

Original Article

Effects of docosahexaenoic acid on large-conductance Ca^{2+} -activated K^+ channels and voltage-dependent K^+ channels in rat coronary artery smooth muscle cells

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Aim: To investigate the effects of docosahexaenoic acid (DHA) on large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels and voltage-dependent K^+ (K_{V}) channels in rat coronary artery smooth muscle cells (CASMCS).

Methods: Rat CASMCs were isolated by an enzyme digestion method. BK_{Ca} and K_{V} currents in individual CASMCs were recorded by the patch-clamp technique in a whole-cell configuration at room temperature. Effects of DHA on BK_{Ca} and K_{V} channels were observed when it was applied at 10, 20, 30, 40, 50, 60, 70, and 80 $\mu\text{mol/L}$.

Results: When DHA concentrations were greater than 10 $\mu\text{mol/L}$, BK_{Ca} currents increased in a dose-dependent manner. At a testing potential of +80 mV, 6.1%±0.3%, 76.5%±3.8%, 120.6%±5.5%, 248.0%±12.3%, 348.7%±17.3%, 374.2%±18.7%, 432.2%±21.6%, and 443.1%±22.1% of BK_{Ca} currents were increased at the above concentrations, respectively. The half-effective concentration (EC_{50}) of DHA on BK_{Ca} currents was 37.53±1.65 $\mu\text{mol/L}$. When DHA concentrations were greater than 20 $\mu\text{mol/L}$, K_{V} currents were gradually blocked by increasing concentrations of DHA. At a testing potential of +50 mV, 0.40%±0.02%, 1.37%±0.06%, 11.80%±0.59%, 26.50%±1.75%, 56.50%±2.89%, 73.30%±3.66%, 79.70%±3.94%, and 78.1%±3.91% of K_{V} currents were blocked at the different concentrations listed above, respectively. The EC_{50} of DHA on K_{V} currents was 44.20±0.63 $\mu\text{mol/L}$.

Conclusions: DHA can activate BK_{Ca} channels and block K_{V} channels in rat CASMCs, and the EC_{50} of DHA for BK_{Ca} channels is lower than that for K_{V} channels; these findings indicate that the vasorelaxation effects of DHA on vascular smooth muscle cells are mainly due to its activation of BK_{Ca} channels.

Keywords: smooth muscle cell; large conductance Ca^{2+} -activated K^+ channel; voltage-gated K^+ channel; docosahexaenoic acid; patch-clamp techniques

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Introduction

ω -3 Polyunsaturated fatty acids (ω -3 PUFAs) consist mainly of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Recently, more attention has been paid to the beneficial effects of ω -3 PUFAs on risk reduction of cardiovascular diseases, especially with regard to vasodilatation, improvement of coronary artery blood flow, anti-arrhythmia, disease prevention, lowering of blood pressure, and other parameters^[1,2]. However, most of the studies mentioned above were performed in clinical practice, and

the molecular mechanisms underlying the cardioprotective effects of ω -3 PUFAs are not fully understood. Studies have shown that dietary DHA in spontaneously hypertensive rats lowers blood pressure and reduces angiotensin II-mediated vasoconstriction. However, the ionic mechanisms driving these effects remain controversial^[3,4].

It has been reported that there are at least four types of potassium channel in vascular smooth muscle cells (VSMCs): the calcium-activated potassium channel (K_{Ca}), the voltage-dependent potassium channel (K_{V}), the ATP-sensitive potassium channel (K_{ATP}), and the inward rectifier potassium channel (K_{ir}). These channels play important roles in cell membrane potential repolarization, resting potential maintenance, cell excitability, and the adjustment

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of VSMC contraction and relaxation. The channels also have important effects on vascular tone. K_V channels are widely distributed in VSMCs, and they regulate the contraction and relaxation of VSMCs through effects on membrane potential and voltage-gated Ca^{2+} channel activity^[5]. BK_{Ca} channels are most plentiful in VSMCs. Due to their large conductance and high density in VSMCs, BK_{Ca} channels are very important in controlling resting membrane potential and vascular tone^[6-8]. It has been reported that the arachidonic acid (AA) pathway and the DHA metabolizer of cytochrome P450 (CYP450) pathway are potent vasodilators that directly activate BK_{Ca} channels in the rat CASMC^[9]. Most importantly, the BK_{Ca} channel was more sensitive to n-3 eposide than to n-6 eposide. Hence, the DHA metabolites of CYP450 are known to be the most potent vascular BK_{Ca} channel activators and vasodilators thus far recognized. It has also been shown that the effects of DHA on K_V channels are controversial in different kinds of cell, including ventricular myocytes, neurons, and vascular smooth muscle cells (VSMCs)^[10, 11]. In this study, we investigated whether DHA had a direct effect on BK_{Ca} channels or other vascular K^+ channels. We examined the effects of DHA on BK_{Ca} channels and voltage-dependent K^+ (K_V) channels from freshly isolated CASMCs of rats, using whole-cell patch-clamp recordings to study the mechanism for DHA-induced vasodilatation.

Materials and methods

Major experimental instruments The instruments included MultiClamp 700B patch clamp amplifier (Axon Instruments, USA), D/A and A/D converter (DigiData 1322, Axon Instruments, USA), Pclamp9.0 pulse software (Axon Instruments, USA), MP-285 motorized micromanipulator (Sutter Instruments, USA), IX71 inverted microscope (Olympus, Japan), SA-OLY/2 and DH-35 culture dish heater (Warner Instruments, USA), and P-97 micropipette puller (Sutter Instruments, USA).

Reagents, solutions and drugs Trypsin inhibitor (type II-S) was from Sigma-Aldrich; papain and dithiothreitol were from Biosharp (Korea); collagenase was from Worthington Biochemicals. The buffer solution contained (in mmol/L) 145.0 NaCl, 4.0 KCl, 0.05 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES, and 10.0 glucose, adjusted to pH 7.2 with NaOH. For the recording of BK_{Ca} currents, the pipette solution contained (in mmol/L) 140 KCl, 0.5 $MgCl_2$, 5.0 Na_2ATP , 0.5 Na_2GTP , 10 HEPES, and 1.0 EGTA, at a pH of 7.2. $CaCl_2$ was added to provide 1.0 $\mu mol/L$ of free Ca^{2+} , and the external solution was the buffer solution. For the recording of K_V current, the pipette solution contained (in mmol/L) 110 KCl, 30

KOH, 10 HEPES, 10 EGTA, 1 $MgCl_2$, 1 $CaCl_2$, 3 Na_2ATP , and 0.5 GTP, adjusted to pH 7.2 with KOH. To minimize the activity of K_{ATP} channels, a high concentration of ATP (3.0 mmol/L) was used. The free $[Ca^{2+}]$ was 20 nmol/L, calculated using the Maxchelator program; the external solution contained (in mmol/L) 134 NaCl, 6 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. To minimize the activity of BK_{Ca} channels, external Ca^{2+} levels were lowered to 100 nmol/L to reduce Ca^{2+} influx. Intracellular Ca^{2+} was buffered to a low level (20 nmol/L) with EGTA. DHA was dissolved in absolute ethanol as a 50 mmol/L stock solution, protected from light, and stored at $-20^\circ C$. On the day of the experiment, portions of these stock solutions were added to the bath solution to obtain the desired final concentrations.

CVSMCs isolation Healthy Sprague-Dawley rats of both sexes, aged 8–12 weeks and weighing approximately 200 g, were provided by the Experimental Animal Center of Soochow University (Suzhou, China). All of the investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the PRC National Department of Health. Animals were anesthetized with pentobarbital sodium intraperitoneally (50 mg/kg, ip), and the heart was rapidly removed from the thorax and placed in a buffer solution. CVSMCs in small arteries were isolated as described previously^[12, 13]. In brief, after being isolated under a dissection microscope, the secondary and tertiary branches of the coronary arteries were incubated at $37^\circ C$ for 10 min in 1.0 mL of buffer solution containing 0.1% bovine serum albumin. Initially, vessels were enzymatically digested for 10 min at $37^\circ C$ in 1.0 mL of fresh buffer solution containing 1.5 mg of papain and 1.0 mg of dithiothreitol. The vessels were further digested for 10 min at $37^\circ C$ in 1.0 mL of fresh buffer solution containing 1.0 mg of collagenase and 1.0 mg of a trypsin inhibitor. To remove the exogenous enzymes, each vessel was transferred three times to 1.0 mL of fresh buffer solution and gently triturated with a fire-polished glass pipette until complete dissociation. The resulting smooth muscle cell suspension was stored at $4^\circ C$ and used within 8 h.

BK_{Ca} and K_V current recording and channel kinetic parameters BK_{Ca} and K_V currents in individual CVSMCs were recorded using a patch-clamp technique following the method described previously^[14-16]. In brief, isolated CVSMCs were placed in a 1.0 mL chamber on the stage of an inverted microscope and perfused with the external solution. Borosilicate glass capillaries were used to fabricate patch pipettes. When filled with internal solution, each electrode had a tip resistance between 4 and 10 M Ω and a typical seal resistance greater than 10 G Ω . BK_{Ca} currents were

recorded using a MultiClamp 700B patch clamp amplifier. Voltage clamp pulses were generated via an IBM-compatible computer connected to a DigiData 1322 D/A and A/D converter. Data acquisition and analyses were performed using pCLAMP9.0 software. The effects of DHA at different concentrations (10, 20, 30, 40, 50, 60, 70, and 80 $\mu\text{mol/L}$) on BK_{Ca} currents were measured by eliciting BK_{Ca} currents in the presence of 1 $\mu\text{mol/L}$ cytoplasmic free Ca^{2+} . Responses were measured from a holding potential of -60 mV to a testing potential of +80 mV at 10 mV increments for 400 ms, and they were repeated at 10-s intervals. To eliminate K_{V} current contamination, the external solution included 3 mmol/L 4-AP, a K_{V} channel blocker. K_{V} currents were generated by stepwise 10 mV depolarizing pulses (400 ms duration, 10-s intervals) from a holding potential of -60 mV to +50 mV in cells dialyzed with 100 nmol/L ionized Ca^{2+} . The effects of DHA on K_{V} channel currents at the concentrations listed above were measured. In addition, 100 nmol/L of iberiotoxin (IBTX), a BK_{Ca} channel blocker, was present in the external solution to block BK_{Ca} channel activities. Currents were normalized to cell capacitance and expressed as pA/pF. All experiments were performed at room temperature (21–23 °C).

Statistical analysis Continuous variables are expressed as mean \pm standard deviation. SPSS11.5 (Chicago, Illinois, USA) was used for the statistical analysis. Comparisons among groups were performed by an analysis of variance (ANOVA) with a least-significant difference contrast. Control and drug data for individual groups were compared using paired *t*-tests. $P < 0.05$ was considered significant. Origin-Pro7.5 software (OriginLab, USA) was utilized to calculate the EC_{50} .

Results

Effects of DHA on BK_{Ca} currents (1) Effects on BK_{Ca} currents and peak currents: at a testing potential of +80 mV, the stable peak current was 513.5 ± 101.96 pA and current density was 48.9 ± 2.45 pA/pF ($n=10$). When DHA was applied at 10, 20, 30, 40, 50, 60, 70, and 80 $\mu\text{mol/L}$, BK_{Ca} current increases of $6.1 \pm 0.3\%$, $76.5 \pm 3.8\%$, $120.6 \pm 5.5\%$, $248.0 \pm 12.3\%$, $348.7 \pm 17.3\%$, $374.2 \pm 18.7\%$, $432.2 \pm 21.6\%$, and $443.1 \pm 22.1\%$, respectively, were observed ($P < 0.05$, $n=10$). Figure 1 shows current traces elicited by depolarization from the -60 mV holding potential (HP) to the +80 mV test potential at different DHA concentrations. The EC_{50} of the BK_{Ca} current, calculated by a curve fit with the concentrations and percentage increases for the currents, was 37.53 ± 1.65 $\mu\text{mol/L}$ (Figure 2). (2) Effects on the cur-

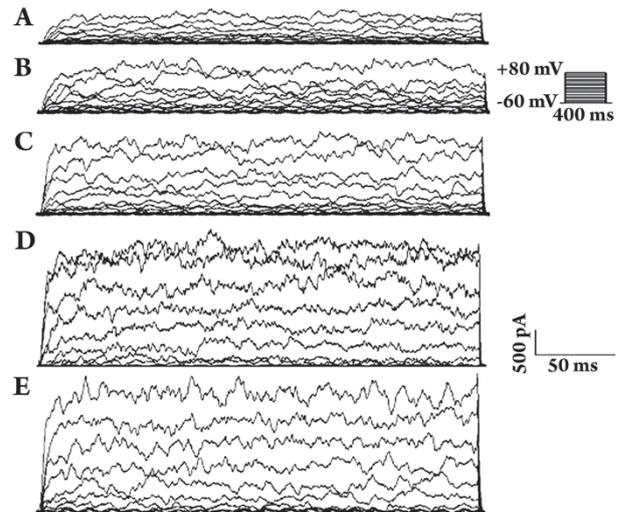


Figure 1. Increase of BK_{Ca} currents at different DHA concentrations. A, B, C, D, and E are DHA at 0, 20, 40, 60, and 80 $\mu\text{mol/L}$, respectively. 4-AP 3 mmol/L was present in the external solution to block K_{V} channel activities.

rent-voltage (*I*-*V*) curves of BK_{Ca} currents: When DHA was used at 20, 40, 60, and 80 $\mu\text{mol/L}$, BK_{Ca} currents and densities increased gradually with increasing DHA concentrations. The *I*-*V* curves were shifted upward (Figure 3).

Effects of DHA on K_{V} currents (1) Effects on K_{V} currents and peak currents: At a testing potential +50 mV, the stable peak current was 460.95 ± 73.01 pA and current density was 43.9 ± 2.32 pA/pF ($n=10$). When DHA was applied at 10, 20, 30, 40, 50, 60, 70, and 80 $\mu\text{mol/L}$, K_{V} currents were blocked by $0.40 \pm 0.02\%$, $1.37 \pm 0.06\%$, $11.80 \pm 0.59\%$, $26.50 \pm 1.75\%$, $56.50 \pm 2.89\%$, $73.30 \pm 3.66\%$, $79.70 \pm 3.94\%$, and $78.10 \pm 3.91\%$, respectively ($P < 0.05$, $n=10$).

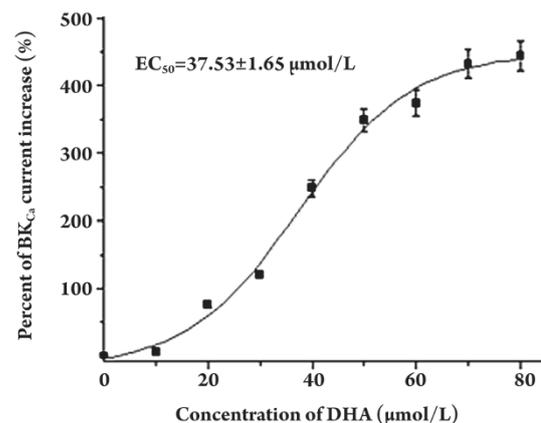


Figure 2. Percentages of BK_{Ca} currents increased at different DHA concentrations.

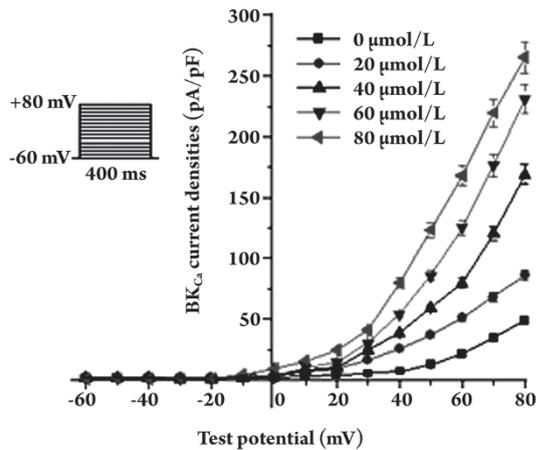


Figure 3. *I-V* curves of BK_{Ca} currents at different DHA concentrations and test potentials.

Figure 4 shows the current traces elicited by depolarization from the -60 mV holding potential to the +50 mV test potential at different DHA concentrations. The EC₅₀ of DHA on K_V currents was 44.20±0.63 μmol/L (Figure 5). (2) Effects on the *I-V* curves of K_V currents: when DHA was used at concentrations of 20, 40, 60, and 80 μmol/L, K_V currents and current densities decreased gradually with increasing DHA concentrations. The *I-V* curves were shifted downward (Figure 6).

Discussion

Many kinds of potassium channels in VSMCs play the important role in regulating membrane potential and vascular tone. Of these potassium channels, BK_{Ca} channels and K_V channels are widely distributed in VSMCs. In particular, BK_{Ca} channels are most widely expressed channels

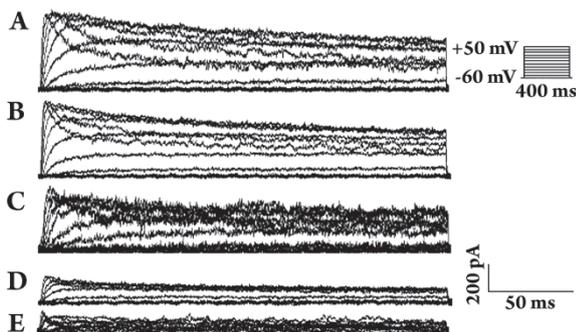


Figure 4. Decrease of K_V currents at different DHA concentrations. A, B, C, D, and E are DHA at 0, 20, 40, 60, and 80 μmol/L, respectively. IBTX 100 nmol/L was present in the external solution to block BK_{Ca} channel activities.

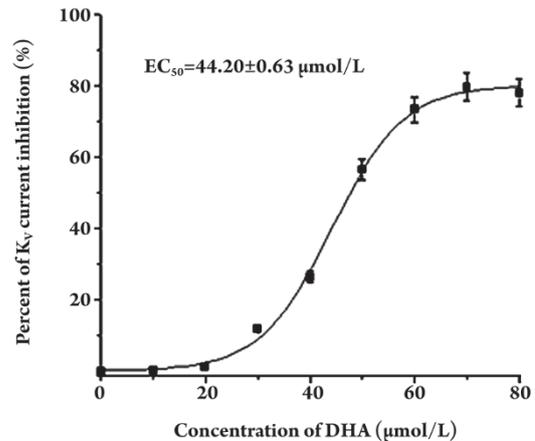


Figure 5. Percentages of K_V currents decreased at different DHA concentrations.

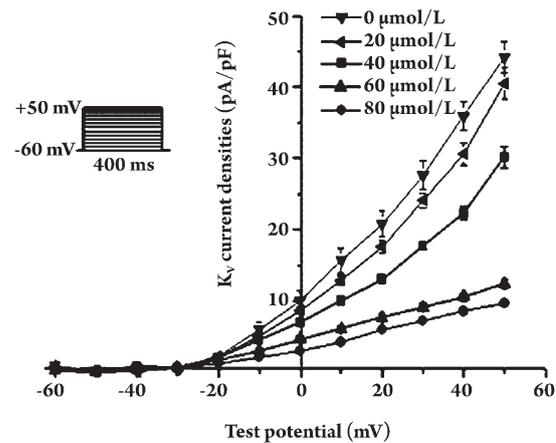


Figure 6. *I-V* curves of K_V currents at different DHA concentrations and test potentials.

in VSMCs. BK_{Ca} channels are composed of a pore-forming α-subunit and regulatory β-subunit. Because of their strong conductance (200–250 pS) and high density (1–4 channels/μm²) in VSMCs, the activity of BK_{Ca} channels is a key determinant in controlling resting membrane potential and vascular tone^[17,18]. BK_{Ca} channels can be activated by either elevation of cytosolic Ca²⁺ concentrations ([Ca²⁺]_i) or cell membrane depolarization^[19,20]. The activation of BK_{Ca} channels repolarizes the cell membrane potential and promotes the closure of voltage-activated Ca²⁺ channels (VACC), opposing the role of VACCs in vasoconstriction. Therefore, BK_{Ca} channels are key channels for the regulation of vascular tone in VSMCs. Interestingly, BK_{Ca} channels are not expressed in cardiomyocytes^[21]. K_V channels are also com-

posed of a pore-forming α -subunit and regulatory β -subunit. To date, more than 30 genes encoding several subfamilies of K_V α -subunits are currently recognized, and each α -subunit is associated with ancillary β -subunits that influence the characteristics of the channel and modify the intrinsic properties of the α -subunits^[22, 23]. Studies have shown that the *Kv1.2*, *Kv1.3*, *Kv1.4*, *Kv1.5*, *Kv4.1*, and *Kv4.2* genes are expressed in rat VSMCs as well as rat ventricular myocytes. With regard to the rat coronary artery, the expression of *Kv1.2* and *Kv1.5* has been found in rat CASMCs^[24–26].

Broad K_V channel distribution has also been detected in VSMCs. Depolarization of the membrane potential in VSMCs leads to an influx of Ca^{2+} through L-type Ca^{2+} channels and activation of the contractile machinery. These changes permit K_V channels to open and allow an efflux of K^+ , resulting in repolarization and the consequent return to the resting membrane potential. Taken together, these findings indicate that the main function of K_V channels is to limit membrane depolarization and maintain resting vascular tone^[22, 27].

Fatty acids, especially ω -3 PUFAs, are one important source of cardiac energy. They have important effects on the electrophysiology and metabolism of cardiomyocytes. It has been reported that a high dietary intake of ω -3 PUFA (more than 4.0 g a day) is linked to favorable physiological alterations, including reduction in triglyceride levels, anti-arrhythmic actions, anti-thrombotic effects, lowering of blood pressure, and enhancement immune function; together, these changes may contribute to a lower risk of cardiovascular diseases^[2, 28, 29]. Although some clinical studies have elucidated that DHA intake accumulates easily in cardiac tissues, leads to the dilation of the coronary artery, and enhances coronary artery blood flow^[30], the mechanisms of these beneficial effects of DHA for the prevention of cardiovascular disease are still not completely clear. To elucidate the mechanisms by which DHA affects vasodilatation, we investigated the effects of DHA on BK_{Ca} and K_V channels in rat CASMCs using the whole-cell patch clamp technique.

Our results have shown that DHA at concentrations greater than 10 μ mol/L activated BK_{Ca} channels, and BK_{Ca} currents increased with the elevation of DHA concentrations. This indicates that DHA has an activating effect on BK_{Ca} channels, which then leads to the efflux of K^+ , closure of Ca^{2+} channels, reduction of influx of Ca^{2+} , acceleration of cell membrane potential repolarization, and vasodilatation. More interestingly, our results have shown that DHA has a completely opposite effect on K_V channels. When DHA concentrations were greater than 20 μ mol/L, K_V currents were gradually blocked by increasing concentrations of DHA; this

finding indicates that DHA can inhibit K_V channels. Theoretically, the inhibition of K_V channels can block the efflux of K^+ and repolarization of the cell membrane potential, which results in vasoconstriction. However, many studies have shown that DHA has the characteristics to induce vasodilatation^[6, 8, 12, 30, 31]. Because 1) the density of BK_{Ca} channels is far greater than that of K_V channels in VSMCs and 2) the EC_{50} of DHA on BK_{Ca} channels is less than that on K_V channels, we suggest that the increased amount of K^+ efflux through activated BK_{Ca} channels is far greater than the reduced amount of K^+ efflux through inhibited K_V channels at a given DHA concentration. Consequently, we conclude that the effect of DHA on vasodilatation is accomplished mainly *via* the activation of BK_{Ca} channels, and that BK_{Ca} channels thus play a key role in vasodilatation as well as in improvement of coronary artery blood flow.

Ye *et al*^[12] tested the potency of docosahexaenoate and its five cytochrome P-450 epoxygenase metabolites, known as the epoxydocosapentaenoates (EDPs), in dilating porcine and rat coronary arterioles. They found that, compared with 13,14-EDP, docosahexaenoate (DHA) weakly activated BK_{Ca} channels in a single channel recording. However, these authors used low concentrations of DHA (below 10 μ mol/L). Another study^[31] has shown that DHA can significantly dilate aortic rings freshly isolated from spontaneously hypertensive rats through the modulation of L-type Ca^{2+} channels and K_{ATP} channels. Due to the absence of a metabolic environment under these conditions, metabolites of DHA are absent; therefore, DHA may have direct effects on K^+ channels or L-type Ca^{2+} channels in rat VSMCs. The results from our study have shown that DHA at concentrations greater than 10 μ mol/L directly and markedly activated BK_{Ca} channels. With regard to the effects of DHA on K_V channels, varying results have been reported. Wu *et al*^[11] demonstrated that the enhancement of outward voltage-gated K^+ (IK_V) currents and inwardly rectifying IK_1 currents could be related to vasorelaxation induced by DHA in human coronary artery smooth muscle, and Poling *et al*^[10] reported that DHA inhibited K_V channels in neurons. Studies have also shown that DHA inhibits K_V channels in ventricular myocytes, in which the expression of $K_V1.1$ - $K_V1.5$ and $K_V4.1$ - $K_V4.2$ has been detected^[32, 33]. Our studies have shown that DHA at concentrations greater than 20 μ mol/L markedly inhibited K_V channels in rat CASMCs.

Studies have shown that several types of vascular K^+ channels, including BK_{Ca} channels and K_V channels, are activated by activating adenylyl cyclase. This activation thereby increases the intracellular concentration of cAMP, which activates a cAMP-dependent protein kinase (PKA). The

activation of PKG can also result in the activation of vascular K^+ channels^[34]. In addition, inhibition of K^+ channels in VSMCs is initiated through the activation of protein kinase C (PKC)^[35, 36]. However, the molecular mechanisms of BK_{Ca} channel activation and K_V channel inhibition by DHA remain unclear.

In our experiments, DHA directly activated BK_{Ca} channels, indicating that DHA may bind on or be close to the α -pores or β -regulatory units of BK_{Ca} channels and induce conformational changes in BK_{Ca} channels. The molecular mechanisms by which BK_{Ca} channels are activated and K_V channels inhibited by DHA require future study.

Because there are still no reports regarding the direct effects of DHA on BK_{Ca} channels and K_V channels in rat CASMCs, the present study has an important implication for the investigation of the mechanisms by which ω -3 PUFA causes vasodilatation and the lowering of blood pressure. However, our studies have some limitations. First, only the effects of DHA on BK_{Ca} and K_V channels were investigated, whereas the effects of DHA on K_{ATP} and K_{ir} channels need to be probed further. Second, because ω -3PUFAs consist mainly of DHA and EPA, the effects of EPA on BK_{Ca} and K_V channels need to be studied further.

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Author contribution

Wen-ping JIANG designed research; Li-hong LAI performed research; Ru-xing WANG contributed new analytical tools and reagents; Li-hong LAI analyzed data and wrote the paper.

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