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Original

Selective and potent inhibitory effect of docosahexaenoic acid (DHA) on U46619-induced contraction in rat aorta

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Abstract

Inhibitory effects of docosahexaenoic acid (DHA) on blood vessel contractions induced by various constrictor stimulants were investigated in the rat thoracic aorta. The inhibitory effects of DHA were also compared with those of eicosapentaenoic acid (EPA) and linoleic acid (LA). DHA exhibited a strong inhibitory effect on the sustained contractions induced by U46619, a TXA_2 mimetic. This inhibitory effect of DHA was not affected by removal of the endothelium or by treatment with either indomethacin or N^{ω}-nitro-L-arginine. DHA also significantly diminished $PGF_{2\alpha}$-induced contraction but did not show any appreciable inhibitory effects on the contractions to both phenylephrine (PE) and high-KCl. Similarly, EPA exhibited significant inhibitory effects against the contractions induced by both U46619 and $PGF_{2\alpha}$ without substantially affecting either PE- or high-KCl-induced contractions. However, both DHA and EPA generated more potent inhibitions against contractions induced by U46619 than those by $PGF_{2\alpha}$. In contrast, LA did not show significant inhibitory effects against any contractions, including those induced by U46619. The present findings suggest that DHA and EPA elicit more selective inhibition against blood vessel contractions that are mediated through stimulation of prostanoid receptors than those through α-adrenoceptor stimulation or membrane depolarization. Although DHA and EPA have similar inhibitory potencies against prostanoid receptor-mediated contractions, they had a more potent inhibition against TXA_2 receptor (TP receptor)-mediated contractions than against $PGF_{2\alpha}$ receptor (FP receptor)-mediated responses. Selective inhibition by either DHA or EPA of prostanoid receptor-mediated blood vessel contractions may partly underlie the mechanisms by which these ω-3 polyunsaturated fatty acids exert their circulatory-protective effects.

Key words: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), ω-3 polyunsaturated fatty acid (ω-3 PUFA), thromboxane A_2 (TXA_2), vascular smooth muscle

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Introduction

Docosahexaenoic acid (DHA; 22:6 n-3) is an ω-3 polyunsaturated fatty acid (PUFA). This PUFA together with eicosapentaenoic acid (EPA; 20:5 n-3), another representative ω-3 PUFA, constitutes the major components of the PUFA contained in fish oil. These ω-3 PUFAs differ only slightly from arachidonic acid (20:4 n-6), which is also a PUFA constituent of fish oil although categorized as an ω-6 PUFA due to its carbon chain length and the number and position of its double bonds. However, it seems that these marginal structural variations lead to remarkable diversity in physiological or pharmacological effects of these PUFAs.

To date, the advantages of the dietary intake of fish or fish oil against circulatory diseases have been suggested as a result of numerous epidemiological studies and clinical trials. For instance, intake of fish/fish oil has been shown to protect against coronary heart disease (1–3), atherosclerosis (4, 5), and stroke (6). Furthermore, a blood pressure-lowering effect was shown in hypertensive patients, but not in normotensive individuals (7–9). These circulatory-protective effects of fish oil may be partly ascribed to the blood vessel relaxation attained with DHA and/or EPA. In support of this presumption, both DHA and EPA were reported to produce relaxant responses in isolated vascular tissues (10–13). However, in these studies, the relaxant effects of DHA and EPA were mainly examined against the contractions induced by α-adrenoceptor stimulation or by depolarizing high-KCl solution. Furthermore, it seems unlikely that the blood vessel relaxant effects of these ω-3 PUFAs reported in these studies adequately explain their blood pressure decreasing effects in hypertensive patients; with a concentration of 10^{-5} M, the 30% reduction in muscle tension would seem to be inadequate to account for the observed blood pressure decrease. Therefore, if the vascular relaxation and subsequent blood vessel dilatation underlie the mechanisms by which DHA and EPA exert their protective effects against cardiovascular diseases, there may be a further yet-to-be-defined mechanism involved that would support this assumption.

With regard to the above mentioned concept, we have previously reported that DHA more selectively diminishes thromboxane A_2 (TXA_2) receptor (TP receptor)-mediated contractions than α-adrenoceptor-mediated responses in guinea-pig aortic smooth muscle (14). However, the detailed mechanisms responsible for this finding are still to be clarified. However, TXA_2, a powerful vasoconstrictor, is suggested to play a causative role in the pathogenesis of hypertension (15, 16), and its production is elevated in hypertension as a result of stimulation by angiotensin II (Ang II) (17). Thus, if selective inhibition of TP receptor-mediated contractions by DHA is not limited to guinea-pig blood vessels and is a common phenomenon attained in all blood vessels, the possible circulatory-protective effects of ω-3 PUFAs including DHA may be strengthened with further experimental evidence obtained from a different preparation from another species. With this background, we carried out this study to determine whether DHA also more selectively suppresses TP receptor-mediated contractions in the rat aorta. In this study, the inhibitory effects of DHA against various vasoconstrictor stimulants were compared with those of EPA, and linoleic acid (LA), an ω-6 PUFA that is abundant in vegetable oils, to reveal whether DHA is more potent than either EPA or LA in suppressing TP receptor-mediated contraction.

Methods

Animals

Male Wistar rats (8 – 9 weeks old, weighing 180 – 230 g, Sankyo Labo Service, Tokyo, Japan) were housed under controlled conditions (temperature 21 – 22°C, relative air humidity 50 ± 5%, fixed 12h-light (08:00 to 20:00)/12h-dark cycle). Food and water were available *ad libitum* to all animals. This study was conducted in

accordance with the Guideline for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (accredited by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan).

Preparation of rat thoracic aortic rings

Wistar rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) and killed by decapitation. A section of the thoracic aorta between the aortic arch and diaphragm was isolated and placed in normal Tyrode's solution (mM): NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0 and glucose, 5.6. The aorta was cleaned of loosely adhering fat and connective tissues, and cut into ring segments of about 2 mm in length. The endothelium was removed by rubbing the intimal surface gently with an eyebrow brush. In some experiments, endothelium-intact ring segments were carefully prepared so that the intimal surface of the blood vessel segments was not damaged.

Measurement of tension changes

The aortic ring segments were mounted using stainless steel hooks (outer diameter, 150 – 200 μm) with an optimal resting tension of 1.0 g in a 5-ml organ bath (UC-5; UFER Medical Instrument, Kyoto, Japan) containing normal Tyrode's solution. Normal Tyrode's solution was continuously gassed with 95% O_2 – 5% CO_2, and kept at 35.0 ± 1.0°C (pH = 7.4). Muscle tension changes were isometrically recorded with a force-displacement transducer (T7-8-240; Orientec, Tokyo, Japan) connected to a minipolygraph (Signal Conditioner: Model MSC-2; Primetech Corp., Tokyo, Japan). Aortic tension changes were recorded with PowerLab/ML-846™ and Chart™ (Version 7.0) software (ADInstruments Japan, Tokyo, Japan). Before starting the tension change experiments by using various chemical stimulants, ring preparations were equilibrated for 60 min with bathing solution (normal Tyrode's solution) being exchanged with a fresh solution every 20 min.

After a 60-min equilibration period, to make sure that aortic preparations were capable of generating normal contractile responses, they were contracted with high-KCl (8×10^{-2} M) Tyrode's solution (mM): NaCl, 82.3; KCl, 80.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0 and glucose, 5.6. Then, high-KCl solution was replaced with normal Tyrode's solution and when the muscle tension returned to a basal tension level, the absence of endothelium was confirmed by the lack of relaxation in response to acetylcholine (ACh, 10^{-5} M) in the preparation pre-contracted with noradrenaline (NA, 3×10^{-7} M). When endothelium-intact preparations were used, they were considered as endothelium-intact if their relaxant responses to 10^{-5} M ACh substantially exceeded 75%. After this procedure, the bathing solution was exchanged with fresh Tyrode's, and the aortic ring preparations subsequently left to re-equilibrate for a further 40 min.

Evaluation of inhibitory effects of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and linoleic acid (LA) on various vascular contractions

To investigate the inhibitory effects of post-treated PUFAs (DHA, EPA and LA), aortic ring preparations were pre-contracted with U46619 (5×10^{-9} M), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (10^{-5} M), phenylephrine (PE) (3×10^{-7} M) or high-KCl (8×10^{-2} M) to produce sustained contractions. After the muscle contractions reached a steady-state level, DHA, EPA or LA (10^{-6} – 3×10^{-5} M for all) was applied to the bath medium at a desired single concentration. At the end of experiments, to confirm the substantially maximal inhibitory response, SQ 29,548 (a TP receptor antagonist, 10^{-7} M) or papaverine (10^{-4} M) was applied. Inhibitory effects of DHA, EPA and LA on the sustained vascular contractions were expressed as a percentage relaxation; they were calculated by considering the tension level just before addition of PUFAs as 0% relaxation, and the basal tension level

before application of vasoconstrictor stimulations (U46619, $PGF_{2\alpha}$, PE, high-KCl) as 100% relaxation. When the sustained muscle tension levels attained with these vasoconstrictor stimulants were required to be shown, they were expressed as relative contraction to the high-KCl-induced muscle tension level obtained at the beginning of the experiments.

When the effects of the pretreatment effects with PUFAs were investigated against the vascular contractions, aortic rings were firstly contracted for 20 min with desired constrictors. Tested constrictors were: U46619 (10^{-8} M), $PGF_{2\alpha}$ (10^{-5} M), PE (3×10^{-7} M), NA (10^{-7} M), 5-hydroxytryptamine (5-HT, 10^{-5} M) and high-KCl (8×10^{-2} M). When the tension levels returned close to basal level, DHA (10^{-5} M) or its vehicle (pure ethanol; final bath concentration less than 0.3%) was applied 40 min before a subsequent second application of the constrictor stimulation. When the pretreatment inhibitory effects of DHA were evaluated, the contractile responses to the second stimulant application in the presence of either the vehicle or DHA (10^{-5} M) were expressed as a % of the contraction produced by the first application of the stimulant.

All experiments to examine the smooth muscle-direct effects of PUFAs with endothelium-denuded preparations were carried out in the presence of indomethacin (Indo) (3×10^{-6} M) to rule out the possible contribution of endogenous prostanoids. In addition, in some experiments using endothelium-intact preparations, to clarify the possible roles for endothelium-derived prostacyclin (PGI_2) and NO in the inhibitory effects of DHA on U46619-induced contraction, the effects of inhibitors which affect their synthesis were investigated; tested inhibitors were Indo (3×10^{-6} M) and N^{ω}-nitro-L-arginine (L-NNA) (5×10^{-5} M).

Drugs

The followings drugs were used: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619), [1S-[1α,2 α(Z),3 α,4 α]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29,548) (Cayman Chemical, Ann Arbor, M.I., USA); L-phenylephrine hydrochloride, indomethacin (Indo), and N^{ω}-nitro-L-arginine (L-NNA) (Sigma-Aldrich Co., St. Louis, M.O., USA); L-noradrenaline (NA) hydrogen tartrate monohydrate, serotonin (5-hydroxytryptamine, 5-HT) creatinine sulfate, papaverine (PPV) hydrochloride (Wako Pure Chemical Industries, Osaka, Japan); acetylcholine (ACh) chloride (Daiichi Sankyo, Tokyo, Japan); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fuji Pharma Co., Ltd, Tokyo, Japan). All other chemicals were commercially available and of reagent grade.

DHA, EPA, and LA were dissolved in pure ethanol as a stock solution at 10^{-2} M, and diluted further with distilled water to the desired concentrations. U46619 was dissolved in 70% ethanol as a stock solution at 10^{-4} M. SQ 29,548 was dissolved in 70% ethanol as a stock solution at 10^{-3} M. Indo was dissolved in pure ethanol as a stock solution at 10^{-2} M. Final ethanol concentration in the bath medium did not exceed 0.3%, which did not affect the vascular responses. NA was dissolved in ascorbic acid solution (10^{-3} M) to prepare solutions of 10^{-5} – 10^{-4} M. All other drugs were prepared as aqueous solution and diluted with distilled water.

Statistical Analysis

Results are presented as mean values ± S.E.M. and *n* refers to the number of experiments. Significance of differences between means was evaluated using either an unpaired Student's *t*-test or unpaired Student's *t*-test with Welch's correction if necessary, or a one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism™ (version 4.00; GraphPad Software, San Diego, C.A., USA). *P* values of less than 0.05 were considered to be statistically significant.

Results

Inhibitory effects of DHA on the sustained contraction to U46619 and the role for endothelium

We first investigated whether DHA was capable of inhibiting TP receptor-mediated contractions in the rat thoracic aorta. Fig. 1A is a typical trace showing the effects of DHA on a sustained contraction resulting from the application of U46619 (5×10^{-9} M) in an endothelium-intact preparation. The functional presence of an endothelium in this preparation was confirmed by the almost full relaxation resulting from ACh (10^{-5} M) being applied against the contraction to NA (3×10^{-7} M). In this preparation, DHA (10^{-5} M) practically abolished the entire sustained contraction induced by U46619 (5×10^{-9} M), and application of SQ 29,548 (10^{-7} M), a TP receptor antagonist, did not elicit any further inhibition. The inhibitory effect against the U46619-induced sustained contraction was not obtained by the vehicle for DHA (data not shown).

Fig. 1B is a trace showing the effects of DHA on a U46619-induced contraction in an endothelium-denuded preparation. The absence of the endothelium in this preparation was ascertained by the disappearance of an ACh-induced relaxation against a NA-induced contraction. In this preparation, DHA (10^{-5} M) also almost entirely abolished the U46619-induced sustained contraction. As shown in the recording (Fig. 1B), DHA-induced inhibitory effects seemed to consist of two relaxant components: a relatively slowly-developing phase that was observed within 10 min after the application of DHA, and a steeply-developing phase that led to the full relaxant response. At present, we cannot explain these phenomena, and thus our data analysis in the present study focused on the full relaxant response that was obtained 60 min after the application of DHA.

The possible contribution of the endothelium to the DHA effect was also examined by investigating the effects of inhibitors that interrupt the synthesis of vaso-relaxant factors on the DHA-induced response, and a representative trace showing the result was illustrated in Fig. 1C. In this experiment, an endothelium-intact preparation was used, and an almost full inhibitory response was found to be attained by DHA even in the combined presence of both Indo (3×10^{-6} M) and L-NNA (5×10^{-5} M). Fig. 1D summarizes the inhibitory effects of DHA (10^{-5} M) on the sustained contractions induced by U46619 (5×10^{-9} M) under three conditions mentioned above. Fig. 1D clearly shows that the DHA-induced inhibitory effect against a U46619-induced contraction was not affected by removal of the endothelium, or by inhibitors of both cyclooxygenase (COX) and NO synthase (NOS).

Investigation of possible role for endothelium in DHA-induced inhibitory effect on the sustained contraction to NA

We have previously shown that DHA diminishes TP receptor-mediated contractions more selectively than α-adrenoceptor-mediated responses in the guinea-pig aorta (14). Hence, it is plausible that the inhibitory effect of DHA against U46619-induced sustained contraction is largely attributed to an interaction with TP receptor-related events including direct antagonistic action against this prostanoid receptor. If this assumption is applicable to the endothelium-intact preparation and such factors predominate, a possible role of the endothelium might not be detected when the blood vessel is contracted with U46619. Therefore, we further investigated this possibility by using an endothelium intact NA-contracted preparation, and results are shown in Fig. 2.

We confirmed that the preparation shown in Fig. 2 was endothelium-intact since an almost full relaxation was attained with ACh (10^{-5} M) against a NA (3×10^{-7} M)-induced contraction. The preparation was contracted again with NA (3×10^{-7} M) and DHA (10^{-5} M) was applied. However, DHA did not show any inhibitory effects against the NA-induced contraction during the 60-min observation period after its application. This preparation could be relaxed by ACh (10^{-5} M) applied at the end of experiment. When seeing the trace in Fig.

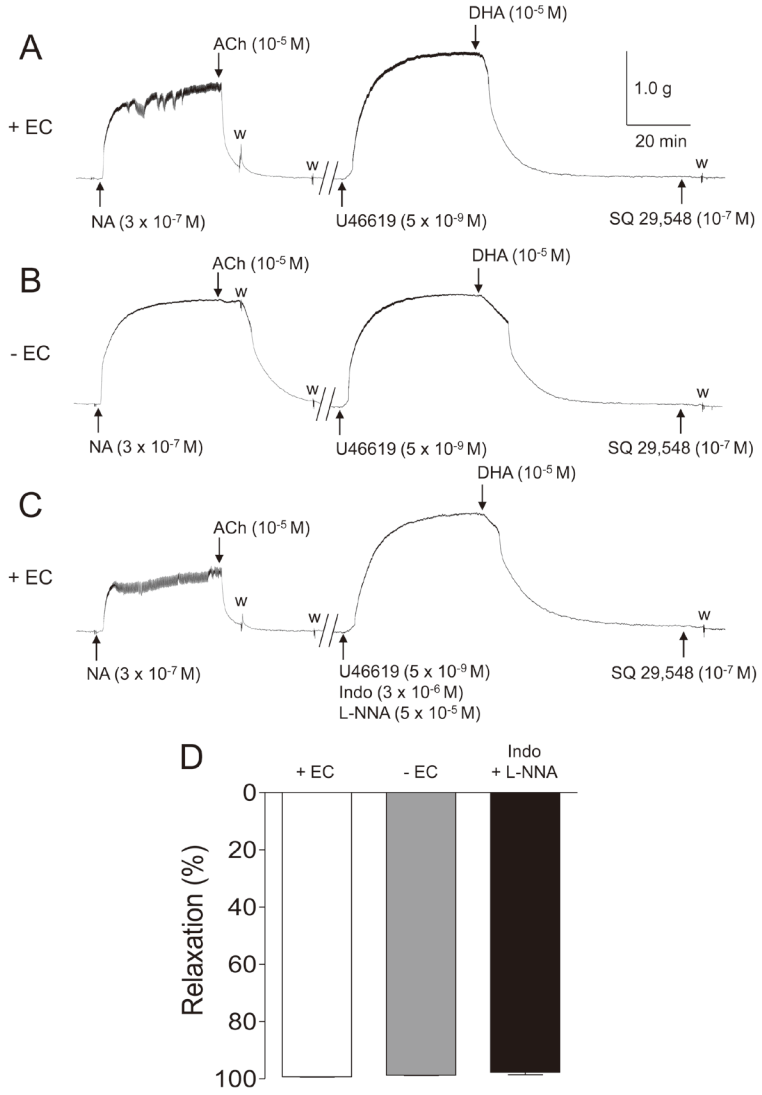

Fig. 1. Inhibitory effects of DHA on the sustained contraction to U46619 in segments of the rat thoracic aorta. A–C: Typical traces showing the inhibitory effects of DHA *vs*. U46619. Firstly, acetylcholine (ACh, 10^{-5} M) was applied during the contraction to noradrenaline (NA, 3×10^{-7} M). The preparations resulting in traces A and C were judged to be endothelium-intact (+EC) since ACh produced an almost full relaxation against the NA-induced contraction. The preparation used for trace B was verified as endothelium-denuded (-EC) by the disappearance of ACh-induced relaxation. Inhibitors were not present in A and B whereas both indomethacin (Indo, 3×10^{-6} M) and N^{ω}-nitro-L-arginine (L-NNA, 5×10^{-5} M) were present in C. w: wash of preparation with fresh medium. D: Summarized data showing the inhibitory effects of DHA *vs*. U46619. Data are shown as mean values ± S.E.M. ($n = 4$ for each). No significant differences were found among the three groups.

2, it appeared that DHA had elicited a further contraction to that induced by NA. However, this tension change was judged not to be produced by DHA itself since a similar tension change occurred in the preparation treated with DHA vehicle (data not shown). In this case, the degree of the second NA-induced contraction was smaller than that of the first contractile response. Although the exact reason explaining this phenomenon is not known, this might be partly related to the fact that endothelium is preserved in this preparation since endothelium-denuded preparation shows relatively similar degree of contractile responses to repeated stimulations with NA.

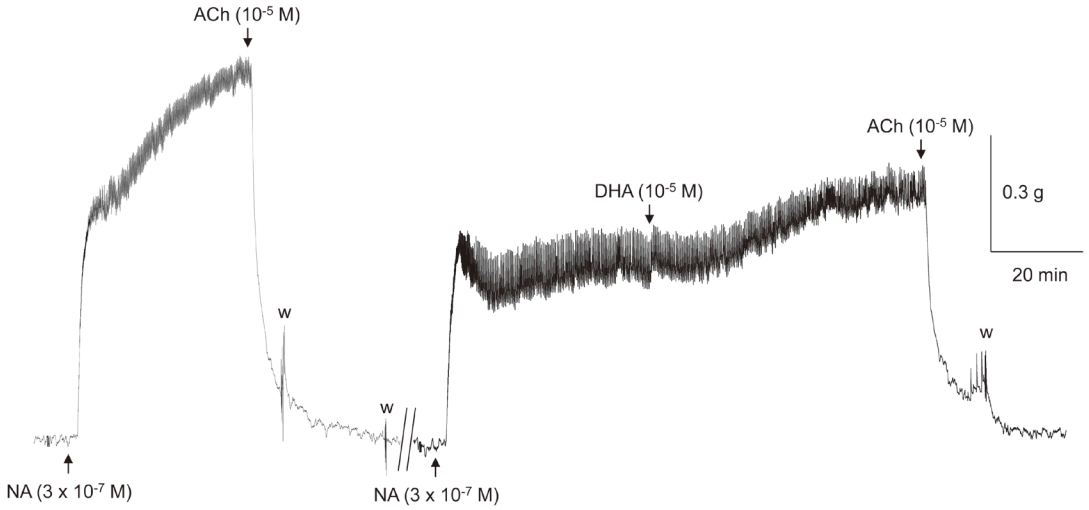

Fig. 2. A typical trace showing the effects of DHA on NA-induced sustained contractions in preparations of the rat aorta with intact endothelium. The preparation used was endothelium-intact since an almost full relaxation was produced by ACh (10^{-5} M) during the sustained contraction to NA (3×10^{-7} M). Sustained contraction was again induced by NA (3×10^{-7} M), and DHA (10^{-5} M) was applied to the bath medium. To examine whether DHA exerted any effects on NA-induced contraction, tension changes were recorded for 60 min after DHA application. To verify the functional integrity of endothelium, appearance of relaxant response to ACh (10^{-5} M) was confirmed at the end of experiments. Tension changes were recorded in the absence of Indo and L-NNA. Similar experiments were performed in total $n = 4$ preparations. w: wash of preparation with fresh medium.

Comparison of the effects of DHA against sustained contractions induced by various chemical stimulants

The next series of experiments were carried out to investigate whether the inhibitory effect of DHA against a sustained contraction is attained exclusively when the blood vessel smooth muscle is stimulated with a TP receptor agonist, or whether this ω-3 PUFA also exhibits inhibition against other stimulants. To this end, we tested the following stimulants: U46619 (TP receptor agonist), $PGF_{2\alpha}$ (FP receptor agonist), phenylephrine (PE, α_1-adrenoceptor agonist), noradrenaline (NA) and high-KCl (depolarizing stimulus). In this series of experiments, 5-hydroxytryptamine (5-HT) was not used as a constrictor. This is because 5-HT did not elicit a stable long-lasting muscle tone that was required to be attained to evaluate appropriately the inhibitory effects of DHA. Endothelium-denuded preparations were used in this series of experiments to clearly detect smooth muscle-direct effect of DHA.

Fig. 3 illustrates typical traces showing the effects of DHA on sustained contractions induced by various stimulants. Similarly against the contraction to U46619 (5×10^{-9} M) (Fig. 3A), DHA at 10^{-5} M strongly diminished a $PGF_{2\alpha}$ (10^{-5} M)-induced sustained contraction (Fig. 3B). However, it appeared that DHA required longer times to reach a steady-state maximum inhibition against a $PGF_{2\alpha}$-induced contraction than against a contraction induced by U46619. To make sure whether this notion was significant, we tentatively calculated the time that was needed to reach 50% response of the maximum inhibition (T_{IC50}). The times were calculated to be 11.6 ± 1.2 min ($n = 4$) *vs.* U46619 and 21.6 ± 2.6 min ($n = 4$) *vs.* $PGF_{2\alpha}$, and shown to be significantly different to each other ($P < 0.05$). In contrast, DHA did not show any appreciable inhibitory effects against the contractions to PE (3×10^{-7} M) (Fig. 3C) and high-KCl (8×10^{-2} M) (Fig. 3D). As shown in Fig. 3C and Fig. 3D, both contractions to PE and high-KCl were diminished to near basal tension level by papaverine (PPV, 10^{-4} M). Fig. 3E summarizes the results shown in Fig. 3A-D together with the results against NA (10^{-7} M)-induced contrac-

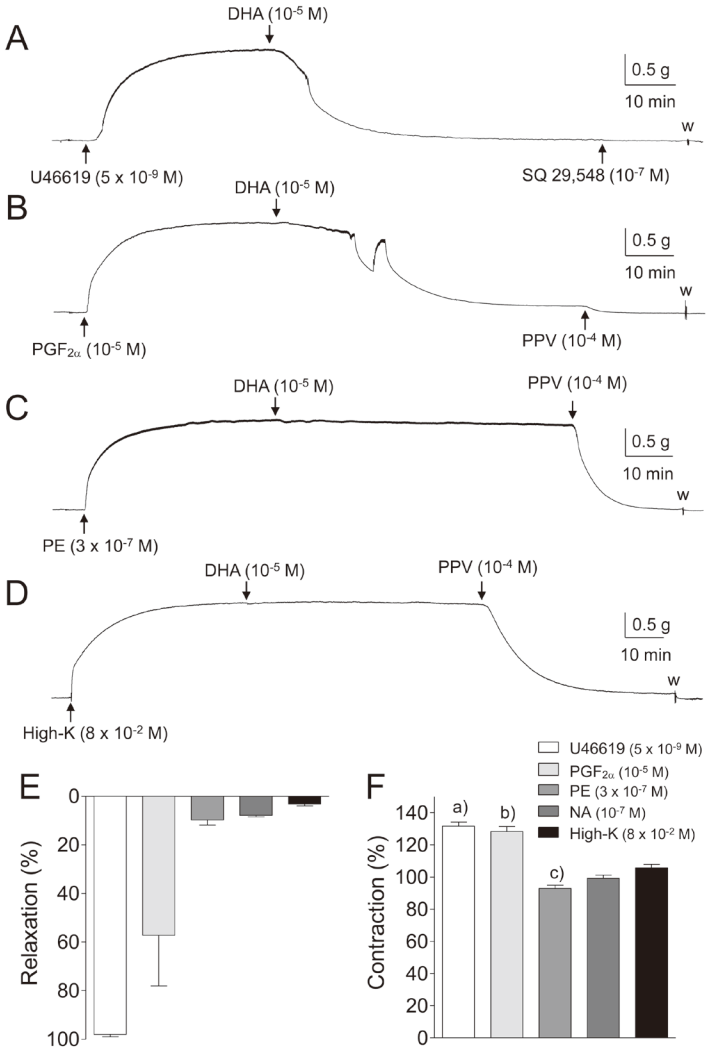

Fig. 3. Effects of DHA on the sustained contractions to various stimulants. A–D: Typical traces showing the effects of DHA (10^{-5} M) on the sustained contractions to U46619 (5×10^{-9} M) (A), $PGF_{2\alpha}$ (10^{-5} M) (B), phenylephrine (PE, 3×10^{-7} M) (C) and high-KCl (8×10^{-2} M) (D). Preparations used were endothelium-denuded, and tension changes were recorded in the presence of Indo (3×10^{-6} M). PPV: papaverine, 10^{-4} M; w: wash of preparation with fresh medium. E: Summarized data showing the inhibitory effects of DHA including those of DHA *vs.* noradrenaline (NA, 10^{-7} M). F: Developed tensions (contraction) attained with tested constrictor stimulations. Muscle tensions were shown being normalized with respect to high-KCl (8×10^{-2} M)-induced muscle tension obtained in the beginning of experiments. Statistical significances were detected between: U46619 *vs.* PE, NA and high-KCl, [a)]$P < 0.01$; $PGF_{2\alpha}$ *vs.* PE, NA and high-KCl, [b)]$P < 0.01$; PE *vs.* high-KCl [c)]$P < 0.05$.

tion. Fig. 3F shows the muscle tension levels attained with tested constrictor stimulants. The muscle tensions that were normalized with respect to the high-KCl (8×10^{-2} M)-induced tension obtained in the beginning of experiments were: 131.5 ± 2.7% for U46619 (5×10^{-9} M), 128.4 ± 3.1% for $PGF_{2\alpha}$ (10^{-5} M), 93.0 ± 2.0% for PE (3×10^{-7} M), 99.2 ± 2.1% for NA (10^{-7} M), 105.7 ± 2.2% for high-KCl (8×10^{-2} M) ($n = 4$ for each). Statistical significances were detected between: U46619 *vs.* PE, NA and high-KCl ($P < 0.01$); $PGF_{2\alpha}$ *vs.* PE, NA and high-KCl ($P < 0.01$); PE *vs.* high-KCl ($P < 0.05$). Since the tension levels elevated with U46619 and $PGF_{2\alpha}$ were higher than those with other stimulants, the pre-contraction amplitude was ruled out as a factor in the generation of the selective inhibition by DHA against TP and FP receptor-mediated contractions.

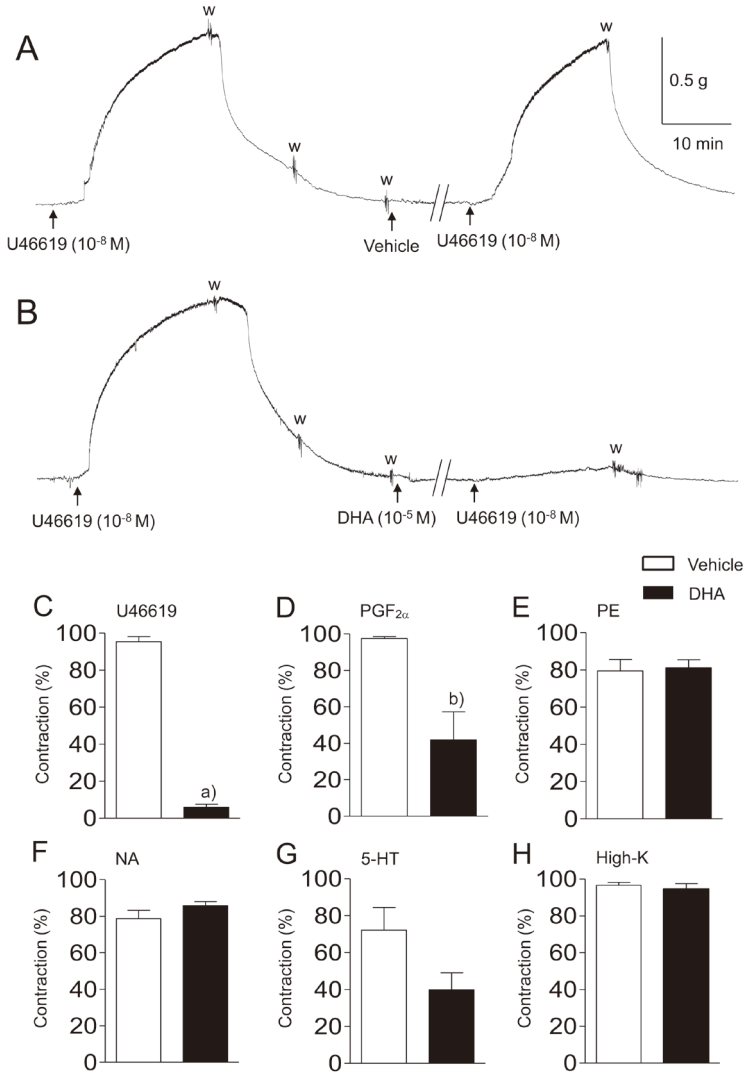

Fig. 4. Pretreatment effects of DHA on the aortic contractions to various stimulants. A, B: Typical traces showing the pretreatment effects of DHA (10^{-5} M) (B) or its vehicle (A) *vs*. U46619 (10^{-8} M). Tension changes were recorded in the presence of Indo (3×10^{-6} M) with endothelium-denuded preparations. w: wash of preparation with fresh medium. C–H: Summarized data showing the pretreatment effects of DHA on various contractions. Contractile stimulants used were: U46619 (10^{-8} M) (C), $PGF_{2\alpha}$ (10^{-5} M) (D), PE (3×10^{-7} M) (E), NA (10^{-7} M) (F), 5-hydroxytryptamine (5-HT, 10^{-5} M) (G) and high-KCl (8×10^{-2} M) (H). Data are shown as mean values ± S.E.M. (n = 4 for each). Significant differences between two groups: [a)] $P < 0.01$, [b)] $P < 0.05$.

Pretreatment effects of DHA on the vascular contractions induced by various stimulants

In order to verify that DHA was capable of diminishing vascular contractions mediated through TP and FP receptors without affecting contractions induced by α_1-adrenoceptor stimulation and membrane depolarization, we next examined the pretreatment effects of DHA. This experimental approach also seemed to be effective in determining whether DHA exhibited inhibitory effects against 5-HT-induced contractions; 5-HT was found not to cause a stable sustained contraction that would allow an analysis of the post-treatment effects of DHA.

Fig. 4A, B illustrate typical traces showing the pre-treatment effects of DHA on the contractions to U46619 (10^{-8} M). U46619-induced contractions were observed for 20-min periods after application, and DHA (10^{-5} M)

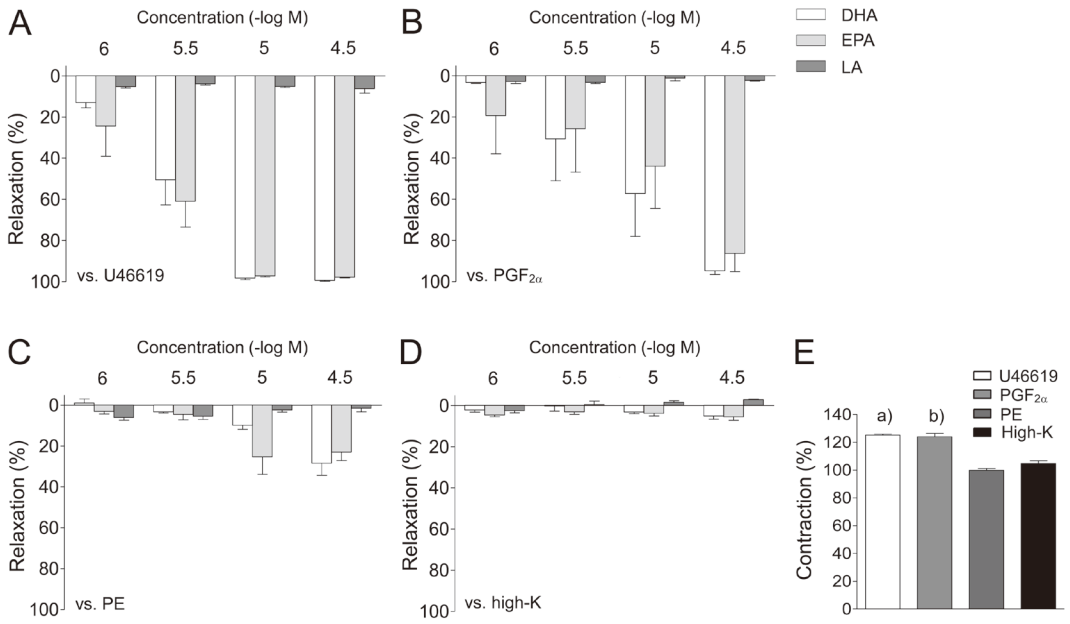

Fig. 5. Comparison of the inhibitory effects of DHA, EPA and LA on the sustained contractions induced by various stimulants. Preparations used were endothelium-denuded, and tension changes were recorded in the presence of Indo (3×10^{-6} M). Sustained contractile stimulants were: U46619 (5×10^{-9} M) (A), $PGF_{2\alpha}$ (10^{-5} M) (B), PE (3×10^{-7} M) (C) and high-KCl (8×10^{-2} M) (D). When the muscle tension increased with vasoconstrictor stimulations reached a steady-state level, each PUFA (DHA, EPA, LA) at a desired concentration was applied to the bath medium. The PUFA concentrations were 10^{-6} – 3×10^{-5} M. The data with 10^{-5} M DHA *vs*. U46619, $PGF_{2\alpha}$, PE and high-KCl are the same shown in Fig. 3. Tension developments attained with tested constrictor stimulants are also shown in E. Data are shown as mean values ± S.E.M. ($n = 4$ for each in A–D, and $n = 16$ for each in E). Statistical significances were detected between: U46619 *vs*. PE and high-KCl, [a)]$P < 0.01$; $PGF_{2\alpha}$ *vs*. PE and high-KCl, [b)]$P < 0.01$.

(Fig. 4B) or its vehicle (Fig. 4A) was applied 40 min before a subsequent second application of U46619. In the preparation pretreated with vehicle, the second application of U46619 elicited a contraction that was virtually comparable to the corresponding control response (Fig. 4A). In contrast, in the preparation pretreated with DHA, the contraction in response to second application of U46619 was almost completely suppressed (Fig. 4B). This pretreatment effect of DHA was consistent with the result that was obtained by the post-treatment with DHA (Fig. 1, 3A). Fig. 4C summarizes the results shown in Fig. 4A, B.

With similar procedures, the pretreatment effects of DHA (10^{-5} M) were also examined against contractions to $PGF_{2\alpha}$ (10^{-5} M), PE (3×10^{-7} M), NA (10^{-7} M), 5-HT (10^{-5} M) and high-KCl (8×10^{-2} M), and their summarized results are shown in Fig. 4D–H. Pronounced and substantial inhibition was obtained against $PGF_{2\alpha}$-induced contractions (Fig. 4D). In contrast, DHA did not show significant inhibitory effects against other contractions to PE (Fig. 4E), NA (Fig. 4F), 5-HT (Fig. 4G) and high-KCl (Fig. 4H).

Comparison of the inhibitory effects of DHA, EPA and LA on sustained contractions

Inhibitory effects of DHA against sustained contractions were compared with those of EPA (another ω-3 PUFA) and LA (an ω-6 PUFA), and the results are shown in Fig. 5. Stimulants employed to obtain sustained contractions were: U46619 (5×10^{-9} M), $PGF_{2\alpha}$ (10^{-5} M), PE (3×10^{-7} M) and high-KCl (8×10^{-2} M).

First of all, it was observed that EPA as well as DHA was able to inhibit U46619- and $PGF_{2\alpha}$-induced contractions, and that the inhibitory potencies of these two ω-3 PUFAs were almost comparable (Fig. 5A, 5B).

However, against contractions induced by U46619, DHA and EPA showed more potent diminishing effects than against those induced by $PGF_{2\alpha}$. This idea was supported by the pIC_{50} values (minus logarithm of IC_{50} values) of DHA and EPA. The pIC_{50} values of DHA *vs*. U46619 and $PGF_{2\alpha}$ were calculated to be 5.56 ± 0.12 (n = 4 from 8 preparations) and 5.04 ± 0.13 (n = 4), and these values were shown to be significantly different (P < 0.05). In addition, the pIC_{50} value of EPA *vs*. U46619 (5.68 ± 0.01, n = 4) was larger than the value for $PGF_{2\alpha}$ (5.34 ± 0.52, n = 4) although these values were not significantly different. In contrast, LA at concentrations up to 3×10^{-5} M did not show any substantial inhibitory effects against contractions to both U46619 and $PGF_{2\alpha}$.

Against PE (3×10^{-7} M)-induced contractions, none of the PUFAs tested showed substantial inhibition at concentrations up to 3×10^{-6} M (Fig. 5C). However, with concentrations over 3×10^{-6} M, DHA and EPA exhibited a weak inhibition on PE-induced contractions. As to LA, it did not show any inhibitory effects against PE-induced contractions even at a concentration of 3×10^{-5} M. None of the three PUFAs showed substantial inhibition against depolarizing contraction to high-KCl (8×10^{-2} M) (Fig. 5D).

Discussion

In the present study using preparations from the rat aorta, we found that DHA strongly diminished contractions mediated through prostanoid receptors (TP and FP receptors) with only a weak inhibition or a substantially negligible effect against contractions mediated through non-prostanoid receptors. Almost similar inhibitory effects were mimicked by another ω-3 PUFA EPA while an ω-6 PUFA LA did not show substantial inhibitory effects against any contractions. Interestingly, inhibitory effects of DHA and EPA were implied to be generated more strongly against contractions mediated through TP receptors rather than those through FP receptors.

To date, many endogenous and exogenous blood vessel relaxant stimulants have been reported to exert their relaxant effects entirely or partly mediated through endothelium-derived relaxants. These mediators include: PGI_2 (18, 19), NO (20–22), and endothelium-derived hyperpolarizing factor (EDHF) (23, 24). However, in regard to DHA, it has not been conclusively determined whether the vascular effects of DHA significantly involve the endothelium. For instance, some groups have reported vascular smooth muscle-direct blood vessel relaxation by DHA (12, 14, 25, 26). In contrast, a significant role for the endothelium has been suggested in DHA-induced vascular relaxation. The significant role for the endothelium includes a stimulating effect due to endothelial NO release (27, 28) and endothelial conversion of DHA to lipoxygenase products (17S-hydroxy DHA) (29). The obligatory roles for the endothelium in EPA-induced relaxation were also reported in both the rabbit and cat aortae (30). Therefore, we determined to investigate this question.

In this regard, we found out that DHA was able to inhibit U46619-induced sustained contraction even in the absence of endothelium or in the combined presence of COX and NOS inhibitors (Fig. 1). Furthermore, we also found that DHA did not produce inhibitory effects on NA-induced contraction in endothelium-intact preparations (Fig. 2). This additional experiment was designed to appropriately evaluate whether the endothelium significantly contributes to DHA vascular effects under conditions in which DHA does not interact with TP receptors. This was necessary because a possible contribution of the endothelium to DHA vascular effects may be masked even if it presents when endothelium-unrelated factors, such as TP receptor interaction, exclusively surmounts the endothelial role. Based on these findings, we concluded that at least in the rat thoracic aorta, the endothelium does not play a significant role in the effect that DHA has against blood vessel tension changes. In this case, in addition to releasing vaso-relaxant substances, the “roles of the endothelium” could include the possible conversion of DHA into more active metabolites. However, we considered that this could

be ruled out as a mechanism that underlies the DHA-induced blood vessel effects.

In earlier studies in which the possible blood vessel relaxant effects of DHA were investigated, relatively well-known stimulants such as NA and high-KCl solution were used as constrictors to evaluate PUFA effects. For instance, the relaxant effects of DHA on NA- and high-KCl-induced contractions were reported in aortic preparations isolated from either normotensive rats (10) or spontaneously hypertensive rats (SHR) (13). These experiments were reasonable since available vasoconstrictors were limited. However, blood vessel relaxant effects attained with ω-3 PUFAs including DHA seem not to be so strikingly robust considering that a concentration of 10^{-5} M was employed to obtain the relaxant effect. In support of earlier reports, our present findings show that very small or marginal inhibitory effects were elicited against PE- and high-KCl-induced contractions by DHA (Figs. 3–5) and EPA (Fig. 5).

In contrast, we incidentally observed that DHA more strongly inhibits TP receptor-mediated contraction than α-adrenoceptor-mediated responses in the guinea-pig aorta (14). The present study with the rat aorta also showed that DHA exhibited a more pronounced inhibition of TP receptor-mediated contractions, and that this effect was conspicuous by its pre-treatment as well as by post-treatment. In support of our findings in rat and guinea-pig aortae, DHA is reported to antagonize prostanoid TP receptor in platelets (31) and inhibit TXA_2-like vasoconstrictor responses of SHR aorta (32). Therefore it may be plausible that selective and potent inhibition by DHA of TP receptor-mediated blood vessel contraction is a common event observed transcending animal species and experimental materials. Since the target receptor for $PGF_{2\alpha}$ (FP receptor) is shown to be similar to the TP receptor in primary structure (33, 34), it was presumed to some degree that $PGF_{2\alpha}$-induced contraction was inhibited by DHA. However, it was likely that the inhibitory effects of DHA against $PGF_{2\alpha}$-induced contractions are faintly less potent than those against U46619-induced contractions. This idea is supported by the longer T_{IC50} of DHA against $PGF_{2\alpha}$ *vs*. U46619, and its smaller pIC_{50} value against $PGF_{2\alpha}$ *vs*. U46619.

Our present study showed that another ω-3 PUFA EPA suppressed both TP and FP receptor-mediated sustained contractions with almost similar potencies to those of DHA. By contrast, an ω-6 PUFA LA did not significantly inhibit TP receptor-mediated contraction even with higher concentrations than those for DHA and EPA (up to 3×10^{-5} M). These findings might imply that specific inhibitions against prostanoid receptor-mediated contractions are commonly attained with ω-3 PUFAs, but this rule cannot be significant for ω-6 PUFAs. However, our unpublished observation showed that arachidonic acid (AA), another kind of ω-6 PUFA, diminished U46619-induced contraction of the rat thoracic aorta with a similar potency to that of both DHA and EPA. Therefore, selective inhibitory effects against prostanoid receptor-mediated vascular contractions are not exerted exclusively by ω-3 PUFAs. A possible alternative explanation for these selective inhibitory effects by PUFAs would be the difference in carbon chain length, but this possibility needs to be more fully investigated. Nevertheless, it should be noted that when a comparison is made between the representative major PUFAs contained in food oils, the inhibitory effect exerted selectively against prostanoid receptor-mediated blood vessel contractions is attained more conspicuously with ω-3 PUFAs (DHA, EPA) rather than ω-6 PUFA (LA).

In contrast to our present finding that LA did not show any substantial inhibitory effects against sustained vascular contractions, LA was reported to cause significant relaxation in aortic smooth muscle (11). This blood vessel relaxant effect of LA seems to be elicited through mechanisms unrelated to COX and lipoxygenase products (11). Another report also showed that LA lowered blood pressure with an equipotent activity to fish oil FAs in the hypertensive rat, where the blood pressure elevation is generated by angiotensin II (Ang II) (35). At present we do not have a clear explanation for the discrepancy between our present results and other reports. Differences in rat strains, experimental protocols and so on might be the cause and require further investigation.

To date, there has been no conclusive explanation given as to why DHA exerts its inhibitory effects selec-

tively against TP receptor-mediated blood vessel contractions. One possibility could be that DHA behaves as an antagonist of TP receptors to counteract blood vessel contractions mediated through prostanoid receptors (14). Strong inhibition by DHA against FP receptor-mediated contraction found in the present study may support this idea. In this regard, Abeywardena and Head (36) propose that DHA is the only PUFA to show competitive antagonism at TP receptors in the rat aorta. In addition, DHA as well as EPA was found to inhibit TP receptor-mediated platelet activation in a competitive fashion, and to interact with platelet TP receptors (31). The activation of plasma membrane K^+ channels including BK channels has been proposed as a mechanism by which DHA exerts its biological actions (37, 38). The possibility that plasma membrane K^+ channel activation triggers DHA-induced blood vessel relaxation is fascinating. However, even if activation of K^+ channels is significant in DHA-induced blood vessel relaxation, it is unclear why this should lead to selective inhibition of TP receptor-mediated blood vessel contraction. Furthermore, significant roles for enzymatic products generated via cytochrome P-450 (CYP) epoxygenase (37) or endothelium-derived lipoxygenase (29) have been recently proposed to have a role in DHA-triggered blood vessel relaxation. At present, we are carrying out further investigations to obtain more detailed information on the mechanisms by which DHA exerts its selective inhibition against TP receptor-mediated vascular contraction by considering possible roles for DHA metabolites.

Our present finding obtained in segments of the rat aorta that DHA inhibits prostanoid receptor-mediated contractions more potently than other contractions leads to the idea that this ω-3 PUFA is effective in overcoming prostanoids-generating cardiovascular abnormalities. The present finding also show that EPA as well as DHA are effective against these diseases. With regard to the DHA concentration in human plasma, it can be elevated by dietary intake of fish oil to $\approx 10^{-5} - 1.5 \times 10^{-5}$ M (10 – 15 μM) from a normal level of around 5×10^{-6} M (5 μM), thus increasing the DHA plasma concentration level by twice to three times the basal level (11). Therefore, the concentrations of DHA and EPA used in the present study to diminish vascular contractions are clinically significant. And our results may partly provide an experimental rationale to the epidemiological studies which demonstrate a significant blood pressure reduction resulting from the intake of fish oil in hypertensive patients but not in normotensive individuals (7–9). There is considerable interest in the recent suggestion that TXA_2 could play a significant role in the pathogenesis of hypertension without contributing to the normal regulation of blood pressure (15). Furthermore, with consideration that the TP receptor antagonist SQ 29,548 is reported to lower elevated blood pressure in rats with hypertension elicited with Ang II-salt (15), it may be plausible that DHA and EPA can act as a TP receptor antagonist to reduce blood pressure. However, in general, DHA is superior to EPA at the levels contained in fish oil (39). Therefore, the possible cardiovascular-protective effects produced by intake of fish/fish oil might reflect that the blood vessel relaxant effects are largely due to DHA rather than EPA.

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Conflict of interest

The authors declare that they have no conflict of interest.

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