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Inhibition by docosahexaenoic acid of receptor-mediated Ca^{2+} influx in rat vascular smooth muscle cells stimulated with 5-hydroxytryptamine

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Abstract

The effect of docosahexaenoic acid treatment on intracellular Ca^{2+} dynamics in rat vascular smooth muscle cells stimulated with 5-hydroxytryptamine (5-HT) has been investigated in order to elucidate one of the mechanisms for its beneficial effect on cardiovascular disorders. The treatment of cells with 30 μM docosahexaenoic acid for 2 days inhibited an increase in intracellular Ca^{2+} concentration induced by 5-HT (10 μM) and a depolarizing concentration of KCl (80 mM). Docosahexaenoic acid treatment significantly inhibited divalent cation influx stimulated by 5-HT and KCl, as measured by Mn^{2+} quenching method, whereas had no effect on 5-HT-induced Ca^{2+} release from the internal stores. Docosahexaenoic acid treatment also significantly inhibited 5-HT receptor-mediated Ca^{2+} influx through Ni^{2+}-insensitive channels that were distinct from store-operated channels. These results suggest that the specific inhibition of intracellular Ca^{2+} dynamics in vascular smooth muscle cells may contribute to the beneficial properties of docosahexaenoic acid on cardiovascular disorders.

Keywords: Docosahexaenoic acid; Smooth muscle cell, vascular; Ca^{2+} concentration, intracellular; Ca^{2+} channels; Cardiovascular protection

1. Introduction

There are a number of evidences indicating that diets rich in fish oils attenuate the progression of several types of human and experimental cardiovascular disorders such as myocardial infarction, arrhythmias, atherosclerosis or hypertension. $n-3$ Polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid contained in fish oils are thought to be the active biological components of these cardioprotective effects (for reviews, Leaf and Weber, 1989; Horrocks and Yeo, 1999). Although the precise mechanisms of beneficial effects of these fatty acids are still unclear, dietary administration of fish oils to genetically hypertensive rats has been shown to reduce the exaggerated contractility of the vasculature in response to sympathetic nerve stimulation or noradrenaline (Head et al., 1991; Yin et al., 1991; Chu et al., 1992; Mano et al., 1995; McLennan et al., 1996). Vasoactive effects of these fatty acids, in particular, eicosapentaenoic acid have been intensively elaborated in vitro with regard to arachidonic acid metabolites, endothelium-derived relaxing factor (EDRF), cyclic nucleotides, or intracellular Ca^{2+} as possible mediators using isolated vessel preparations (Juan and Sametz, 1986; Lawson et al., 1991; Engler, 1992a; Engler et al., 1999). However, dietary docosahexaenoic acid seems to be more effective than eicosapentaenoic acid in suppressing arrhythmias induced by ischaemia in rats, inhibiting thromboxane-like vasoconstrictor responses in aorta from spontaneously hypertensive rats (SHR), and retarding hypertension development in hypertensive rats (McLennan et al., 1996). Further, although in vitro studies have shown that both eicosapentaenoic acid and docosahexaenoic acid have vasorelaxant effects on isolated rat aortic rings (Juan and Sametz, 1986; Engler, 1992a,b; Engler et al., 1999), the effect of docosahexaenoic acid is greater than that of eicosapentaenoic acid (Engler, 1992a). Since intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle cells has a pivotal role as a second messenger in the mechanism of vasoconstriction (Karaki et al., 1997), the vasorelaxant effects of docosahexaenoic acid could be, at least in part, attributed to the effect on intracellular Ca^{2+} dynamics in vascular smooth muscle cells.

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We previously reported that dietary administration of semi-purified docosahexaenoic acid to young stroke-prone spontaneously hypertensive rats (SHRSP) for 14 weeks remarkably inhibited the development of hypertension in a dose-dependent manner (Kimura et al., 1995). Further, the $[Ca^{2+}]_i$ in vascular smooth muscle cells stimulated by 5-hydroxytryptamine (5-HT), angiotensin II and a depolarizing concentration of KCl was higher in cells isolated from SHRSP than control Wistar Kyoto rats, and was inhibited by docosahexaenoic acid treatment, although cells of SHRSP were refractory to the inhibitory effect (Hirafuji et al., 1998b). These results suggest that the inhibitory effect of docosahexaenoic acid on $[Ca^{2+}]_i$ may explain a part of the beneficial effects on cardiovascular disorders. However, its precise mechanism has been not yet fully understood. Therefore, in the present study, the effect of docosahexaenoic acid on intracellular Ca^{2+} dynamics has been further elucidated using rat vascular smooth muscle cells stimulated with 5-HT, which is considered to be associated with cardiovascular diseases because of its strong vasoconstrictive property (Frishman et al., 1995).

2. Materials and methods

2.1. Cell culture

Vascular smooth muscle cells were isolated from aortic media of male Wistar rats (6–7 weeks old) as described previously (Hirafuji et al., 1999). Briefly, the thoracic aortic media was incubated with collagenase (1.0 mg/ml) and elastase (0.5 mg/ml) for 90 min at 37 °C. Isolated cells were suspended in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and seeded on coverglasses (8 × 16 mm). Cells were cultured in the culture medium for 6–8 days with medium change every 2–3 days, and then in the culture medium containing 2% fetal calf serum for 2 days to render them quiescent. Primary cultured cells were used throughout this study.

Docosahexaenoic acid was dissolved as sodium salt in physiological saline containing 0.1% bovine serum albumin as carrier protein, further diluted and added to the culture medium. Cells were cultured for 2 days in the presence of docosahexaenoic acid.

2.2. Experimental protocols for measurement of $[Ca^{2+}]_i$

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described previously (Hirafuji et al., 1999). As the standard experimental protocol for the measurement of $[Ca^{2+}]_i$, cells untreated (control) or treated with docosahexaenoic acid were loaded with 5 μM fura-2 acetoxymethyl ester for 45 min at room temperature in Hanks' balanced salt solution containing 0.1% bovine serum albumin and 10 mM HEPES (HBSS; pH 7.4). The cells were washed three times with HBSS, and then continuously perfused at 1 ml/min with HBSS in a fluorescence spectrophotometer (Hitachi F-4000, Japan). Cells were exposed to test drugs added to HBSS, and the fluorescence of fura-2 at 505 nm emission wavelength alternately excited at 340 and 380 nm was measured.

For the measurement of Ca^{2+} release from the intracellular stores, cells loaded with fura-2 were first perfused for 3–5 min with HBSS containing 1.3 mM Ca^{2+}. The cells were then perfused with Ca^{2+}-free HBSS containing 1 mM EGTA for 3 min, and stimulated with 5-HT added to the Ca^{2+}-free buffer at least for 5 min.

For the measurement of $[Ca^{2+}]_i$ in the presence of Ni^{2+}, cells loaded with fura-2 were first perfused for 3–5 min with HBSS. The cells were then perfused with HBSS containing 1 mM Ni^{2+} for 3 min, and stimulated with 5-HT added to the same buffer at least for 5 min.

After these protocols, R_{max}, the maximal fluorescence ratio, was measured by exposing cells to 10 μM ionomycin in the presence of 5 mM Ca^{2+}, followed by perfusion with Ca^{2+}-free HBSS containing 1 mM EGTA to obtain R_{min}, the minimum ratio. The cells were finally exposed to 0.05% Triton X-100 to obtain the autofluorescence. After the subtraction of autofluorescence, $[Ca^{2+}]_i$ was calibrated according to the equation of Grynkiewicz et al. (1985), assuming the K_d of the Ca^{2+}-fura-2 interaction to be 225 nM in the cytosolic environment.

2.3. Measurement of Mn^{2+} influx

Since Mn^{2+} seems to enter the cytosol through the same pathways as Ca^{2+}, and quenches the fura-2 fluorescence, the Mn^{2+}-induced decrease in fura-2 fluorescence at 360 nm excitation wavelength, which is independent of Ca^{2+} concentration, is often used as a surrogate of Ca^{2+} to trace Ca^{2+} influx mechanisms (Simpson et al., 1990; Monteith et al., 1997). Cells loaded with fura-2 were exposed to 0.1 mM Mn^{2+} 2 min before and during stimulation with 5-HT and KCl. The decreases in 505 nm emission wavelength of fura-2 fluorescence in single cells were monitored in a digital video-imaging microscopy system (Argus 50/CA, Hamamatsu Photonics, Japan). Results were expressed as percentage change observed for 2 min before and after the stimulation, taking the initial fluorescence intensity as 100%.

2.4. Materials

Fetal calf serum, penicillin, streptomycin and Dulbecco's modified Eagle medium were obtained from Gibco; fura-2 acetoxymethyl ester, nickel chloride and HEPES from Dojin; ionomycin from Calbiochem; docosahexaenoic acid, 5-HT creatinine sulfate, collagenase and elas-

tase from Sigma; bovine serum albumin (fraction V) from Boehringer Mannheim.

2.5. Statistical analysis

Results are expressed as mean ± S.E. of replicate experiments. Statistical analysis of the results was performed using Student's *t*-test for unpaired data, Kruskal–Wallis rank test for concentration–response relationships, and Newman–Keuls multiple range test for multiple comparisons. P values less than 0.05 were considered as significant.

3. Results

3.1. Effect of docosahexaenoic acid treatment on intracellular Ca^{2+} dynamics

Vascular smooth muscle cells isolated from rat aorta were cultured for 2 days in the culture medium containing 30 μM docosahexaenoic acid, and the $[Ca^{2+}]_i$ in cells stimulated with 5-HT and a depolarizing concentration of KCl was determined. Fig. 1 demonstrated the representative tracings of intracellular Ca^{2+} dynamics induced by these stimuli. 5-HT at a concentration of 10 μM induced a biphasic intracellular Ca^{2+} dynamics, i.e., a rapid and transient increase in $[Ca^{2+}]_i$ followed by a smaller sustained increase. Depolarizing concentration of KCl at 80 mM induced a gradual increase in $[Ca^{2+}]_i$. As summarized in Fig. 2, the treatment with docosahexaenoic acid significantly inhibited the resting $[Ca^{2+}]_i$ before stimulation (Basal), the highest $[Ca^{2+}]_i$ in response to stimulation (Peak), and $[Ca^{2+}]_i$ at 5 min after stimulation (At 5 min) with 10 μM 5-HT and 80 mM KCl. The concentration-dependent effects of docosahexaenoic acid treatment on the intracellular Ca^{2+} dynamics induced by 10 μM 5-HT were demonstrated in Fig. 3. All of the three parameters in intracellular Ca^{2+} dynamics were significantly ($P < 0.05$) inhibited by docosahexaenoic acid treatment in a concentration-dependent manner.

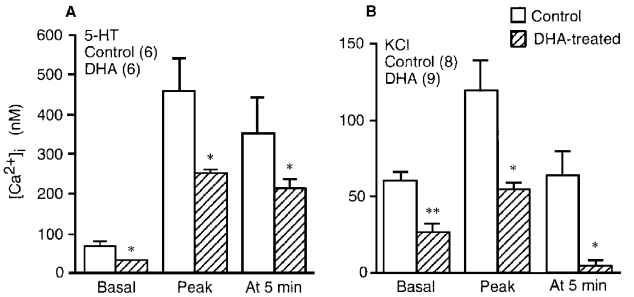

Fig. 2. Effect of docosahexaenoic acid treatment on $[Ca^{2+}]_i$ in rat vascular smooth muscle cells stimulated with 5-HT (A) and KCl (B). Cells treated without (control) or with docosahexaenoic acid (30 μM) for 2 days were stimulated with 5-HT (10 μM) or a depolarizing concentration of KCl (80 mM). Basal: the basal $[Ca^{2+}]_i$ before stimulation; Peak: the peak $[Ca^{2+}]_i$ after stimulation; After 5 min: $[Ca^{2+}]_i$ 5 min after stimulation. Each column represents mean ± S.E. of (n) experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. each control.

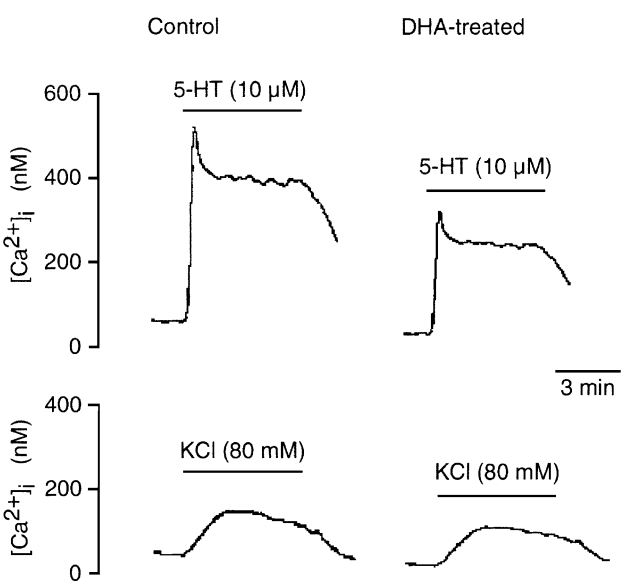

Fig. 1. Effect of docosahexaenoic acid treatment on 5-HT and KCl-induced intracellular Ca^{2+} dynamics in rat vascular smooth muscle cells in culture. Cells treated without (control) or with docosahexaenoic acid (30 μM) for 2 days were stimulated with 5-HT (10 μM) or a depolarizing concentration of KCl (80 mM). Tracings of a representative experiment of each stimulus.

3.2. Effect of docosahexaenoic acid treatment on Ca^{2+} release from internal stores

Fig. 4 demonstrated the effect of docosahexaenoic acid (30 μM) treatment on Ca^{2+} release from the internal stores in vascular smooth muscle cells. As shown in Fig. 4A, 5-HT (10 μM) induced a small transient increase in

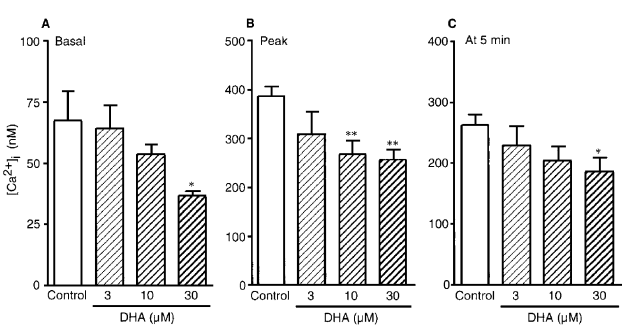

Fig. 3. Concentration-dependent effect of docosahexaenoic acid treatment on $[Ca^{2+}]_i$ in rat vascular smooth muscle cells stimulated with 5-HT. Cells treated without (control) or with the indicated concentration of docosahexaenoic acid for 2 days were stimulated with 5-HT (10 μM). Basal (A): the basal $[Ca^{2+}]_i$ before stimulation; Peak (B): the peak $[Ca^{2+}]_i$ after stimulation; After 5 min (C): $[Ca^{2+}]_i$ 5 min after stimulation. Each column represents mean ± S.E. of 6, 4, 6 and 6 experiments for control, 3, 10 and 30 μM docosahexaenoic acid, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. each control.

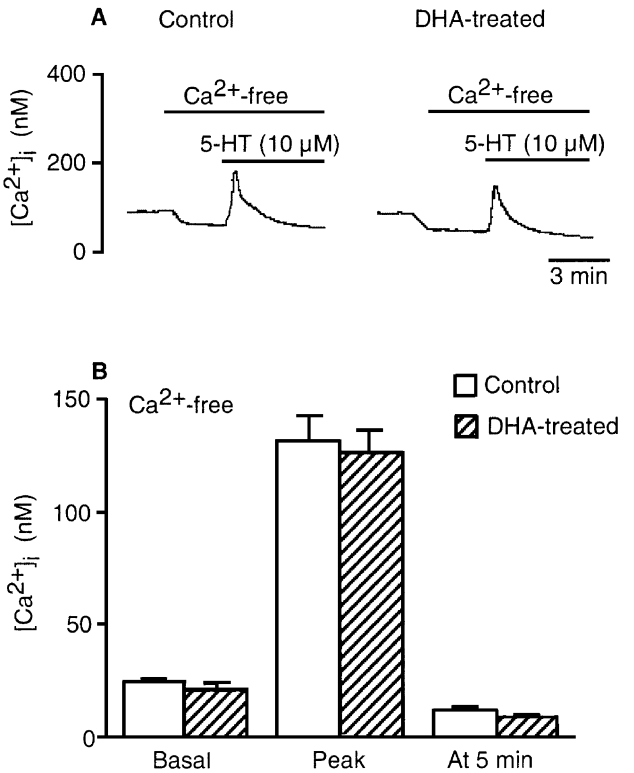

Fig. 4. Effect of docosahexaenoic acid treatment on Ca^{2+} mobilization from internal stores in rat vascular smooth muscle cells stimulated with 5-HT. Cells treated without (control) or with docosahexaenoic acid (30 μM) for 2 days were preincubated with Ca^{2+}-free buffer (+1 mM EGTA) 3 min before and during stimulation with 5-HT (10 μM). (A) Representative tracings showing the Ca^{2+} release from the internal stores. (B) Summary of the effect of docosahexaenoic acid treatment on intracellular Ca^{2+} dynamics. Basal: the basal $[Ca^{2+}]_i$ before stimulation; Peak: the peak $[Ca^{2+}]_i$ after stimulation; After 5 min: $[Ca^{2+}]_i$ 5 min after stimulation. Each column represents mean ± S.E. of 12 and 10 experiments for control and docosahexaenoic acid, respectively.

$[Ca^{2+}]_i$ in Ca^{2+}-free HBSS containing 1 mM EGTA in both control and docosahexaenoic acid-treated cells. There are no significant differences in intracellular Ca^{2+} dynamics between these groups, as summarized in Fig. 4B.

3.3. Effect of docosahexaenoic acid treatment on Mn^{2+} influx

The effects of docosahexaenoic acid treatment on extracellular Ca^{2+} influx into vascular smooth muscle cells stimulated with 5-HT and KCl were then investigated by Mn^{2+} quenching method. As shown in Fig. 5, when cells were stimulated with 10 μM 5-HT and 80 mM KCl in the presence of Mn^{2+} (0.1 mM), the rate of Mn^{2+} influx as estimated by the decrease in the fura-2 fluorescence was significantly increased, indicating the activation of cation channels. Docosahexaenoic acid (30 μM) treatment significantly inhibited the rate of Mn^{2+} influx induced by these stimuli.

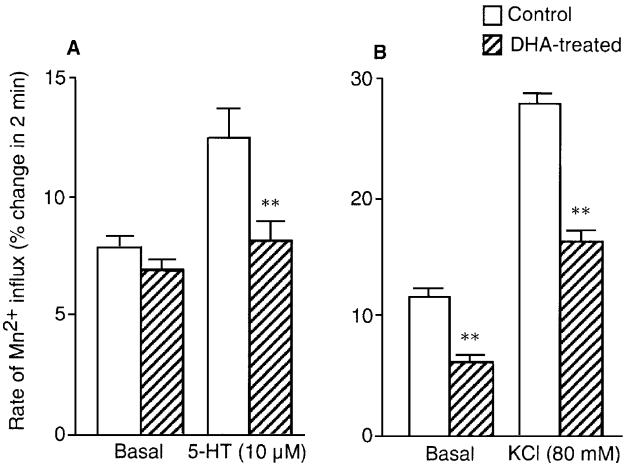

Fig. 5. Effect of docosahexaenoic acid treatment on rate of Mn^{2+} influx into rat vascular smooth muscle cells stimulated with 5-HT and KCl. Cells treated without (control) or with docosahexaenoic acid (30 μM) for 2 days were stimulated with 5-HT (10 μM) or a depolarizing concentration of KCl (80 mM). Rate of Mn^{2+} influx was expressed as percentage change in fura-2 fluorescence intensity (505 nm) at 360 nm excitation wavelength, taking the initial fluorescence level as 100%. Each column represents mean ± S.E. of randomly selected 14 single cells. $^{**}P < 0.01$ vs. each control.

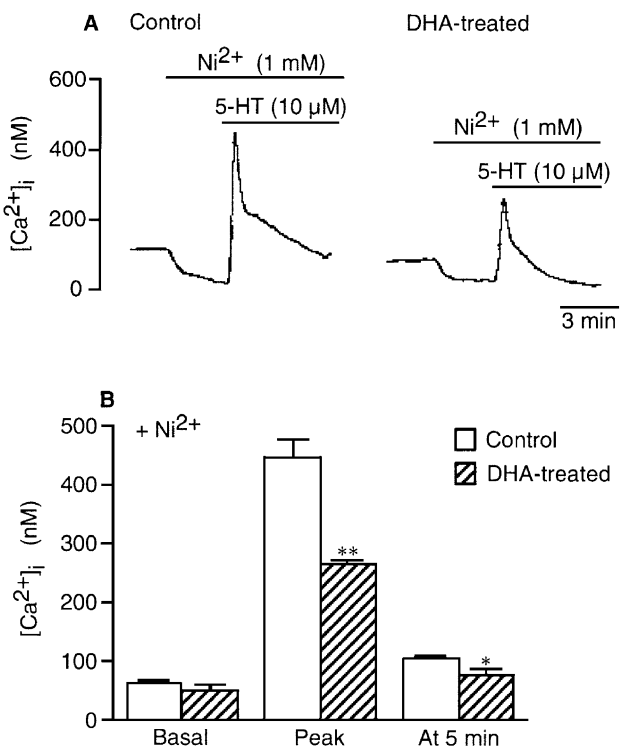

Fig. 6. Effect of docosahexaenoic acid treatment on Ni^{2+}-insensitive Ca^{2+} channels in rat vascular smooth muscle cells stimulated with 5-HT. Cells treated without (control) or with docosahexaenoic acid (30 μM) for 2 days were preincubated with HBSS containing Ni^{2+} (1 mM) 3 min before and during stimulation with 5-HT (10 μM). (A) Representative tracings showing intracellular Ca^{2+} dynamics in the presence of Ni^{2+}. (B) Summary of the effect of docosahexaenoic acid treatment. Basal: the basal $[Ca^{2+}]_i$ before stimulation; Peak: the peak $[Ca^{2+}]_i$ after stimulation; After 5 min: $[Ca^{2+}]_i$ 5 min after stimulation. Each column represents mean ± S.E. of five and six experiments for control and docosahexaenoic acid, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. each control.

3.4. Effect of docosahexaenoic acid treatment on Ni^{2+}-insensitive Ca^{2+} channels

As demonstrated in Fig. 6A, in both control and docosahexaenoic acid-treated cells, 5-HT induced a transient Ca^{2+} influx through Ni^{2+}-insensitive Ca^{2+} channels, which were distinguishable from the voltage-dependent or store-operated Ca^{2+} channels in vascular smooth muscle cells (Hirafuji et al., 1999). Fig. 6B summerized the effects of docosahexaenoic acid (30 μM) treatment on intracellular Ca^{2+} dynamics observed in the presence of 1 mM Ni^{2+}. Docosahexaenoic acid treatment significantly inhibited the peak response and the $[Ca^{2+}]_i$ level at 5 min after the 5-HT stimulation, indicating that docosahexaenoic acid inhibited Ca^{2+} influx through the Ni^{2+}-insensitive Ca^{2+} channels.

4. Discussion

In the present study, we have investigated the effect of docosahexaenoic acid treatment on intracellular Ca^{2+} dynamics induced by 5-HT in rat vascular smooth muscle cells in culture. When cells were pretreated with docosahexaenoic acid added to the culture medium for 2 days, the resting $[Ca^{2+}]_i$ before 5-HT stimulation, peak response of $[Ca^{2+}]_i$, and $[Ca^{2+}]_i$ at 5 min after the stimulation were significantly inhibited in a concentration-dependent manner. Receptor stimulation with 5-HT of vascular smooth muscle cells induces both the release of Ca^{2+} from intracellular store(s) and the influx of extracellular Ca^{2+} through Ca^{2+} channels on the cell membrane (Capponi et al., 1987; Wang et al., 1991; Hirafuji et al., 1998c, 1999). Docosahexaenoic acid treatment has no inhibitory effect on the 5-HT-induced inositol 1,4,5-triphosphate (IP_3) production by vascular smooth muscle cells (Hirafuji et al., 1998b). The present study also demonstrated that docosahexaenoic acid had no effect on the Ca^{2+} transient induced by 5-HT in the absence of extracellular Ca^{2+}. Therefore, it is ruled out the possibility that the inhibitory effect of docosahexaenoic acid is due to the inhibition of Ca^{2+} release from the intracellular store(s).

5-HT has been shown to stimulate voltage-dependent L-type Ca^{2+} channels in vascular smooth muscle cells, by using the whole-cell voltage clamp method (Hirakawa et al., 1995). Since docosahexaenoic acid treatment inhibited the KCl-induced intracellular Ca^{2+} dynamics and Mn^{2+} influx, it is suggested that docosahexaenoic acid inhibited receptor-mediated Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels. In consistency with our result, the inhibition of voltage-dependent L-type Ca^{2+} current or $^{45}Ca^{2+}$ uptake by docosahexaenoic acid has been reported not only in vascular smooth muscle cells (Asano et al., 1997), but also in tracheal smooth muscle cells (Hazama et al., 1998) and cardiac myocyte (Hallaq et al., 1992; Pepe et al., 1994; Xiao et al., 1997). However, the voltage-dependent L-type Ca^{2+} channels in vascular smooth muscle cells seem to be involved mainly in the sustained Ca^{2+} influx, since L-type Ca^{2+} channel blockers such as diltiazem, nifedipine or Ni^{2+}, a non-selective cation channel blocker, hardly suppress the 5-HT-induced transient Ca^{2+} influx (Capponi et al., 1987; Wang et al., 1991; Hirafuji et al., 1998c, 1999). Therefore, our result indicates that the inhibitory effect of docosahexaenoic acid on the Ca^{2+} transient induced by 5-HT is due to the inhibition of Ca^{2+} influx through the voltage-independent channels.

Voltage-independent Ca^{2+} channels have been classified in two main channels, i.e., receptor-operated channels and store-operated channels in vascular smooth muscle cells (Hughes, 1995; Karaki et al., 1997). Docosahexaenoic acid seems to have no effect on store-operated Ca^{2+} channels, since this fatty acid has no effect on the $[Ca^{2+}]_i$ change induced by thapsigargin (Hirafuji et al., 1998a), which induces Ca^{2+} influx through store-operated Ca^{2+} channels by inhibiting sarcoplasmic reticulum Ca^{2+}-ATPase (Xuan et al., 1992). Our recent study has shown that 5-HT, but not angiotensin II, induces transient Ca^{2+} influx through Ni^{2+}-insensitive Ca^{2+} channels, which are distinguishable from the voltage-dependent or store-operated Ca^{2+} channels (Hirafuji et al., 1999). Since this effect is mediated via 5-HT_2 receptor subtype coupled to a G protein, the Ni^{2+}-insensitive channels may be one of receptor-operated Ca^{2+} channels, which appear to comprise a heterogeneous family of channels regulated by a variety of second messengers such as IP_3, inositol tetraphosphates (IP_4), cyclic GMP, protein kinase C or Ca^{2+} itself (Hughes, 1995). In a recent electrophysiological study, docosahexaenoic acid, when applied acutely to the cells, has been also shown to effectively inhibit receptor-mediated non-selective cation currents, which are resistant to Ca^{2+} channel blockers, in rat A7r5 vascular smooth muscle cells stimulated with vasopressin and endothelin (Asano et al., 1997). In contrast to 5-HT, vasopressin and endothelin have been shown to promote a transient Ca^{2+} influx mainly through the non-L-type and receptor-mediated Ca^{2+} entry pathways, which are inhibited by Ni^{2+} in vascular smooth muscle cells (Simpson et al., 1990). Taken together with these studies, our result with Ni^{2+} suggests that 5-HT receptor-stimulated Ca^{2+} channels inhibited by docosahexaenoic acid treatment are not the same subclass as the cation channels opened by stimulation of vasopressin and endothelin receptors.

It is shown that acutely applied docosahexaenoic acid inhibits the L-type Ca^{2+} currents induced by vasopressin and endothelin (Pepe et al., 1994; Asano et al., 1997). These inhibitory effects of docosahexaenoic acid are acutely irreversible after washing out by a buffer containing bovine serum albumin. It is suggested that the primary site of the acute inhibitory action of $n-3$ polyunsaturated fatty acids is the Ca^{2+} channel protein itself or some site near the channels (Asano et al., 1997). In the present study, after the treatment of cells with docosahexaenoic

acid in the culture medium, the cells were washed out, incubated in HBSS containing 0.1% bovine serum albumin to load fura-2, and superfused with the HBSS before 5-HT stimulation. Therefore, the mechanism of inhibitory effect of docosahexaenoic acid added to the culture is possibly different from that of acutely applied docosahexaenoic acid. When added to the culture medium, docosahexaenoic acid can be easily incorporated into the membrane phospholipids (Morisaki et al., 1985), and increases membrane cholesterol efflux (Dusserre et al., 1995) and membrane fluidity (Hashimoto et al., 1999) in vascular smooth muscle or endothelial cells. These alterations would directly or indirectly affect downstream signalling pathway after 5-HT receptor stimulation or expression of ion channel proteins. Docosahexaenoic acid treatment has no effect on $[Ca^{2+}]_i$ change induced by thapsigargin (Hirafuji et al., 1999a), suggesting that the effect of docosahexaenoic acid is not due to the nonspecific inhibitory effects on membrane properties. Nor is it a cytotoxic effect, since docosahexaenoic acid at 30 μM shows no morphological changes and no effect on cell proliferation and size in our experimental condition (Hirafuji et al., 1998a). Finally, the maximal concentration (30 μM) of docosahexaenoic acid used in the present study is close to that in the plasma of rat fed diet containing 5% docosahexaenoic acid for 14 weeks (Minami et al., 1997).

In conclusion, the present study demonstrated that, when added to the culture medium, docosahexaenoic acid inhibited Ca^{2+} influx through Ni^{2+}-insensitive receptor-operated Ca^{2+} channels in addition to the voltage-dependent channels in rat vascular smooth muscle cells stimulated with 5-HT. Since the intracellular Ca^{2+} in vasuclar smooth muscle cells plays an important role in regulating vascular tone, it is suggested that the specific inhibition of intracellular Ca^{2+} dynamics in these cells may contribute to the beneficial properties of docosahexaenoic acid on cardiovascular disorders.

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