Eicosapentaenoic acid (EPA) induces Ca^{2+}-independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation

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Abstract Eicosapentaenoic acid (EPA), but not its metabolites (docosapentaenoic acid and docosahexaenoic acid), stimulated nitric oxide (NO) production in endothelial cells in situ and induced endothelium-dependent relaxation of bovine coronary arteries precontracted with U46619. EPA induced a greater production of NO, but a much smaller and more transient elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), than did a Ca^{2+} ionophore (ionomycin). EPA stimulated NO production even in endothelial cells in situ loaded with a cytosolic Ca^{2+} chelator 1,2-bis-*o*-aminophenoxythamine-*N*′,*N*′,*N*′-tetraacetic acid, which abolished the $[Ca^{2+}]i$ elevations induced by ATP and EPA. The EPA-induced vasorelaxation was inhibited by N^{ω}-nitro-L-arginine methyl ester. Immunostaining analysis of endothelial NO synthase (eNOS) and caveolin-1 in cultured endothelial cells revealed eNOS to be colocalized with caveolin in the cell membrane at a resting state, while EPA stimulated the translocation of eNOS to the cytosol and its dissociation from caveolin, to an extent comparable to that of the eNOS translocation induced by a $[Ca^{2+}]i$-elevating agonist (10 μM bradykinin). Thus, EPA induces Ca^{2+}-independent activation and translocation of eNOS and endothelium-dependent vasorelaxation.

Key words: Eicosapentaenoic acid; Nitric oxide; Endothelial cell; Vasorelaxation; Smooth muscle; Cytosolic Ca^{2+}

1. Introduction

The dietary intake of large amounts of marine oils rich in ω-3 polyunsaturated fatty acids (PUFA) has been suggested to account for the low incidence of coronary artery disease among Greenland Eskimos [1]. Dietary supplementation with fish oil or eicosapentaenoic acid (EPA, C20:5*n*-3), a PUFA of the ω-3 series, augments endothelium-dependent relaxations in porcine coronary arteries and also reduces contractile responses evoked by noradrenaline and arachidonic acid in the rat aorta [2–6]. In addition, chronic exposure of cultured endothelial cells to EPA potentiates the release of an endothelium-derived relaxing factor (nitric oxide, NO) in response to receptor agonists, such as purines and kinins [7].

Because NO production by the endothelium induces vasorelaxation and a reduction in platelet aggregation [8], it therefore has beneficial effects for subjects with vascular diseases [9–11]. Overcoming endothelial dysfunction by PUFA (especially EPA) contributes to a suppression of atherogenic processes and of ischemic heart disease, whereas a recent randomized clinical trial showed that dietary intake of PUFA has modest effects on coronary atherosclerosis [12] and it was also reported that docosahexaenoic acid (DHA), but not EPA, has a blood pressure-lowering effect in hyperlipidemic men [13,14]. However, the cellular mechanism by which PUFA potentiates endothelial function (NO production) has remained to be clarified. The direct effects of PUFA on NO production and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in endothelial cells in situ have apparently never been documented. NO is synthesized from the amino acid L-arginine by NO synthases (NOSs), comprised of Ca^{2+}-dependent constitutive NOSs (endothelial and neuronal NOS) and Ca^{2+}-independent inducible NOS (iNOS) [15]. In unstimulated endothelial cells, endothelial NOS (eNOS) is targeted to specific microdomains in the plasma membrane termed caveolae, where eNOS is associated with a scaffold protein caveolin, resulting in tonic inhibition of the enzyme activity [16–20]. The elevation of $[Ca^{2+}]i$, induced by Ca^{2+}-elevating agonists such as bradykinin (BK) and ATP, stimulates the binding of calmodulin (CaM) to eNOS and the dissociation of the enzyme from caveolin, thereby activating eNOS [20]. In addition to the Ca^{2+}-dependent activation of eNOS, Mizuno et al. reported the agonist-dependent modulation of the relationship between $[Ca^{2+}]i$ and NO production in endothelial cells [21]. It was also reported that shear stress or ceramide can stimulate NO production with no detectable increase in $[Ca^{2+}]i$ in endothelial cells [22,23], thereby indicating the Ca^{2+}-independent activation of eNOS by these stimulations. However,

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Abbreviations: EPA, eicosapentaenoic acid; NO, nitric oxide; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; L-NAME, N^{ω}-nitro-L-arginine methyl ester; eNOS, endothelial nitric oxide synthase; PUFA, polyunsaturated fatty acids; BAPTA, 1,2-bis-*o*-aminophenoxythamine-*N*′,*N*′,*N*′-tetraacetic acid; NOS, nitric oxide synthase; CaM, calmodulin; HUVEC, human cultured umbilical vein endothelial cells; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; DAN, 2,3-diaminonaphthalene; TRIM, 1-(2-trifluoromethylphenyl) imidazole; BK, bradykinin; iNOS, inducible NOS; PSS, physiological salt solution; ION, ionomycin

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Ca^{2+}-dependency of the PUFA-induced NO production in endothelial cells has heretofore remained unknown.

Using endothelial cells in situ which only express the eNOS isoform, instead of cultured endothelial cells which may also possibly express Ca^{2+}-independent iNOS by stimulators included in the growth medium, we examined effects of PUFA on $[Ca^{2+}]i$ and NO production. We report here the first evidence that EPA induces Ca^{2+}-independent activation and translocation of eNOS in endothelial cells in situ, events linked to endothelium-dependent vasorelaxation which was blocked by a NOS blocker.

2. Materials and methods

2.1. Materials

EPA, docosapentaenoic acid (DPA), DHA, ATP and U-46619 were purchased from Sigma (St. Louis, MO, USA). Fura-2/AM and 2,3-diaminonaphthalene (DAN) were from Dojindo (Kumamoto, Japan). 1,2-Bis-*o*-aminophenoxythamine-*N*′,*N*′,*N*′-tetraacetic acid (BAPTA)/AM was from Molecular Probes (OR, USA). N^{ω}-Nitro-L-arginine methyl ester (L-NAME) was from Wako Pure Chemical (Osaka, Japan), and 1-(2-trifluoromethylphenyl) imidazole (TRIM) was from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Katayama Chemical (Osaka, Japan). The composition of the normal physiological salt solution (PSS) was (in mM); 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose. All solutions were gassed with a mixture of 5% CO_2 and 95% O_2 (pH adjusted to 7.4).

2.2. Measurement of $[Ca^{2+}]i$ of the endothelial cells in situ

The changes in $[Ca^{2+}]i$ of the endothelial cells in situ were measured using fluorometry of fura-2, as described [24–27]. Briefly, the bovine aortic valvular strips, obtained from a local slaughter-house, were incubated in PSS containing 25 μM fura-2 AM for 90 min at 37°C. The fluorescence intensities at alternating 340 nm (F_{340}) and 380 nm (F_{380}) excitation and their ratio ($R = F_{340}/F_{380}$) were monitored at 510 nm emission, using a spectrofluorometer (Hitachi F2000) which operates on our own program [27]. Double staining of fura-2 and fluorescent acetylated low-density lipoprotein revealed that fura-2 signals exclusively arose from the monolayered endothelial cells on the surface of the aortic side of the aortic valves [24–27]. Before starting the experimental protocol, all strips were stimulated with 10 μM ATP for 1 min to obtain a reference response. The fluorescence ratio values were normalized by assigning values in normal PSS and at the peak response to 10 μM ATP to be 0 and 100%, respectively.

2.3. Measurements of NO production of the endothelial cells in situ

To determine NO production in endothelial cells in situ, we measured the concentration of nitrite in PSS, using a DAN assay, as described [27]. PUFA was applied to the aortic valve pinned in the Sylgard chamber by exchanging the solutions. At the end of the 5 min treatment, the solution aspirated from the chamber was used for NO measurements. To determine the basal NO production in endothelial cells in situ at rest, fresh PSS (600 μl) was applied to the aortic valve for 5 min and was collected for NO measurements before the start of the experiments.

The intensity of the fluorescent signal of 1-(H)-naphthotriazole, the product of reactions between nitrite and DAN, was measured with excitation at 365 nm and emission at 405 nm, using a spectrofluorometer (Hitachi, F2000) with a detection limit of 10 nM. Standard sodium nitrite solutions (10 nM–1 mM) were made fresh to obtain a standard curve, before each measurement. The level of NO production was expressed as a percentage, assigning value at the 'zero' level (= background fluorescence of solution only, without the aortic valves) and the value obtained from the aortic valves at rest (treated with fresh PSS for 5 min) to be 0 and 100%, respectively.

2.4. Force recording of arterial strips with or without endothelium

Isometric contractions of the bovine coronary arterial rings without adventitia, obtained from a local slaughter-house, were measured using a force transducer (TB-611T, Nihon-Kohden, Japan), as described [25–27]. Briefly, after recording steady responses to repeated applications of 118 mM K^+-depolarization, the effects of PUFA on the force were examined at the plateau phase of the sustained contraction induced by 100 nM U-46619. The concentration of U-46619 (100 nM) was selected to obtain the plateau phase of the sustained contraction which is needed for the quantitative observation of the endothelium-dependent vasorelaxation [26]. In some strips, the endothelium was removed by rubbing with a cotton swab and complete removal of the endothelium was confirmed by the absence of relaxation induced by 1 μM BK.

2.5. Cell culture and immunostaining

For confocal cell imaging studies, human cultured umbilical vein endothelial cells (HUVEC; American Type Culture Collection) were seeded onto a gelatin-coated cell disk (Sumitomo, Japan) and subcultured for three to six passages. Immunocytochemistry of the serum-deprived (24 h) HUVEC was done, as described previously [28]. The cells were fixed with 4% paraformaldehyde for 10 min and subsequently permeabilized with 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) for 5 min. After incubation with 10% BSA (1.5 h), the cells were incubated with monoclonal mouse anti-eNOS (Transduction Laboratories) and rabbit anti-caveolin-1 (Santa Cruz Biotechnology) antibodies at 1:100 dilution for 1.5 h. The cells were then incubated with Cy-3-conjugated anti-mouse and Cy-2-conjugated anti-rabbit antibodies at 1:500 dilution for 1.5 h. Immunofluorescence images of endothelial cells were observed under a confocal laser scan microscope (LSM510, Zeiss).

To quantitatively analyze eNOS translocation, at least 100 cells on each coverslip were evaluated for eNOS staining at the cell membrane by an examiner who had been blinded to the culture conditions. The proportion of cells exhibiting eNOS staining at the cell membrane was quantitated for each experimental treatment: the data were expressed as a percentage, assigning the value in the control cells (without stimulation) to be 100%, as described previously [28].

2.6. Statistical analysis

The measured values were expressed as mean ± S.E.M. Student's *t*-test was used to determine the statistical significance between two groups. An analysis of variance and multiple comparison test (Fisher's protected least significant difference; PLAD) were used to determine the statistical significance of the effects of various concentrations of the drugs. *P*-values < 0.05 were considered to be significant.

3. Results

3.1. Effects of PUFA on $[Ca^{2+}]i$ elevation and NO production of the endothelial cells in situ

Since eNOS is a Ca^{2+}/CaM-dependent enzyme, we first investigated effects of PUFA on $[Ca^{2+}]i$ levels in endothelial cells in situ by comparison with a Ca^{2+} ionophore, ionomycin (ION), which simply elevates $[Ca^{2+}]i$ without the activation of any specific signal transduction. As shown in Fig. 1A, ION induced a sustained elevation of $[Ca^{2+}]i$. In contrast, EPA induced a slight and transient elevation of $[Ca^{2+}]i$ with no detectable sustained elevation. Fig. 1B shows concentration-dependent elevations of $[Ca^{2+}]i$ induced by EPA and by ION. 60 μM EPA and 0.03 μM ION had the same degree of potency in elevating $[Ca^{2+}]i$ in endothelial cells in situ (Fig. 1B). Other PUFA, EPA metabolites with a structure similar to that of EPA (DHA and DPA), had no effect on $[Ca^{2+}]i$ levels (data not shown), thereby supporting the specificity of EPA in inducing the $[Ca^{2+}]i$ elevations.

Fig. 2A shows the concentration-dependent elevation of NO production induced by EPA. In contrast, only 0.3 μM, but not 0.03 μM, ION potentiated NO production. DHA (up to 60 μM) had no effect on the NO production. DPA (60 μM) unexpectedly and significantly inhibited NO production.

Since recent studies have suggested the presence of a Ca^{2+}-independent activation of eNOS [21–23], we next examined the relationship between NO production and $[Ca^{2+}]i$. EPA

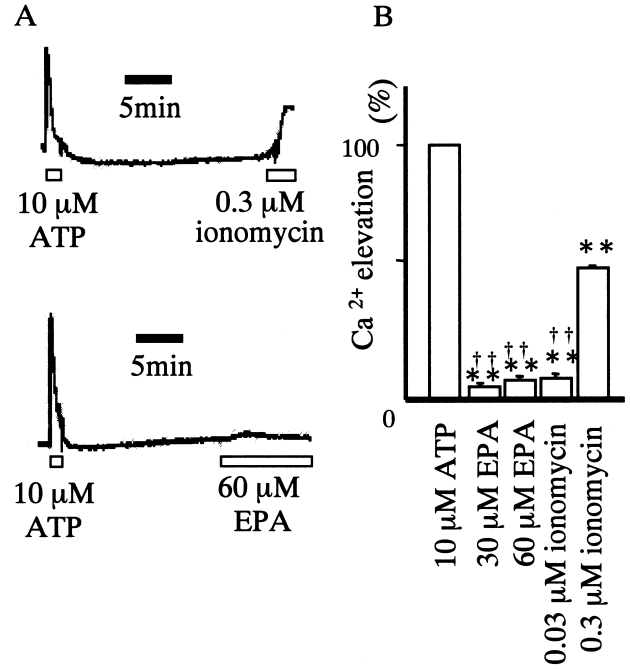

Fig. 1. A: Representative recordings showing changes in $[Ca^{2+}]i$ induced by 10 μM ATP (as a reference), 0.3 μM ION and 60 μM EPA. B: $[Ca^{2+}]i$ elevation was expressed as a percentage, assigning the value induced by 10 μM ATP to be 100%. Data were obtained from four separate experiments and expressed as mean ± S.E.M. $^{**}P < 0.01$ versus ATP, $\dagger\dagger P < 0.01$ versus 0.3 μM ION.

(30 and 60 μM) induced $[Ca^{2+}]i$ elevation as much as or less than seen with 0.03 μM ION (Fig. 1B), whereas 0.03 μM ION caused no significant stimulation of NO production (Fig. 2A), indicating that the extent of the EPA-induced $[Ca^{2+}]i$ elevations is within subthreshold levels for the stimulation of NO production. However, EPA markedly stimulated NO production (Fig. 2A), thereby suggesting the involvement of Ca^{2+}-independent NO production in the EPA-induced response. Fig. 2B shows the relationship between $[Ca^{2+}]i$ elevation (abscissa) and NO production (ordinate). EPA concentration-dependently shifted to the left and upward of the control curve seen with ION stimulation, which suggested that EPA induces Ca^{2+}-independent NO production.

3.2. Effect of PUFA on endothelium-dependent vasorelaxation

To determine the physiological role of NO released from endothelial cells induced by PUFA, we examined the effects of PUFA on the contractility of rings of bovine coronary artery, with and without the endothelium (Fig. 3). EPA (60 μM) relaxed the bovine coronary artery precontracted with U-46619 (a thromboxane A2 analogue): the force was decreased to 20.6 ± 7.1% ($n = 16$; $P < 0.01$) of the U-46619-induced contraction (= 100%) by EPA. The EPA-induced vasorelaxation was inhibited either by the removal of the endothelium (Fig. 3B) or by an inhibitor for all isoforms of NOS (100 μM L-NAME)[8] but not by a specific inhibitor for iNOS (100 μM TRIM) [29] (Fig. 3A): the EPA-induced relaxation (18.9 ± 3.5% of the U-46619-induced contraction, $n = 5$) was unchanged in the presence of TRIM (18.2 ± 6.5%, $n = 5$; $P > 0.05$) and decreased to 2.9 ± 7.6% ($n = 5$; $P < 0.01$) in the presence of L-NAME. The presence of endothelium was confirmed by the addition of 1 μM BK at the end of each experiment. In contrast to EPA, DPA or DHA (up to 60 μM) had no effect on the contraction (data not shown), suggesting that neither DPA nor DHA can induce endothelium-dependent relaxation. These results suggest that EPA specifically induces endothelium-dependent vasorelaxation, which may be mediated by NO released from endothelial cells in situ through the activation of eNOS, but not of iNOS.

3.3. Effect of EPA on translocation of eNOS

Immunostaining of HUVEC using monoclonal eNOS and caveolin-1 antibodies revealed a robust pattern of immunofluorescence in resting cells (Fig. 4A); when primary antibody was not used, there was no staining (data not shown). Caveolin-1 and eNOS shared a similar and overlapping pattern of distribution in endothelial cells, with staining for the two proteins seen at the plasma membrane. Importantly, when endothelial cells were treated with BK (1 μM, 15 min) or EPA (30 μM, 15 min), eNOS was translocated from the plasma membrane to the cytosol, while caveolin-1 immunostaining was not affected (Fig. 4A). These results are compatible with the dissociation of eNOS from caveolin-1 resident in the plasma membrane, as induced by either BK or EPA. The extent of the 60 μM EPA-induced translocation of eNOS was the same as the extent of the eNOS translocation induced by 1 μM BK (Fig. 4B).

3.4. Effect of BAPTA on production of NO induced by EPA

To determine if a slight and transient elevation of $[Ca^{2+}]i$ induced by EPA was required for the production of NO, we investigated the effect of chelation of cytosolic Ca^{2+} on NO

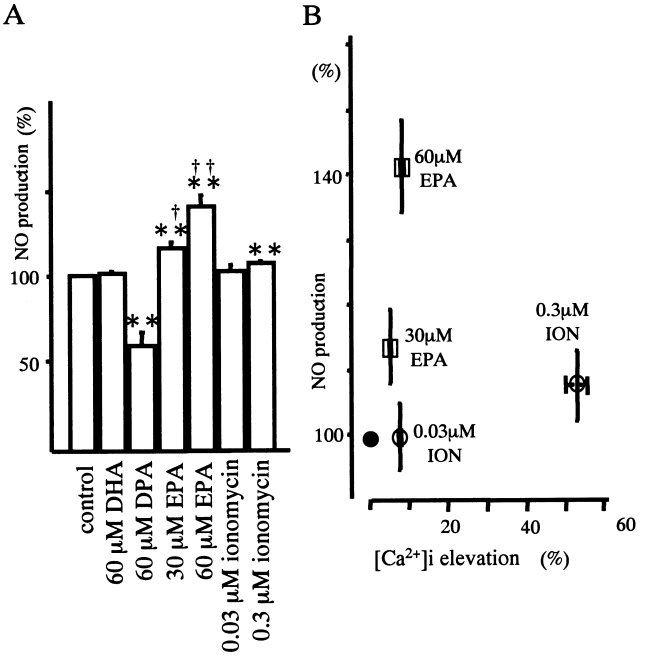

Fig. 2. A: The extent of the NO production induced by PUFA and ION. NO production was expressed as a percentage, assigning the value obtained at rest (= basal NO production by endothelial cells in situ) to be 100% ($n = 4$). Data shown as mean ± S.E.M. $^{**}P < 0.01$ versus control, $\dagger$ $P < 0.05$ versus 0.3 μM ION and $\dagger\dagger P < 0.01$ versus 0.3 μM ION, respectively. B: Relationship between $[Ca^{2+}]i$ elevation and NO production. The extent of $[Ca^{2+}]i$ elevation and NO production was normalized and expressed as a percentage, as described in Section 2. Closed circle represents the value obtained from the cells at rest.

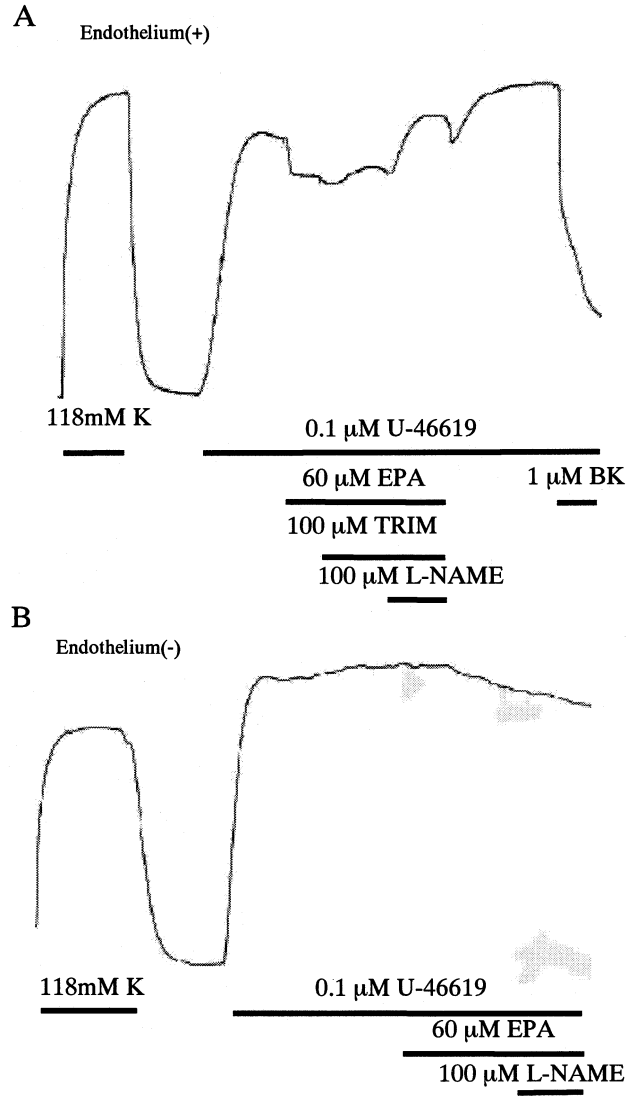

Fig. 3. Representative recordings showing effects of 60 μM EPA on contractions of the bovine coronary artery, with (A) and without (B) endothelium, precontracted by 100 nM U-46619.

production in endothelial cell in situ, which were loaded with fura-2 and a cytosolic Ca^{2+} chelator, BAPTA. As shown in Fig. 5A, neither ATP (10 μM) nor EPA (60 μM) can cause an increase in $[Ca^{2+}]i$. The unresponsiveness to ATP or EPA is not due to either cell toxicity or deterioration of fura-2 signal, because a Ca^{2+} ionophore, ION (5 μM), induced $[Ca^{2+}]i$ elevation although the rate of the $[Ca^{2+}]i$ elevation was slower than in the control (without BAPTA), such being compatible with the chelation of cytosolic Ca^{2+}. In contrast, 60 μM EPA stimulated NO production even in the BAPTA-loaded endothelial cells in situ, to an extent comparable to that seen in control cells (without BAPTA) (Fig. 5B). These results suggest that EPA stimulates NO production with no detectable $[Ca^{2+}]i$ elevation in endothelial cells in situ.

4. Discussion

The novel findings of this study are that EPA induces Ca^{2+}-independent activation of eNOS and the translocation and dissociation from caveolin-1 of eNOS in endothelial cells in situ, which express only eNOS, but not Ca^{2+}-independent iNOS. EPA-induced NO production is linked to physiological functions, namely endothelium-dependent vasorelaxation. This is also the first report to demonstrate rapid and direct effects of EPA not only on NO production and $[Ca^{2+}]i$ but also their relationships in endothelial cells in situ, although there is documentation on the beneficial effects of either dietary supplementation with PUFA or chronic exposure to PUFA on endothelial dysfunction and on abnormal vascular contractions induced by vasospastic agonists [2–7] and a recent study is rather controversial and shows that DHA, but

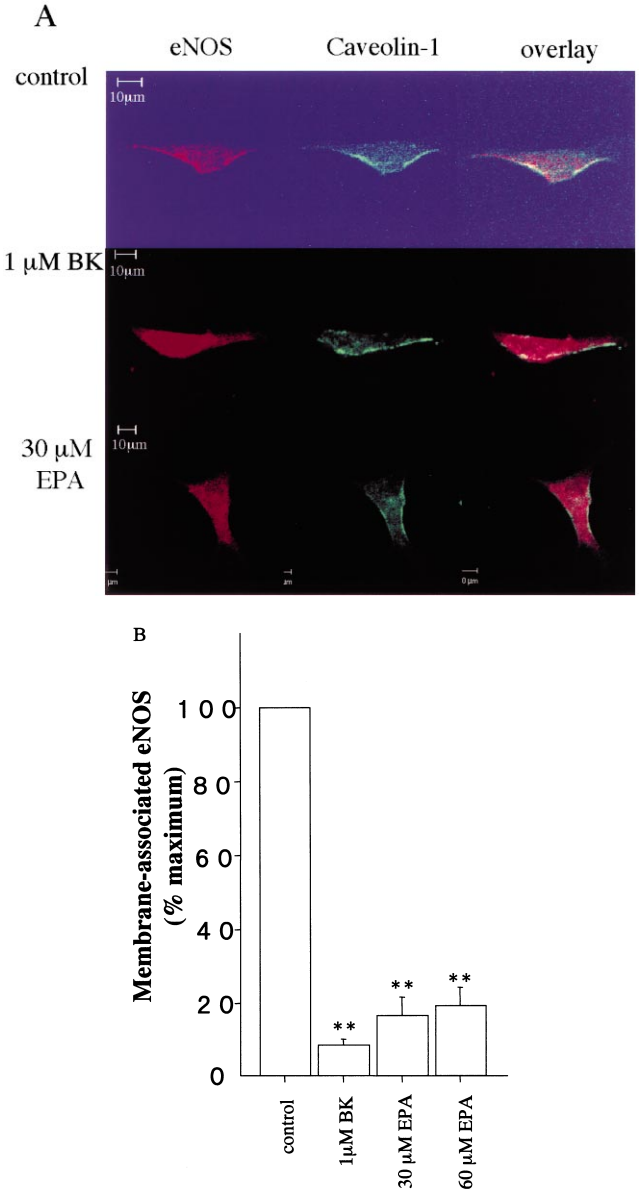

Fig. 4. Translocation and dissociation from caveolin-1 of eNOS in endothelial cells in response to BK and EPA (15 min). A: Representative confocal images showing the immunostaining of eNOS (labeled with Cy-3) and caveolin-1 (labeled with Cy-2) and overlay view of both in endothelial cells which were stimulated by 1 μM BK and 30 μM EPA. B: The extent of the translocation of eNOS in endothelial cells induced by 1 μM BK and 30 and 60 μM EPA. The proportion of cell with eNOS staining at the cell membrane was determined, as described in Section 2. The data represent the mean ± S.E.M. derived from six independent culture preparations. $^{**}P < 0.01$ versus control.

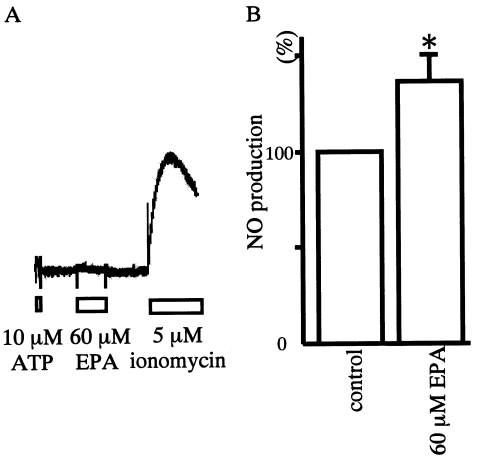

Fig. 5. A: Representative recordings showing changes in $[Ca^{2+}]i$ induced by 10 μM ATP, 60 μM EPA and 5 μM ION in endothelial cell in situ, loaded with fura-2 and cytosolic Ca^{2+} chelator, BAPTA. B: Summary of the repeated experiments shown in (A). NO production on BAPTA (20 μM)-loaded endothelial cells in situ was expressed as a percentage, assigning the value obtained at rest to be 100% ($n=4$). Data are shown as mean ± S.E.M. $*P<0.05$ versus control.

not EPA, reduces ambulatory blood pressure in mildly hyperlipidemic men [13] and stimulates vasodilator response and inhibits constrictor response in the forearm microcirculation [14].

To explore Ca^{2+}-dependency in the activation of eNOS while avoiding possible interference by the Ca^{2+}-independent activity of iNOS, we used endothelial cells in situ, which express only eNOS, but not iNOS [27], instead of using cultured endothelial cells which are grown in the presence of several growth factors included in the medium that may possibly stimulate the induction of iNOS. In the present study, we proposed the Ca^{2+}-independent activation of eNOS by EPA, based on the following evidence: (1) the extent of the 60 μM EPA-induced $[Ca^{2+}]i$ elevation was comparable to the extent of 0.03 μM ION which is a subthreshold level of $[Ca^{2+}]i$ for NO production (Fig. 1); (2) the extent of NO production induced by EPA was much larger than that expected from the extent of the EPA-induced $[Ca^{2+}]i$ elevation, as compared with stimulation with a Ca^{2+} ionophore ION (Fig. 2B); (3) the chelation of cytosolic $Ca^{2+}$with BAPTA abolished the $[Ca^{2+}]i$ elevations induced by EPA or ATP. However, EPA still stimulated NO production even after the chelation of cytosolic Ca^{2+} (Fig. 5). (4) EPA induced transient elevation of $[Ca^{2+}]i$ without the sustained elevation (Fig. 1A). These observations also support the Ca^{2+}-independent activation of eNOS, because sustained elevation of $[Ca^{2+}]i$, but not transient elevation of $[Ca^{2+}]i$, plays a major role in the Ca^{2+}-dependent activation of eNOS [30]. However, the involvement of fura-2-undetectable and BAPTA-insensitive Ca^{2+} pools was not ruled out in the present study. Further experiments are required to obtain more conclusive results: for example, it may be helpful to show the differential sensitivity to BAPTA between EPA and BK responses.

It is unlikely that the Ca^{2+}-independent activity of iNOS is involved in the observed Ca^{2+}-independent NO production by EPA, for the following reasons: (1) the EPA-induced endothelium-dependent vasorelaxation was interfered with by an inhibitor for both eNOS and iNOS (L-NAME), but not by a selective inhibitor of iNOS (TRIM) (Fig. 3); (2) Western blot analysis showed that endothelial cells in situ used in the present study expressed only eNOS, not iNOS, and (3) the rapid response to EPA is not compatible with the de novo induction of iNOS.

The effects of EPA are specific, because its metabolites (DPA and DHA) with a structure similar to that of EPA had no effect on $[Ca^{2+}]i$ elevation, NO production or vascular contraction. However, the molecular mechanisms by which EPA stimulates NO production and translocation of eNOS remain unknown. The following two possibilities may account for the EPA action on eNOS. (1) It was recently reported that EPA inhibits the palmitoylation of Src family kinase such as Fyn and the kinase translocation to detergent-resistant microdomains of the cell membrane (probably caveolae) [31]. Endothelial NOS is also both myristoylated and palmitoylated [28,32–34]. The acylation of eNOS is required for targeting of the enzyme to caveolae [35]. It was reported that eNOS palmitoylation is dynamically regulated and the agonist-induced depalmitoylation of the enzyme results in its translocation to the cytosol [36]. Therefore, EPA may inhibit the palmitoylation of eNOS and its targeting to caveolae and caveolin, the result being eNOS translocation to the cytosol and thereby activation of the enzyme, in a Ca^{2+}-independent manner. (2) It is well documented that caveolin blocks eNOS activity by its association with the enzyme in caveolae [17,20]. Caveolin is also a fatty acid (FA)-binding protein and has an important role in FA transport between membrane compartments [37,38]. Because EPA is also a FA, it is likely that EPA can bind to caveolin, which may in turn assist caveolin to associate with eNOS and thereby activate the eNOS activity. However, in this case, it is still unclear why EPA may activate eNOS and its closely-related metabolites (DPA and DHA) do not.

In conclusion, this is the first documentation that EPA induces Ca^{2+}-independent activation and translocation of eNOS and endothelium-dependent vasorelaxation. It is possible that EPA may be actively incorporated into membrane phospholipid and therefore the free pool as a free fatty acid (FFA) fraction may be rather small. However, the concentration (30 and 60 μM) of EPA used in the present study seems to be physiological, since it was reported that the plasma concentration of EPA in a FFA fraction is 39.4 and 280 μM in healthy and diabetic human subjects, respectively [39,40].

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