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Membrane Cholesterol Determines the Stimulatory Effect of Omega-3 PUFA on BK Channel Activity

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ABSTRACT

Background: The reported ability of omega-3 PUFA to improve cardiovascular function is associated with amelioration of endothelial function. Because the latter is largely controlled by endothelial electrical signaling, the impact and underlying mechanisms of action of docosahexaenoic acid (DHA), a main component of fish oil, on endothelial electrical responses was explored. **Methods:** Patch-clamp registration of the membrane potential, whole-cell and single currents in endothelial cell line derived from human umbilical vein. **Results:** In whole-cell configuration, DHA (3 μM) produced membrane hyperpolarization and stimulation of paxilline-sensitive and GDP S-insensitive outwardly-rectifying current. In inside-out configuration, DHA stimulated single channel BK activity without changing the single channel amplitude. The effect of DHA was absent after depletion of the membrane cholesterol level by preincubation with methylcyclodextrin. **Conclusion:** Membrane cholesterol is an essential component in BK channel sensitivity to DHA. Alterations in cholesterol concentrations within individual lipid rafts may represent a potential mechanism by which omega-3 PUFA affect cellular signaling and function via modulation of BK channels.

Key words: Omega-3 polyunsaturated fatty acids, endothelial cells, large conductance Ca^{2+}-dependent K^+ channels

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INTRODUCTION

Health benefits of dietary supplementation with omega 3 polyunsaturated fatty acids (omega-3 PUFA) are associated with a reduced incidence of several cardiovascular disorders (Chan and Cho, 2009). A beneficial effect of omega 3 PUFA is manifested by protection of myocardium against Ischaemia-Reperfusion (IR) injury (Demaison *et al*., 2001), alleviation of mitochondrial damage induced by exposure to Ca^{2+} (Panasiuk *et al*., 2013) and improvement of mitochondrial function (Khairallah *et al*., 2012). In addition to the well-documented cardioprotective effects, in experimental animal models dietary omega-3 PUFA capable to promote endothelium-dependent relaxation (Kim *et al*., 1992; Shimokawa *et al*., 1988) via increased NO production from endothelial cells (Christon, 2003). Dietary omega-3 PUFA were suggested to ameliorate endothelial dysfunction by correcting the imbalance between endothelium-derived factors (Matsumoto *et al*., 2009).

The reported ability of omega 3 PUFA to improve distinct aspects of cardiovascular function has fueled an interest towards identification of the molecular mechanisms underlying these effects. Endothelial cell function is largely controlled by electrical responses of endothelial cells. Clear correlation between endothelial dysfunction and altered endothelial cell signaling has been reported in different animal models of vascular pathologies (Bondarenko *et al*., 2004, 2012; Bondarenko and Sahach, 2002; Feletou, 2009; Grgic *et al*., 2009; Kohler and Ruth, 2010; Sagach *et al*., 2006). An increased endothelial NO production is accompanied by a rise in endothelial cell Ca^{2+} (Luckhoff and Busse, 1990) and sustained endothelial cell hyperpolarization due to activation of Ca^{2+}-dependent K^+ channels (K_{Ca}) as well as other electrogenic ion transport mechanisms, including Na^+-Ca^{2+} exchanger (Bondarenko, 2004; Bondarenko *et al*., 2013) and Na^+-K^+ ATPase

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(Bondarenko, 2006). Identification of the impact of acutely applied omega-3 PUFA and PUFA-rich diet on distinct ion transport mechanisms in health and disease represents an important aim resolution of which is essential for our understanding of the mechanisms by which PUFA affect cardiovascular function.

Recently, G-protein coupled receptor 120 (GPR120) was identified as omega-3 fatty acid receptor (Oh *et al.*, 2010). While the role of GPR120 in vascular function/signaling is obscure, the receptor was reported to induce angiogenesis (Wu *et al.*, 2013). Another target for omega-3 PUFA is the large-conductance Ca^{2+}-activated K^+ (BK) channels (Lai *et al.*, 2009; Wang *et al.*, 2011) abundantly expressed in smooth muscle cells. While little expressed in healthy endothelium of majority of vascular beds, the BK channels are widely distributed across the vascular wall and represent one of the key determinants regulating vascular function. In vascular smooth muscle cells, the BK channel stimulation counteracts depolarization during myogenic tone development, thus, limiting voltage-dependent Ca^{2+} entry and opposing vasoconstriction. In endothelial cells, the expression of BK channels is believed to be increased under pathological conditions (Sandow and Grayson, 2009) and hence, the channels represent an attractive target for correction of vascular function. Although prior studies performed on smooth muscle cells showed the stimulatory effect of omega-3 FUFA on BK channels (Hoshi *et al.*, 2013; Lai *et al.*, 2009; Wang *et al.*, 2011), there is no consensus on the mechanisms underlying this effect. Either direct interaction with the channel protein complex consisting of pore-forming Slo1 and auxiliary β1 subunits (Hoshi *et al.*, 2013) or involvement of cytochrome P450 metabolites in stimulatory effect of omega-3 PUFA on BK channels (Wang *et al.*, 2011) have been proposed. Accordingly, in the present study, using EA.hy926 endothelial cell line, a cell model which lacks the regulatory subunit (Papassotiriou *et al.*, 2000), it was aimed to investigate the impact of omega-3 PUFA and the associated mechanisms on the activity BK channels.

MATERIALS AND METHODS

Cell culture: The human umbilical vein derived endothelial cell line, EA.hy926 at passage >45 was grown in DMEM containing 10% FCS and 1% HAT (5 mM hypoxanthine, 20 μM aminopterin, 0.8 mM thimidine) and were maintained in an incubator at 37°C in 5% CO_2 atmosphere. For experiments cells were plated on glass cover slips.

Patch clamp recordings: Cells were superfused with a bath solution containing (mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, 2.4 $CaCl_2$. Patch pipettes pulled from glass capillaries after fire-polishing had a resistance of 3–5 MΩ for whole-cell recordings and 5-7 MΩ for single-channel recordings. For whole-cell recordings, the pipette solution contained (mM) 100 K-aspartate, 40 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA and a free $[Ca^{2+}]$ was adjusted to 100 nM by adding 1,924 $CaCl_2$ calculated by the program CaBuf. Single-channel recordings were obtained from excised inside-out membrane patches in symmetrical solutions. The pipettes were filled with (mM) 140 KCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 4,931 $CaCl_2$ with pH 7.2 by adding KOH.. Following gigaseal formation, bath solution was switched to the following (mM) 140 KCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA and 0.3 μM free Ca^{2+} concentration which was adjusted by adding different amounts of $CaCl_2$ calculated by the program CaBuf. pH was adjusted to 7.2 by adding KOH. Single-channel activity was obtained from >20 sec of continuous recording under each experimental condition. Currents were recorded using a patch-clamp amplifier (EPC7, List Electronics, Darmstadt, Germany) at a bandwidth of 3 kHz. The signals obtained were low pass filtered at 1 kHz and digitized with a sample rate of 10 kHz using a Digidata 1200A A/D converter (Axon Instruments, Foster City, CA, USA). Data collection and analysis were performed using Clampex and Clampfit software of pClamp (V9.0, Axon Instruments).

Membrane cholesterol was depleted by preincubation of the cells with 0.5% methyl-β-cyclodextrin (MCD) at 37°C for 1 h.

RESULTS

Docosahexaenoic acid hyperpolarizes endothelial cells and potentiates whole cell paxilline-sensitive outward currents: The resting membrane potential of endothelial cells averaged -32.6±2.1 mV (n = 17). Administration of 3 μM docosahexaenoic acid (DHA) to the bath solution produced a slowly developing membrane hyperpolarization with the mean amplitude 9.1±1.5 mV (n = 5) (Fig. 1a). In voltage-clamp mode, administration of 3 μM DHA potentiated the outwardly rectifying currents elicited by voltage ramps delivered from -90 to +80 mV (n = 5, Fig. 1b, c). The evoked currents were blocked by 1 μM paxilline, a selective BK channel inhibitor (Fig. 1b, c), pointing for BK channel stimulation.

$G_{i/o}$ protein-coupled receptor 120 (GPR120) was recently identified as an omega 3 PUFA receptor/sensor

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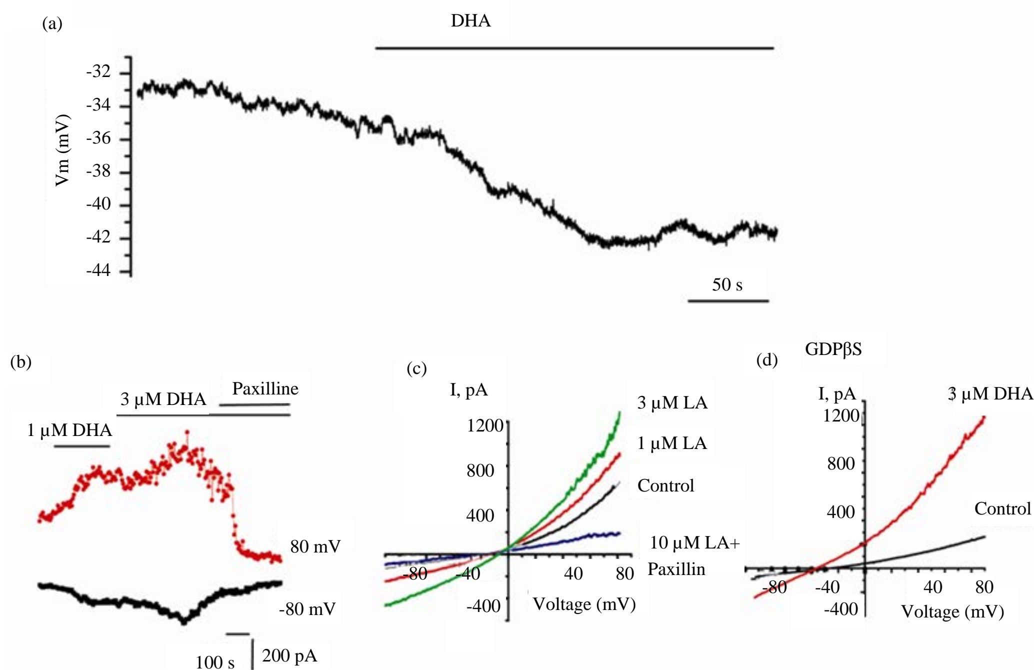

Fig. 1(a-d): Effect of docosahexaenoic acid (DHA) on electrical responses of EA.hy926 endothelial cells, (a) Effect of DHA (3 μM) on endothelial cell membrane potential, (b, c) Representative (n = 5) whole-cell membrane currents evoked by voltage ramps before (control) and after administration of 1 and 3 μM DHA either alone or in combination with 2 μM paxilline, (d) Effect of intracellular perfusion with GDPβS, a G protein inhibitor, on DHA-evoked potentiation of whole-cell membrane currents elicited by voltage ramps (n = 4)

(Oh *et al*., 2010). To explore the mechanisms underlying the BK channel potentiation and to address the role of $G_{i/o}$protein-coupled receptors (GPCR) in this effect, the cells were dialyzed with a pipette solution containing 1 mM GDP S, a G-protein inhibitor. Under these conditions, the potentiating effect of DHA on the outward currents persisted (n = 4, Fig. 1d), indicating that GPR120 and other GPCR are not required for the stimulatory effect of DHA on endothelial BK channels.

DHA potentiates the BK channel activity in cell-free inside-out patches: Because facilitation of whole cell BK currents by DHA may be caused either by an increase in subplasmalemmal Ca^{2+} due to Ca^{2+} entry or by changes in Ca^{2+} or voltage sensitivity of the channel, the impact of DHA on single channel activity in cell-free inside-out patches under fixed Ca^{2+} and voltage was explored next. Figure 2a illustrates the BK single channel activities recorded in excised patches held at +40 mV in the presence of 0.3 μM free Ca^{2+} before (left) and after (right) administration of 1 μM DHA to the inner surface of the patch. Under these conditions, DHA still increased NPo without significant effect on single channel amplitude. The effect of DHA on BK activity was observed also in patches contained only one active patch (Fig. 2b) and the degree of NPo increase was similar to the increase in Po, indicating that the increase in NPo in multichannel patches is rather due to an increase in open probability (Po) of individual patches rather that an increase in the number of active patches. The increase in NPo upon DHA addition was also observed at negative voltages (Fig. 2c) and was sustained and reversible (Fig. 2c) following a 10 min washout.

Cholesterol depletion prevents the DHA-evoke BK potentiation: It was further sought to determine the mechanism through which DHA activates BK channel activity. DHA may modulate the BK function via direct interaction with the channel protein itself or through modulation of biophysical properties of the lipid bilayer.

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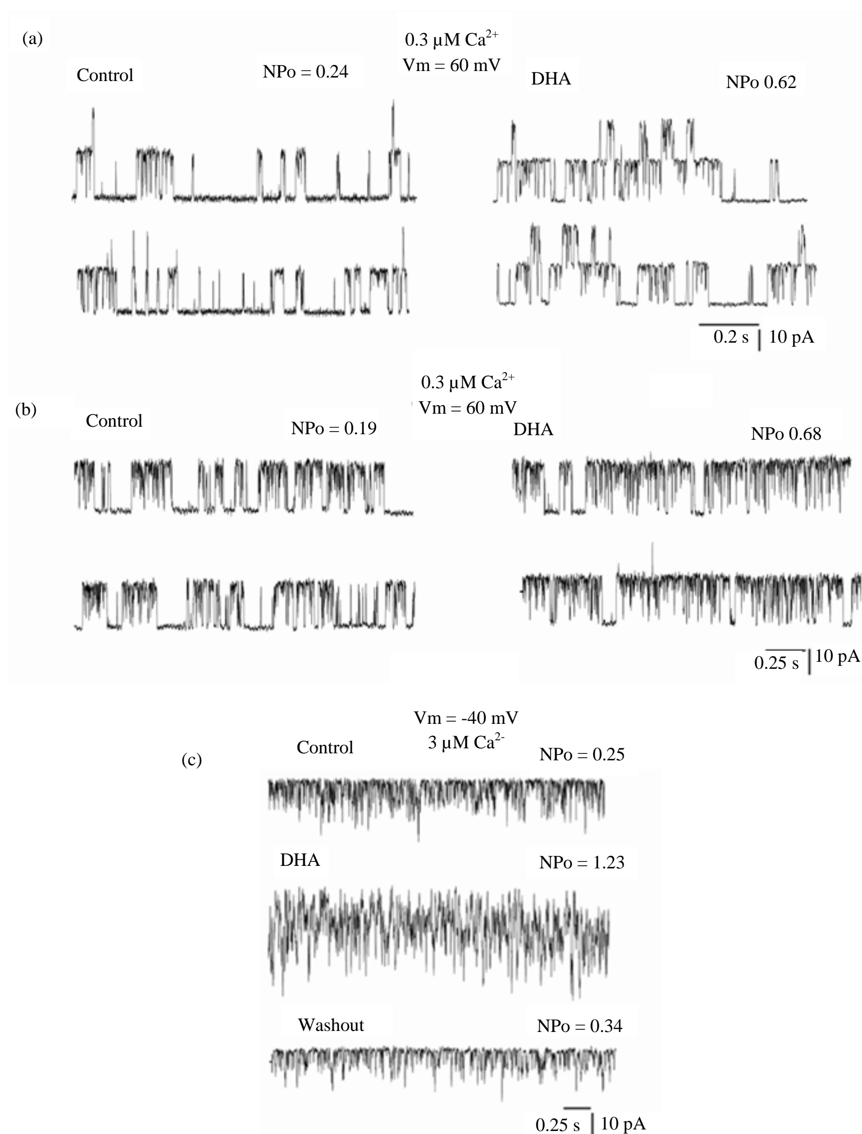

Fig. 2(a-c): DHA potentiates single channel activity of BK channels in excised patches, (a) Representative record showing the effect of 3 μM DHA on single channel activity of BK channels in inside-out patch. Bath solution contained 0.3 μM free Ca^{2+} and membrane patch was maintained at +60 mV, (b) Representative record showing stimulatory effect of 3 μM DHA on BK single channel activity in inside-out patch contained only one active channel. Bath solution contained 0.3 μM free Ca^{2+} and membrane patch was maintained at +60 mV and (c) Effect of 3 μM DHA on single channel activity on BK channels in inside-out patch held at -40 mV in the presence of 3 μM free Ca^{2+}

To discriminate between these possibilities, the impact of disruption of the lipid rafts with MCD DHA on BK activity following was examined. Membrane cholesterol is an important regulator of ion channel activity, including the activity of BK channels (Bukiya *et al*., 2011). Following 1 h preincubation of the cells with MCD, DHA failed to increase the BK activity (Fig. 3, n = 5). These experiments suggest that DHA facilitate

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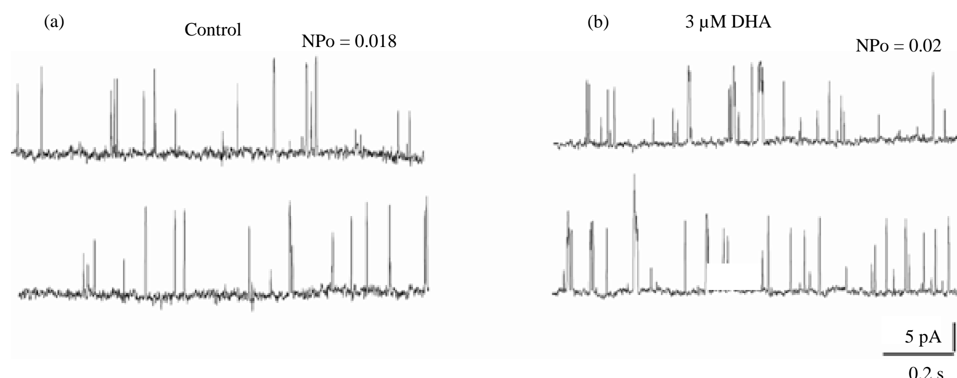

Fig. 3(a-b): Cholesterol depletion prevents the stimulatory effect of DHA (3 μM) on BK channel activity. Representative records of single BK channel activity in inside-out patch exposed to 0.3 μM free Ca^{2+} and held at 40 mV (a) Before and (b) After administration of 3 μM DHA. Cells were preincubated with methylcyclodextrin (MCD)

the BK channel activity through modification of the lipid rafts but not due to interaction with the channel protein itself.

DISCUSSION

The results of the present study demonstrate that administration of DHA, a major constituent of fish oil diets, to endothelial cells produces stimulation of outwardly rectifying paxilline-sensitive current accompanied by cell hyperpolarization. Because endothelial cell function is largely controlled by Ca^{2+} influx driven by membrane hyperpolarization, the latter represents a key signaling event in initiating endothelium-dependent relaxation, whether it is underpinned by Nitric Oxide (NO) or Endothelium-Derived Hyperpolarizing Factor (EDHF). Considering that BK channels are abundantly expressed in vascular smooth muscle cells, activation of BK channels by omega-3 fatty acids and resulting endothelial hyperpolarization may contribute both to endothelium-dependent and endothelium-independent relaxation. As shown previously, different settings of endothelial dysfunction, such as hypertension (Bondarenko and Sagach, 2002) and diabetes (Bondarenko *et al*., 2004) are accompanied by depolarized endothelial membrane potential due to decreased Na^+-K^+-ATPase activity. Because BK channels are regulated by both Ca^{2+} and voltage, depolarization of endothelial cells by ~10 mV observed in experimental hypertension and diabetes, will likely result in a higher sensitivity to omega-3 fatty acids due to facilitated BK opening at depolarized potentials. Accordingly, direct regulation of BK channels by omega-3 fatty acids may represent an important mechanism of restoration of amelioration of endothelial function under pathological conditions.

Omega 3 fatty acids have recently been proposed to act via GPR120 to mediate potent anti-inflammatory and insulin-sensitizing effects (Oh *et al*., 2010). To address possible interaction of DHA with GPR120 or other G-protein coupled receptors and the development of GPR120-dependent BK stimulation, the cell were dialyzed with 1 mM GDP S, a widely used a G-protein inhibitor (Bondarenko *et al*., 2010; Kim *et al*., 2006). Under these conditions, DHA still produced similar degree of potentiation of the outwardly rectifying current evoked by voltage ramps, indicating for GPCR-independent mechanism. As an alternative approach to explore the role of GPCR, the impact of DHA on single channel activity in excised inside out-patches was investigated. Under this configuration, the cytosolic messengers do not control channel function. Consistent with GPCR-independent mechanism, in excised inside-out patches, DHA administered to the inner surface of the membrane increased the open probability of BK opening, indicating that intracellular signal transduction cascade is not required for the DHA effect on the BK channels. Importantly while the channel open probability was increased, the single channel amplitude remained unaffected, indicating that DHA does not alter BK channel function by modifying the conformation of the channel protein itself.

Recent study showed that in HEK cells transiently expressing BK channels formed by pore forming Slo1 and the regulatory β1 subunits, DHA reversible stimulated single channel activity (Hoshi *et al*., 2013)

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likely due to DHA binding to the Slo1 channel complex. Because in endothelial cells, the BK channels consist only from pore-forming alpha subunit (Papassotiriou *et al*., 2000), our results indicate that the auxiliary β1 subunit is not required for the DHA effect. To further address the mechanism of BK potentiation by DHA, the impact of membrane cholesterol depletion in the effect of DHA was examined. Cholesterol, an essential component of cell membranes in eukaryotes, plays a critical role in regulating the activity of membrane-spanning proteins, including ion channels and transporters (Abramov *et al*., 2011; Singh *et al*., 2011). In our experiments, depletion of membrane cholesterol with MCD prevented the stimulatory effect of DHA on BK channels, suggesting that membrane lipid composition determines the stimulatory effect of DHA on BK channels.

CONCLUSION

DHA produces endothelial cell hyperpolarization via stimulation of BK channels, thus, enhancing the driving force for Ca^{2+} influx. Cellular sensitivity to DHA is mediated by membrane cholesterol and alterations in cholesterol concentrations within individual lipid rafts may represent a potential mechanism by which omega-3 PUFA affect endothelial cell signaling and function.

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