Effect of Docosahexaenoic Acid on Voltage-Independent Ca\(^{2+}\) Entry Pathways in Cultured Vascular Smooth Muscle Cells Stimulated with 5-Hydroxytryptamine

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Note

We previously reported that docosahexaenoic acid (DHA) inhibits an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in cultured rat vascular smooth muscle cells (VSMCs) through a mechanism involving mainly voltage-dependent Ca\(^{2+}\) channels; however, the effect of DHA on voltage-independent pathways, such as store-operated and receptor-operated Ca\(^{2+}\) entry, and Ca\(^{2+}\) entry through Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), has not been clarified. In the present study, we investigated the effect of DHA treatment on the expression of transient receptor potential canonical (TRPC) channels, capacitative Ca\(^{2+}\) entry, and Ca\(^{2+}\) entry through NCX in rat cultured VSMCs stimulated with 5-hydroxytryptamine (5-HT). RT-PCR analysis detected TRPC1, TRPC4, and TRPC6 mRNA in cultured VSMCs. DHA treatment for 2 d slightly but significantly decreased TRPC1, but not TRPC4 and TRPC6, mRNA expression. Sarpogrelate, a selective serotonin 5-HT\(_{2A}\) receptor inhibitor, completely inhibited the 5-HT-induced increase in [Ca\(^{2+}\)]\(_i\) in cultured VSMCs. Ca\(^{2+}\) inflow by adding extracellular Ca\(^{2+}\) (1.3 mm) to the Ca\(^{2+}\)-free condition in the presence of 5-HT was partially but significantly inhibited by sarpogrelate. DHA treatment for 2 d had no effect on Ca\(^{2+}\) inflow when extracellular Ca\(^{2+}\) was added to the Ca\(^{2+}\)-free condition in the presence of either 5-HT alone or 5-HT with sarpogrelate. KB-R7943, a selective inhibitor of reverse mode NCX, significantly suppressed the 5-HT-induced increase of [Ca\(^{2+}\)]\(_i\). Furthermore, DHA treatment for 2 d significantly decreased NCX1 mRNA expression. These results suggest that DHA seems to have little effect on capacitative Ca\(^{2+}\) entry. Through decreasing NCX1 expression, DHA may suppress the 5-HT-induced increase in [Ca\(^{2+}\)]\(_i\).

Key words docosahexaenoic acid; store-operated Ca\(^{2+}\) entry; receptor-operated Ca\(^{2+}\) entry; 5-hydroxytryptamine; Na\(^+\)/Ca\(^{2+}\) exchanger; vascular smooth muscle cell

A large number of studies have shown that n-3 polyunsaturated fatty acids, including docosahexaenoic acid (DHA) and eicosapentaenoic acid, protect against several types of cardiovascular diseases, such as myocardial infarction, arrhythmia, atherosclerosis, and hypertension. Although the precise mechanisms are unclear, the protective effects of DHA and eicosapentaenoic acid are attributable to their direct effects on the function of vascular endothelial and smooth muscle cells (VSMCs). Furthermore, it is believed that DHA has more potent and beneficial effects on cardiovascular diseases than eicosapentaenoic acid. In fact, DHA activates large-conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK\(_{ca}\)), and this effect is suggested to reduce blood pressure.

Our laboratory previously reported that treatment of cultured VSMCs with DHA significantly suppresses the increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) induced by 5-hydroxytryptamine (5-HT) and angiotensin II. S-HT and angiotensin II stimulate voltage-dependent Ca\(^{2+}\) channels in VSMCs. Furthermore, DHA treatment of VSMCs inhibits KCl (80 mm)-induced intracellular Ca\(^{2+}\) mobilization and Mn\(^{2+}\) inflow. Thus, it is likely that DHA suppresses receptor-mediated Ca\(^{2+}\) inflow through voltage-dependent Ca\(^{2+}\) channels. However, the effect of DHA on voltage-independent Ca\(^{2+}\) entry is still largely unknown.

Transient receptor potential canonical (TRPC) proteins have been shown to be Ca\(^{2+}\) channels activated by store depletion and/or receptor stimulation. TRPC1 and TRPC6 have been demonstrated to be expressed abundantly in primary culture of VSMCs isolated from rats. Although the function and mechanism of Ca\(^{2+}\) entry via TRPC proteins are not fully defined, TRPC1 is recognized to have an important role in store-operated Ca\(^{2+}\) -entry (SOCE) rather than receptor-operated Ca\(^{2+}\) entry (ROCE). Conversely, TRPC6 is predicted to function in ROCE.

In the present study, we investigated the effect of DHA on SOCE and ROCE, including TRPC expression, to understand further the detailed mechanisms of DHA action on intracellular Ca\(^{2+}\) mobilization in VSMCs.

MATERIALS AND METHODS

Materials Fetal calf serum (FCS), penicillin, streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Life Technologies (Grand Island, NY, U.S.A.). Fura-2 acetoxyethyl ester (fura-2/AM) and ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) were from Dojindo (Kumamoto, Japan). Sarpogrelate was a kind gift from Mitsubishi Tanabe Pharma (Osaka, Japan). DHA was from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents were purchased from standard suppliers.

Cell Culture VSMCs were enzymatically isolated from the aortic media of 6–7-week-old Wistar rats using collagenase and elastase and cultured in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, as

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described previously.\( ^{11} \) To measure \([\text{Ca}^{2+}]_i\), the cells were seeded and grown on coverglasses (8×16 mm; Matsunami Glass, Osaka, Japan). The cells were then cultured in DMEM containing 2% FCS in the presence or absence of 30 \( \mu \text{M} \) DHA. Primary VSMCs were used throughout the experiments. VSMCs for the experimental groups (shown as patterned columns in all figures) and control groups (shown as open columns in all figures) were obtained from the same animals so all experiments could be performed in a paired fashion. This study was conducted in accordance with the Care and Use of Laboratory Animals of the Animal Research Committee of Health Sciences University of Hokkaido.

**RNA Extraction and RT-PCR** TRPC channels and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), which are expressed in cultured VSMCs, were identified by qualitative RT-PCR. Total RNA in vascular media and cultured VSMCs was extracted using TRI Reagent\(^{6}\) (Sigma-Aldrich) according to the manufacturer’s instructions. One-step RT-PCR were carried out using a QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The thermocycling program consisted of 30 min at 50°C, 15 min at 95°C, and 43 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and a final extension for 10 min at 72°C in a thermal cycler (TaKaRa PCR thermal cycler Dice; TaKaRa Bio, Shiga, Japan). The RT-PCR products were separated and visualized on an ethidium bromide-stained 1.0% agarose gel.

mRNA expression in VSMCs was also quantified by real-time RT-PCR. PCR amplification was performed for 43 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and a final extension for 10 min at 72°C in a thermal cycler (TaKaRa PCR thermal cycler Dice; TaKaRa Bio, Shiga, Japan). The RT-PCR products were separated and visualized on an ethidium bromide-stained 1.0% agarose gel.

**Statistical Analysis** Statistical analysis of the results was performed using Student’s unpaired \( t \)-test for unpaired data and ANOVA followed by Dunnett’s test for multiple comparisons. \( p \)-Values less than 0.05 were considered significant.

**RESULTS**

**Effect of DHA on TRPC Expression**

The expression profiles of TRPC mRNA in cultured VSMCs were examined by RT-PCR. PCR amplification was performed for 43 cycles in order to reach saturation. mRNAs of TRPC1, TRPC4, and TRPC6 were clearly detected in cultured VSMCs, whereas mRNAs of TRPC2, TRPC3, TRPC5 and TRPC7 were barely detected (Fig. 1A).

The expression of TRPC mRNA in cultured VSMCs was then investigated by real-time RT-PCR. As shown in Fig. 1B, TRPC1 mRNA expression was slightly but significantly decreased by DHA (30 \( \mu \text{M} \)) treatment for 2 d. DHA had no effect on TRPC4 and TRPC6 mRNA expression (Figs. 1C, D).

**Role of 5-HT in SOCE and ROCE** As shown in Figs. 2A and B, 5-HT at 10 \( \mu \text{M} \) induced a rapid and transient increase in \([\text{Ca}^{2+}]_i\) in cultured VSMCs. Peak \([\text{Ca}^{2+}]_i\) and \([\text{Ca}^{2+}]_o\) at 5 min after stimulation by 5-HT were 230.29±47.20 and 117.71±14.5 nm, respectively, (mean±standard error of the mean (S.E.M.)) These increases were completely blocked by

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**Table 1. Oligonucleotide Sequences of Primers Used for RT-PCR**

| Oligonucleotide Sequence | Sense/Antisense |
|--------------------------|-----------------|
| TRPC1                    | 5’-CTGCCACAGATGTTACAAAGATTTTGGG-3’/5’-GGCGAATTCTCACCTTATCTCTGAT-3’ |
| TRPC2                    | 5’-CAGTTTTCACCGATGTTGCTGATG-3’/5’-CTTGGGGCAGTTACGATTCT-3’ |
| TRPC3                    | 5’-CTCTGGAAGAGTCAACTCCAC-3’/5’-CCACTCTGACATCGTACGTCC-3’ |
| TRPC4                    | 5’-GGCTTACACCTTCAAGGCTTACG-3’/5’-CTTGGGGCAGTTACGATTCT-3’ |
| TRPC5                    | 5’-CTATCAGACAGGCTTATCTAGT-3’/5’-CTACCAAGGAGGATGACGTTTGTATG-3’ |
| TRPC6                    | 5’-GTTGCCAAGTCTCAAGGGCTTACG-3’/5’-CTTGGGGCAGTTACGATTCT-3’ |
| TRPC7                    | 5’-ACCTTACAGACTCACCCAAAC-3’/5’-AGAAAGCTGAGGACACAAAG-3’ |
| NCX1                     | 5’-TAAAAACCATTGAAAGGGACAGC-3’/5’-ACTGCAAGCTTGTGGTGTTGC-3’ |
| NCX2                     | 5’-TCTCTTCCAGGACGCTGTC-3’/5’-GGGCTTCTCTCTCTCTGC-3’ |
| NCX3                     | 5’-ACTTTTGAATGTGATACCATTCCAT-3’/5’-TTGGCCATCTCTCTCTCTGC-3’ |

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**For RT-PCR**

**For real-time RT-PCR**

TRPC1 96 5’-CTTCTTTGAATGTGATACCATTCCAT-3’/5’-TTGGCCATCTCTCTCTCTGC-3’
TRPC4 118 5’-TGACGGAGGGAGAATGTTATCTG-3’/5’-CGCGTGTGGCTGACTTATT-3’
TRPC6 126 5’-ACTGCTTGGTGTCTGCT-3’/5’-TGACGGAGGGAGAATGTTAAGG-3’
NCX1 102 5’-AGCAAGGGCCGCTTCCTTCTTT-3’/5’-GCTGTGGTTGTGGTGGTGTTG-3’
\( \beta \)-Actin 151 5’-CTGGCCAGGACCTGAGACA-3’/5’-GCCGACTGCGGCCATCCTAT-3’
sarpogrelate (1 µM), a selective serotonin 5-HT2A receptor antagonist (Figs. 2A, B).

When extracellular Ca2+ (1.3 mM) was removed, the basal level of [Ca2+]i decreased, and a small transient increase by 5-HT stimulation was observed (Fig. 2C). Figure 2D shows the mean±S.E.M. values of peak [Ca2+]i after the addition of extracellular Ca2+ to the Ca2+-free condition in the presence of 5-HT. This increase in [Ca2+]i was partially but significantly inhibited by sarpogrelate.

**Effect of DHA on 5-HT-Induced SOCE and ROCE**

The effect of DHA on 5-HT-induced SOCE and ROCE was then investigated. As shown in Figs. 3A and C, DHA treatment for 2 d had no effect on the 5-HT-induced increase in [Ca2+]i under the Ca2+-free condition, as described previously.6) DHA treatment also had no effect on the [Ca2+]i change induced by the addition of extracellular Ca2+ to the Ca2+-free condition in the presence of 5-HT without (Figs. 3A, B) or with (Figs. 3C, D) sarpogrelate.

**Role of NCX on the 5-HT-Induced [Ca2+]i Change and the Effect of DHA on NCX mRNA Expression**

As shown in Figs. 4A and B, KB-R7943 (10 µM), a specific inhibitor of reverse mode NCX, significantly inhibited both peak [Ca2+]i and [Ca2+]i at 5 min after stimulation with 5-HT. The reduction rate at peak [Ca2+]i by KB-R7943 was almost the same as that after treatment with DHA for 2 d, and KB-R7943 had no effect on DHA-treated cells (Fig. 4C).

The effect of DHA on NCX mRNA expression was then investigated. In rat cultured VSMCs, NCX1 mRNA, but not NCX2 and NCX3, was expressed abundantly (Fig. 5A). As shown in Fig. 5B, NCX1 mRNA expression was slightly but significantly decreased by DHA (30 µM) treatment for 2 d.

**DISCUSSION**

In the present study, we investigated the effect of DHA on SOCE and ROCE in cultured rat VSMCs. We first showed that cultured VSMCs express TRPC1, 4, and 6 mRNA, which is consistent with a previous report by Poburko et al.15) In intact vascular media before enzymatic digestion, abundant TRPC3 mRNA expression in addition to TRPC1 and TRPC6 mRNA expression was detected, whereas TRPC4 mRNA expression was barely detected (data not shown). Therefore, the process of cell culture may affect the expression pattern of TRPCs in vascular media cells. DHA treatment for 2 d significantly inhibited the expression of TRPC1 mRNA, but not TRPC4 and TRPC6 mRNA. This condition of DHA treatment was the same as in our previous reports showing that DHA significantly inhibits the 5-HT- and angiotensin II-induced increase in [Ca2+]i in the presence of extracellular Ca2+.5,6,8)

Since TRPC1 has an important role in not only SOCE but also ROCE in VSMCs,8) we then investigated the effect of 5-HT on SOCE and ROCE. The 5-HT-induced rapid and transient elevation of [Ca2+]i was completely inhibited by sarpogrelate, suggesting that serotonin 5-HT2A receptor activation mediates Ca2+ mobilization. Sarpogrelate also partially but significantly inhibited Ca2+ entry when extracellular Ca2+ was added to the Ca2+-free condition in the presence of 5-HT, suggesting that this capacitative Ca2+ entry is also partially dependent on the stimulation of serotonin 5-HT2A receptors. Therefore, our result indicates that Ca2+ entry when extracellular Ca2+ is added to the Ca2+-free condition in the presence of 5-HT consists of both SOCE (i.e., which was not blocked by sarpogrelate) and ROCE (i.e., which was blocked by sarpogrelate). In contrast to 5-HT-induced capacitative Ca2+ entry,
olmesartan (100 nM), a selective angiotensin II AT1 receptor antagonist, has no effect on Ca$^{2+}$ entry when extracellular Ca$^{2+}$ is added to the Ca$^{2+}$-free condition in the presence of 100 nM angiotensin II, although olmesartan completely inhibits the angiotensin II-induced increase in [Ca$^{2+}$]$_i$ in the presence of extracellular Ca$^{2+}$ (unpublished data).

Although DHA treatment for 2 d significantly inhibited TRPC1 mRNA expression, it had no impact on 5-HT-induced SOCE and ROCE. We also confirmed that DHA had no impact on angiotensin II-induced SOCE (unpublished data). In astrocytes, it has been reported that acute DHA treatment inhibits SOCE.16) In VSMCs, the addition of DHA at the time of agonist stimulation has no effect on intracellular Ca$^{2+}$ mobilization, even in the presence of extracellular Ca$^{2+}$.5) Therefore,
the inhibitory effect of DHA on SOCE may depend on cell type, being different between vascular and other cell types. Ye et al.\(^{17}\) showed that DHA treatment for 3 d suppresses the TRPC1-mediated Ca\(^{2+}\) signaling pathway by partially displacing TRPC1 from membrane caveolar lipid rafts in vascular endothelial cells. Therefore, it is possible that DHA may affect not only TRPC1 mRNA expression but also TRPC1 distribution in the membrane of VSMCs. However, even if DHA modulates TRPC1 function in VSMCs, it seems to have little effect on SOCE and ROCe, as indicated in our present study. Yet, there is increasing evidence of a role for TRPC1 in VSMCs. Kwan et al.\(^{18}\) reported that TRPC1 and BK Ca co-localize in VSMCs, and Ca\(^{2+}\) influx through TRPC1 activates BKCa to induce membrane hyperpolarization. Ávila-Medina et al.\(^{19}\) reported that TRPC1 and Orai1 co-localize with voltage-dependent Ca\(^{2+}\) L-type Ca\(^{2+}\) channels, and 5-HT-induced TRPC1-dependent SOCE can trigger the activation of voltage-dependent Ca\(^{2+}\) L-type Ca\(^{2+}\) channels. Therefore, it might still be worthwhile continuing to determine the effect of DHA on TRPC1 function in VSMCs.

In fact, we confirmed the abundance of NCX1, but not NCX2 and NCX3, expression in rat VSMCs (Fig. 5A). Although the precise mechanism by which the reverse mode of NCX is activated by 5-HT is still unclear, it was reported recently that the reverse mode of NCX1 contributes to angiotensin II-induced Ca\(^{2+}\) influx in rat VSMCs.\(^{23}\) In the present study, we also showed that KB-R7943 significantly suppressed the 5-HT-induced increase in [Ca\(^{2+}\)]\(_i\), suggesting the involvement of reverse mode NCX in Ca\(^{2+}\) influx by 5-HT stimulation. Furthermore, KB-R7943 had no effect in DHA-treated cells. Therefore, it is possible that DHA suppresses the reverse mode function of NCX. There are three NCX isoforms (NCX1, NCX2, and NCX3); NCX1 is widely expressed in the heart, kidney, brain, arteries, and other organs, whereas NCX2 and NCX3 expression is limited mainly to the brain and skeletal muscle.\(^{22}\) In fact, we confirmed the abundance of NCX1, but not NCX2 and NCX3, expression in rat VSMCs (Fig. 5A). Although the precise mechanism by which the reverse mode of NCX is activated by 5-HT is still unclear, it was reported recently that the reverse mode of NCX1 contributes to angiotensin II-induced Ca\(^{2+}\) influx in VSMCs.\(^{23}\) The authors made the point that angiotensin II-induced SOCE may increase the concentration of intracellular Na\(^+\) through mechanisms involving the TRPC family, and this intracellular Na\(^+\) may lead to the activation of the reverse mode of NCX. Therefore, the suppression of NCX1 expression by DHA treatment may con-
tribute to the inhibition of Ca\(^{2+}\) influx in VSMCs. Thus, it is worthwhile continuing to investigate the effect of DHA on the reduction of [Ca\(^{2+}\)], via NCX in our future studies.

In conclusion, DHA may suppress the 5-HT-induced increase in [Ca\(^{2+}\)], through decreasing NCX1 expression, in addition to suppressing receptor-mediated Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels. Inhibition of SOCE and ROCE could be excluded as the inhibitory mechanism of receptor-mediated Ca\(^{2+}\) influx by DHA treatment in VSMCs.

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Conflict of Interest The authors declare no conflict of interest.

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