca} channels in bovine coronary SMCs under control conditions or treated with 17S-HDHA (10^{-7} \text{ M}) at voltage of -40 \text{ mV} (o: open; c: close). B, summarized data showing the effects of 17S-HDHA on NPo of the BK_{Ca} channels in the absence or presence of iberiotoxin (IBTX, 100 \text{ nM}) in the patch pipette (n = 6). * P <0.05, significant difference compared with the values from vehicle-treated patches; # P
< 0.05, significant difference compared with the values from patches treated with 10^{-7} M 17S-HDHA.
Figure 1

A  Control  

Radioactivity (CPM)  

Time (min)  

14C-DHA metabolites  

CDC  

Radioactivity (CPM)  

Time (min)  

14C-DHA metabolites  

B  

Radioactivity of 14C-DHA metabolites (CPM)  

Control  Indo  CDC  

C  

Conversion rate of DHA into hydroxy metabolites (pmol/min/µg protein)  

Control  SMC  EC  

D  

15-LO  76kDa  

Control  SMC  EC
Figure 3

A

![Graph A showing %Dilation vs. Log M for 17S-HDHA and DHA](image)

B

![Graph B showing %Dilation vs. Log M for Control, CDC, and EC denuded](image)
Figure 5

A

![Graph A: Signal (pA) vs. Time (ms)]

- Vehicle
  - +60 mV
  - -60 mV

B

![Graph B: Current density (pA/pF) vs. Voltage (mV)]

- Vehicle
- 17S-HDHA

* indicates significance.
**Figure 8**

**A** Vehicle Inside-Out

![Graph A]

**B**

![Graph B]

- **NP** \(_0\) in inside-out patches
- **17S-HDHA** [Log M]
- **Vehicle**, **-9**, **-8**, **-7**, **IBTX**