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# Mechanism Underlying Endothelium-Dependent Relaxation by 2-Methylthio-ADP in Monkey Cerebral Artery

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**Abstract.** We recently reported endothelium-dependent relaxation caused by nucleotides in the non-human primate cerebral artery. Here, we investigated the endothelium-dependent, nitric oxide- and prostanoid-independent relaxation induced by 2-methylthio-ADP (2MeSADP) in monkey cerebral artery. Mechanical responses of isolated monkey cerebral arteries to the agents were isometrically recorded. In endothelium-intact arterial strips treated with indomethacin plus $N^G$-nitro-L-arginine and partially contracted with prostaglandin $F_{2\alpha}$, 2MeSADP (1 nM – 10 $\mu$M) induced concentration-dependent relaxation that was abolished by removal of endothelium but was not influenced by either carboxy PTIO or 18$\alpha$-glycyrrhetinic acid. The 2MeSADP-induced relaxation was inhibited by MRS2179 and U73122. The relaxation was markedly suppressed by exposure of the strips to high $K^+$ media, but was not affected by glibenclamide. Combination of charybdotoxin plus apamin markedly suppressed the relaxation, whereas iberiotoxin partially attenuated it. Relaxation induced by 2MeSADP was inhibited by arachidonyl trifluoromethyl ketone, ketoconazole, and 14,15-epoxyeicosa-5(Z)-enoic acid. The inhibitors that affected the 2MeSADP-induced relaxation did not influence relaxation caused by sodium nitroprusside or forskolin. These findings indicate that 2MeSADP elicits 'endothelium-derived hyperpolarizing factor (EDHF)-type' relaxation via stimulation of endothelial $P2Y_1$ receptors in monkey cerebral artery. Furthermore, phospholipase $A_2$, cytochrome P450–derived epoxyeicosatrienoic acids and $Ca^{2+}$-activated $K^+$ channels appear to be involved in the relaxation.

***Keywords***: $Ca^{2+}$-activated $K^+$ channel, endothelium-dependent relaxation, epoxyeicosatrienoic acid, monkey cerebral artery, $P2Y_1$ receptor

## Introduction

Nucleotides play important roles not only as an intracellular source for energy and nucleic acid synthesis but also as intercellular signaling molecules when released to the extracellular space. Once released, nucleotides elicit a variety of biological responses to regulate cellular function (1, 2). Endogenous nucleotides were found to be released from different brain regions, and cerebral blood vessels were found to be more sensitive to nucleotides than non-cerebral vessels (3, 4). Furthermore, in response to hypoxia and shear stress, nucleotides are released from erythrocytes, leukocytes, platelets, and activated endothelial cells. Therefore, nucleotides have an important role in the physiological control of cerebrovascular tone and may relate to brain disorders of vascular origin such as migraine, stroke, and vasospasm after subarachnoidal hemorrhage (1, 2).

Nucleotides are extracellular ligands for the P2-receptor family that, so far, is subdivided into P2X ($_{1–7}$)–receptor subtypes that form ligand-gated ion channels and P2Y ($_{1,2,4,6,11–14}$)–receptor subtypes that are G protein–coupled receptors. Nucleotide-induced regulation of vascular tone is mediated by several P2-receptor subtypes that may reside on either smooth muscle cells or endothelial cells (2, 4). Activation of vascular endothelial $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2X_4$ receptors results in the release of endothelium-derived relaxing factors with subsequent vasodilatation (5). The $P2Y_1$ receptor seems to be of major importance in most vascular beds and has been

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reported to mediate early reactive hyperemia after reperfusion (6). ADP and ATP are natural ligands for $P2Y_1$ receptor, but the synthetic analogue 2-methylthio-ADP (2MeSADP) was found to be a more stable and selective agonist for the receptor, which mimics the natural nucleotides (2, 5).

Cerebral hemodynamics must be properly regulated and coupled with metabolic needs to maintain steady brain function. Although several mechanisms regulate the cerebrovascular tone, nitric oxide (NO) derived from endothelial cells and nitrergic nerves plays a physiological role to maintain adequate cerebral blood flow (7). On the other hand, agonist-induced endothelium-dependent cerebral vasodilations are reportedly mediated by prostanoids, NO, and/or endothelium-derived hyperpolarizing factor (EDHF) in various mammals (8). Although the molecular identity and signaling pathway(s) for EDHF are not fully understood, a contributive role for 'EDHF-type' relaxation has been established in the control of vascular tone in conduit and resistant arteries of many species including humans (9). However, information about this role in the cerebral arteries is limited in the primates. Previously, we examined effects of ATP and ADP on monkey cerebral artery in normal (10) and hyperlipidemic conditions (11), showing a relaxation that was partially dependent on the endothelium. In monkey cerebral artery, we recently reported that pyrimidine (UTP) and purine (2MeSADP) nucleotides cause endothelium-dependent relaxation where inhibition of both cyclo-oxygenase (COX) and nitric oxide synthase (NOS) only partially attenuated the response (12). Endothelium-dependent relaxation resistant to COX and NOS inhibitors is produced more prominently by 2MeSADP than by UTP. Hence, the aim of the present study was to further investigate the mechanism underlying the 'EDHF-type' relaxation induced by 2MeSADP under inhibition of COX and NOS in monkey cerebral artery.

## Materials and Methods

The Animal Care and Use Committee at Shiga University of Medical Science approved the use of monkey blood vessels along with the experimental protocol in the present study.

*Preparation*

Japanese monkeys (*Macaca fuscata*) of either sex, weighing 6 – 12 kg, were used in the present study. Animals anesthetized with both ketamine (15 mg/kg, i.m.) and sodium pentobarbital (30 mg/kg, i.v.) were killed by bleeding from the carotid arteries. The brain was rapidly removed, and cerebral arteries were isolated and cleaned. The arteries were helically cut into strips with special care being taken to preserve the endothelium. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Each specimen was vertically fixed between hooks in a muscle bath (10-mL capacity) containing modified Ringer-Locke solution of the following composition: 120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25.0 mM $NaHCO_3$, and 5.6 mM glucose. The bathing media were maintained at a temperature of 37 ± 0.3°C, aerated with a mixture of 95% $O_2$ and 5% $CO_2$, and the pH was 7.36 – 7.43. Before the start of the experiments, the arterial strips were allowed to equilibrate for 60 – 90 min in the bathing media; during that time, the fluids were replaced every 10 – 15 min.

*Recordings of mechanical responses*

The hook fixing the upper end of the strips was connected to the lever of a force-displacement transducer (TB-611T; Nihon-Kohden Kogyo, Tokyo) connected to an amplifier (AP-621G, Nihon-Kohden Kogyo). Isometric contractions and relaxations were displayed on a pen recorder. The resting tension applied to each preparation was adjusted to 1 g, which was optimal for inducing the maximal contraction (13). At the start of the experiment, the contractile response to 30 mM KCl was obtained first. Then, the strips were repeatedly washed with fresh medium and equilibrated. The strips were precontracted with prostaglandin $(PG)F_{2\alpha}$ in a concentration range of 0.1 – 1 $\mu$M, and integrity of the endothelium was determined by checking the relaxation caused by 10 $\mu$M histamine (10) under treatment with famotidine (10 $\mu$M, Fig. 1A). At the end of each series of experiments, papaverine (0.1 mM) was applied to attain the maximal relaxation, which was taken as 100% relaxation.

*Experimental design*

As reported previously, 2MeSADP produced an endothelium-dependent relaxation in monkey cerebral artery (12). The tracing of the 2MeSADP-induced relaxation is shown in Fig. 1B. In preliminary experiments, relaxation induced by 2MeSADP (1 nM – 10 $\mu$M) was found to be decreased with repeated applications, probably due to desensitization of endothelial $P2Y_1$ receptor (14). Therefore, we decided to compare the initial response to 2MeSADP in order to analyze the underlying mechanism as reported previously (15). To this end, a concentration–response curve for 2MeSADP was obtained in the presence of either indomethacin (10 $\mu$M) plus $N^G$-nitro-L-arginine (L-NA; 10 $\mu$M) alone (control) or in the presence of additional blocking agents (experimental). In separate series of experiments, relaxations by NS-309 [a small ($SK_{ca}$)- and intermediate ($IK_{ca}$)-conductance $Ca^{2+}$-activated $K^+$-channel opener, 0.1 – 3 $\mu$M],

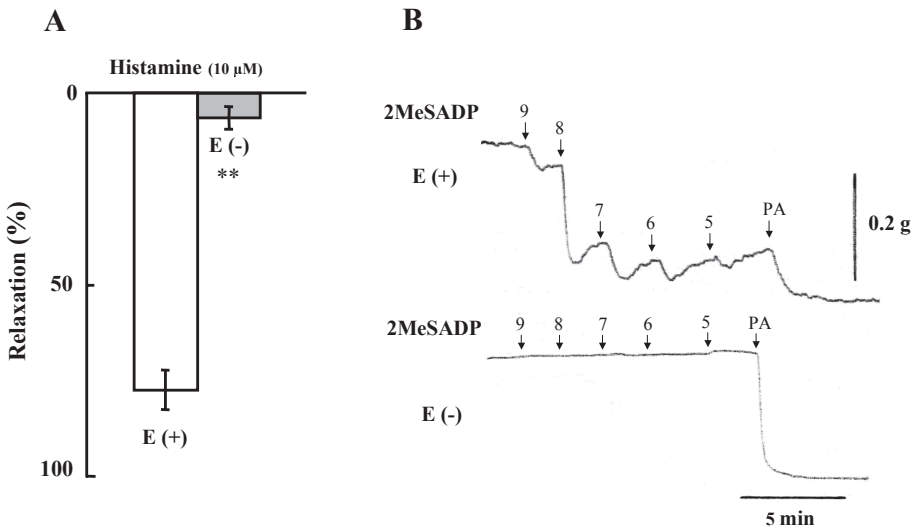


**Fig. 1.** Relaxant responses to histamine (10 $\mu$M) under treatment with famotidine (10 $\mu$M, panel A) and a tracing of relaxation caused by 2MeSADP (1 nM – 0 $\mu$M, panel B) in monkey cerebral artery strips with [E (+)] and without [E (−)] the endothelium in the presence of indomethacin (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$. Relaxation induced by papaverine (PA, 0.1 mM) was taken as 100% on the ordinate of panel A. Concentrations from 9 to 5 indicate $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M, respectively. Vertical bars represent S.E.M. Significantly different from E (+), $^{**}P < 0.01$.

sodium nitroprusside (SNP; a guanylyl cyclase activator, 0.1 nM – 1 $\mu$M) or forskolin (an adenylyl cyclase activator, 1 nM – 1 $\mu$M) were obtained under the same experimental protocol. The strips were incubated with blocking agents for at least 30 min before the concentration–response curve for an agonist was obtained. Concentrations of agonists and blocking agents were expressed as their final concentrations in the organ bath.

*Drugs*

The drugs used were L-NA, apamin, charybdotoxin, iberiotoxin (Peptide Institute, Minoh); 2MeSADP, NS-309, SNP, forskolin, indomethacin, famotidine, MRS2179, suramin, 18$\alpha$-glycyrrhetinic acid, U73122, U73343, glibenclamide, and ketoconazole (Sigma, St. Louis, MO, USA); 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and arachidonyl trifluoromethyl ketone (ATK; Cayman Chemical, Ann Arbor, MI, USA); $PGF_{2\alpha}$ (Pharmacia-Upjohn, Tokyo); histamine (Kanto Chemicals Co., Tokyo); ketamine (Sankyo, Tokyo); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide sodium salt (carboxy PTIO; Dojindo Laboratories, Kumamoto); pentobarbital and papaverine hydrochloride (Dainippon-Sumitomo Pharma Co., Osaka). Dimethylsulfoxide was used as a solvent for NS-309, forskolin, 18$\alpha$-glycyrrhetinic acid, U73122, U73343, and glibenclamide. Ethanol was used for ATK, 14,15-EEZE, and ketoconazole. Indomethacin and $PGF_{2\alpha}$ were dissolved in sodium bicarbonate buffer (pH 9.2). These solvents at the concentrations used in the present study did not show significant influence on the vascular response. Distilled water was used to dissolve all other drugs and to prepare serial dilutions, as required, from stocks on the day of the experiment.

*Statistics*

Relaxations induced by agonists were calculated as a percentage of those induced by papaverine. The results shown in the text and figures are expressed as mean values ± S.E.M. Statistical analyses were made by Student's unpaired *t*-test for two groups and by Tukey's test after one-way analysis of variance for three or more groups. A value of $P < 0.05$ was considered to be statistically significant.

## Results

In monkey cerebral arterial strips treated with indomethacin (10 $\mu$M) plus L-NA (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$, the addition of 2MeSADP (1 nM – 10 $\mu$M) produced a concentration-related relaxation (Fig. 2A). Removal of the endothelium abolished the relaxation induced by 2MeSADP (Fig. 2A), but not that by SNP (Fig. 2B). Carboxy PTIO (0.3 mM), an NO scavenger, did not inhibit the response to 2MeSADP (Fig. 2A), but significantly attenuated the response to SNP (Fig. 2B, Table 1). 18$\alpha$-Glycyrrhetinic acid (0.1 mM), a gap junction inhibitor, did not alter the relaxation induced by 2MeSADP or by SNP (Fig. 2: A and B).

Treatment with MRS2179 (a selective $P2Y_1$-receptor antagonist, 10 $\mu$M) significantly inhibited the relaxation induced by 2MeSADP but did not abolish it (Figs. 3A and 4A). No significant further inhibition was obtained by the addition of suramin (a non-selective P2Y-receptor antagonist; 100 $\mu$M, Figs. 3A and 4A), whereas treatment with a higher concentration of MRS2179 (100 $\mu$M) almost abolished the relaxation (Figs. 3A and 4A). MRS2179 did not significantly modify the relaxation induced by NS-309 (Fig. 3B), SNP (Fig. 3C and Table

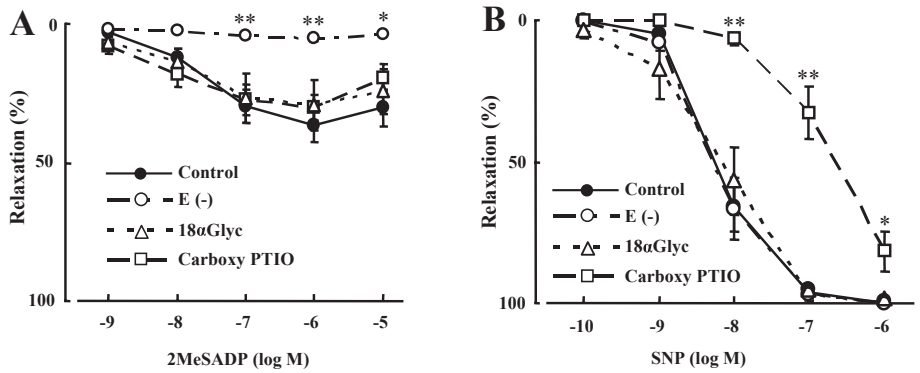


**Fig. 2.** Modifications of concentration–response curve for 2MeSADP (1 nM – 10 $\mu$M, panel A) and SNP (0.1 nM – 1 $\mu$M, panel B) in monkey cerebral artery strips with the endothelium (control) by endothelial denudation [E (−)], carboxy PTIO (0.3 mM), and 18$\alpha$-glycyrrhetinic acid (18$\alpha$Glyc: 0.1 mM). Strips were pretreated with indomethacin (10 $\mu$M) plus L-NA (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$. Relaxation induced by papaverine (0.1 mM) was taken as 100% on the ordinate. Vertical bars represent the S.E.M. of 6 – 8 experiments. Significantly different from the control, *$P < 0.05$, **$P < 0.01$.

**Table 1.** Modifications by inhibitors of the relaxation induced by SNP (100 nM) in monkey cerebral artery strips treated with indomethacin plus L-NA and partially contracted with $PGF_{2\alpha}$

| Treatment | *n* | Relaxation (%)[a] |
|---|---|---|
| Control | 8 | 94.6 ± 1.7 |
| Carboxy PTIO (0.3 mM) | 7 | 32.7 ± 9.4** |
| U73122 (10 $\mu$M) | 5 | 93.8 ± 3.6 |
| MRS2179 (10 $\mu$M) | 7 | 94.1 ± 2.5 |
| ATK (10 $\mu$M) | 6 | 98.2 ± 1.0 |
| Ketoconazole (10 $\mu$M) | 5 | 81.9 ± 13.3 |
| 14,15-EEZE (1 $\mu$M) | 5 | 98.0 ± 1.3 |

*n*, Number of strips; [a]relaxations relative to those induced by 0.1 mM papaverine; **significantly different from the control, $P < 0.01$ (unpaired *t*-test).

1), or forskolin (Figs. 3D and 4B). The 2MeSADP-induced relaxation was significantly inhibited by U73122 (a phospholipase C inhibitor; 10 $\mu$M, Fig. 4C), but not by its inactive analogue U73343 (10 $\mu$M, Fig. 4C). U73122 did not influence the relaxation by SNP (Table 1) or forskolin (Fig. 4D).

Relaxation by 2MeSADP was markedly suppressed by exposure to high-$K^+$ solution (30 mM, Fig. 5A), but was not significantly inhibited by glibenclamide (an ATP-sensitive $K^+$-channel blocker; 1 $\mu$M, Fig. 5A). Treatment with iberiotoxin alone [a selective large ($BK_{ca}$)-conductance $Ca^{2+}$-activated $K^+$-channel blocker, 0.1 $\mu$M] only partially inhibited the relaxation by 2MeSADP (Fig. 5B). Iberiotoxin did not significantly alter the relaxation by NS-309 (Fig. 5C), SNP (Fig. 5D), or forskolin (Table 2). On the other hand, blockade of $K_{ca}$ channels with the combination of charybdotoxin (0.1 $\mu$M) plus apamin (1 $\mu$M) markedly suppressed the relaxation induced by 2MeSADP (Fig. 5B), to an extent similar to suppression exerted by high-$K^+$ solution. The combination of charybdotoxin plus apamin inhibited the relaxation by NS-309 (Fig. 5C), whereas it did not affect the relaxation by SNP (Fig. 5D) or forskolin (Table 2).

Treatment with ATK (a phospholipase $A_2$ inhibitor, 10 $\mu$M) significantly attenuated the relaxation by 2MeSADP (Fig. 6A), but not that by NS-309 (Fig. 6B), SNP (Table 1), or forskolin (Table 2). Ketoconazole [a cytochrome P450 (CYP) inhibitor, 10 $\mu$M] significantly inhibited the relaxation by 2MeSADP (Fig. 6A), but not that by SNP (Table 1) or forskolin (Table 2). Treatment with 14,15-EEZE [an epoxyeicosatrienoic acids (EETs) antagonist, 1 $\mu$M] significantly attenuated the relaxation by 2MeSADP (Fig. 6A), but not that by NS-309 (Fig. 6B), SNP (Table 1), or forskolin (Table 2).

## Discussion

In the present study, 2MeSADP elicits a concentration-related, endothelium-dependent relaxation in monkey cerebral artery under inhibition of COX and NOS. In order to test the possibility of involvement of residual NO derived from the preformed store in the relaxation or incomplete blockade of NOS, effect of carboxy PTIO was examined, but the NO scavenger did not affect the relaxation indicating that NO is not involved in the relaxation at all. Direct communication with electric coupling between vascular endothelium and the underlying smooth muscle through the gap junction has been reported in some vessels (16), but this was not the case for the 2MeSADP-induced relaxation in monkey cerebral artery

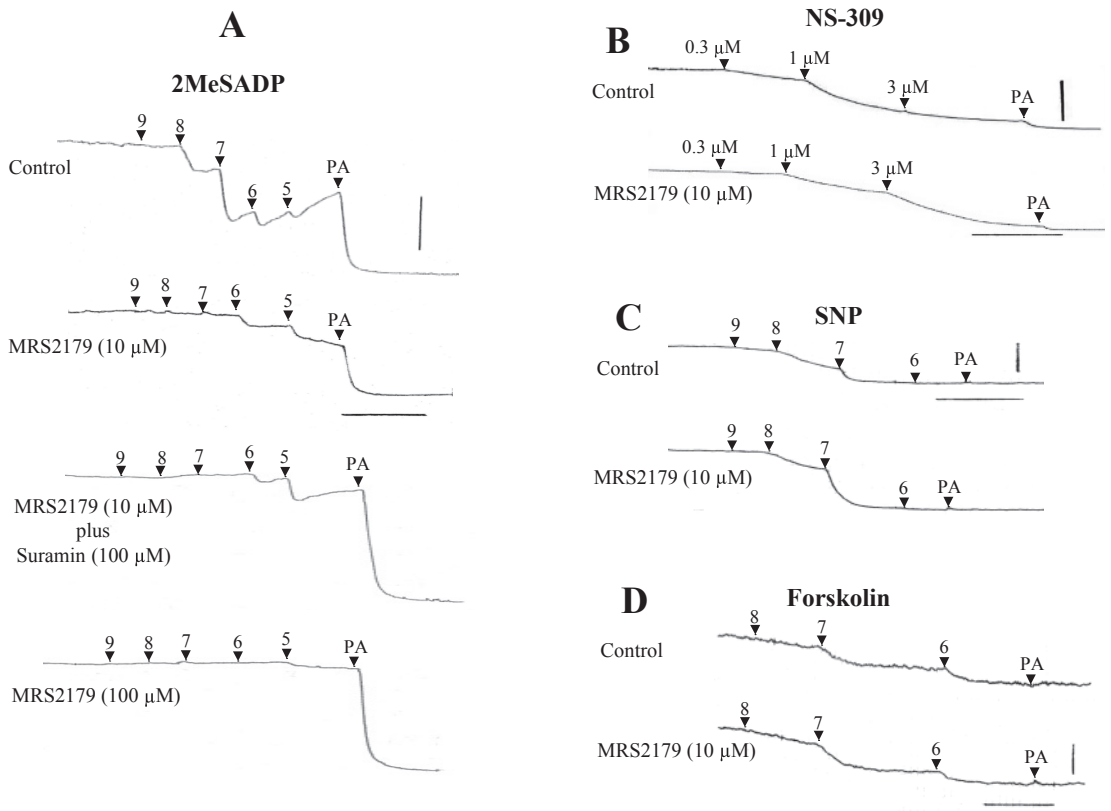


**Fig. 3.** Tracings of the response to 2MeSADP (A) in the absence (control, top) and presence of MRS2179 (10 $\mu$M, second), MRS2179 (10 $\mu$M) plus suramin (100 $\mu$M, third), and MRS2179 (100 $\mu$M, bottom); and tracings of the response to NS-309 (B), SNP (C), and forskolin (D) in the absence (control) and presence of MRS2179 (10 $\mu$M). Concentrations from 9 to 5 indicate $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M, respectively. Papaverine (0.1 mM, PA) produces the maximal relaxation. Vertical lines indicate 0.2 g. Horizontal lines indicate 5 min.

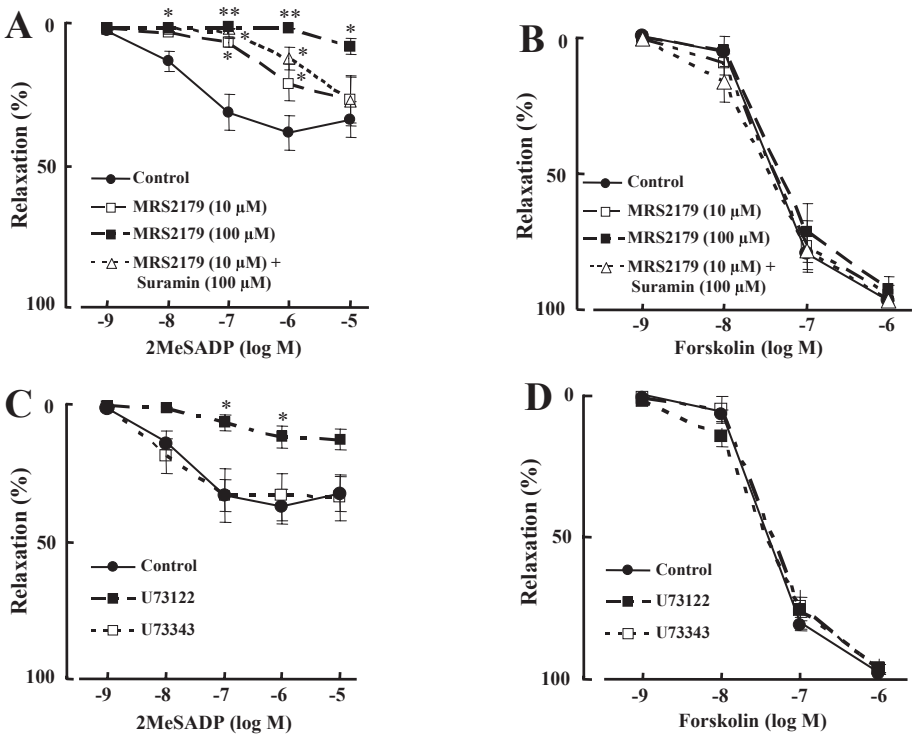


**Fig. 4.** Modifications of the concentration-response curve for 2MeSADP (1 nM – 10 $\mu$M, panels A and C) and forskolin (0.1 nM – 1 $\mu$M, panels B and D) in monkey cerebral artery strips with the endothelium (control) by MRS2179 (10 $\mu$M, panels A and B), MRS2179 (10 $\mu$M) plus suramin (100 $\mu$M, panels A and B), MRS2179 (100 $\mu$M, panels A and B), U73122 (10 $\mu$M, panels C and D), and U73343 (10 $\mu$M, panels C and D). Strips were pretreated with indomethacin (10 $\mu$M) plus L-NA (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$. Relaxation induced by papaverine (0.1 mM) was taken as 100% on the ordinate. Vertical bars represent the S.E.M. of 5 – 7 experiments. Significantly different from the control, $^*P < 0.05$, $^{**}P < 0.01$.

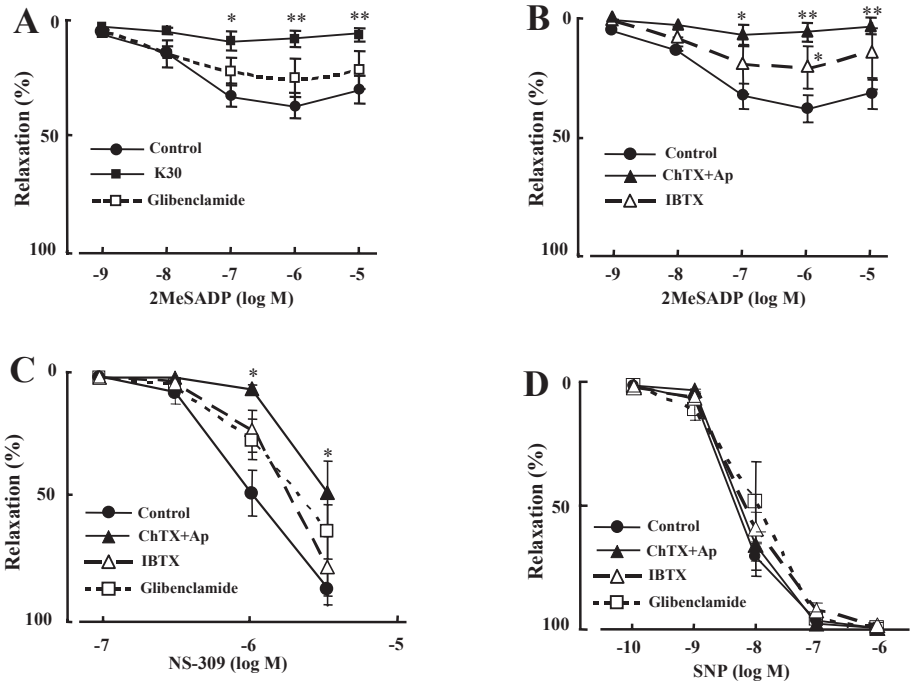


**Fig. 5.** Modifications of concentration–response curve for 2MeSADP (1 nM – 10 $\mu$M, panels A and B), NS-309 (0.1 – 3 $\mu$M, panel C), and SNP (0.1 nM – 1 $\mu$M, panel D) in monkey cerebral artery strips with the endothelium (control) by high-$K^+$ solution (K30, 30 mM; panel A), glibenclamide (1 $\mu$M; panels A, C, and D), a combination of charybdotoxin (0.1 $\mu$M) plus apamin (1 $\mu$M) (ChTX + Ap; panels B – D), and iberiotoxin (IBTX, 0.1 $\mu$M; panels B – D). Strips were pretreated with indomethacin (10 $\mu$M) plus L-NA (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$. Relaxation induced by papaverine (0.1 mM) was taken as 100% on the ordinate. Vertical bars represent the S.E.M. of 4 – 7 experiments. Significantly different from the control, $*P < 0.05$, $**P < 0.01$.

**Table 2.** Modifications by inhibitors of the relaxation induced by forskolin (1 $\mu$M) in monkey cerebral artery strips treated with indomethacin plus L-NA and partially contracted with $PGF_{2\alpha}$

| Treatment | *n* | Relaxation (%)[a] |
|---|---|---|
| Control | 7 | 95.3 ± 1.3 |
| Glibenclamide (1 $\mu$M) | 4 | 96.4 ± 1.3 |
| Charybdotoxin (0.1 $\mu$M) + Apamin (1 $\mu$M) | 6 | 96.3 ± 1.9 |
| Iberiotoxin (0.1 $\mu$M) | 5 | 94.5 ± 3.6 |
| ATK (10 $\mu$M) | 5 | 94.8 ± 1.6 |
| Ketoconazole (10 $\mu$M) | 5 | 93.6 ± 1.9 |
| 14,15-EEZE (1 $\mu$M) | 6 | 98.5 ± 1.0 |

*n*, Number of strips; [a]relaxations relative to those induced by 0.1 mM papaverine.

because 18$\alpha$-glycyrrhetinic acid, a gap-junction blocker, did not suppress the relaxation. However, the relaxation seems to be caused by hyperpolarization because depolarization by high-$K^+$ solution abolished the endothelium-dependent relaxation by 2MeSADP. Combination of charybdotoxin plus apamin also abolished the relaxation, indicating the involvement of small- and intermediate-conductance $Ca^{2+}$-activated $K^+$ channels in the hyperpolarization induced by 2MeSADP. Similar results have been reported in monkey lingual artery (17). These results suggest that 2MeSADP elicits an 'EDHF-type' relaxation that involves a diffusible endothelium-derived mediator(s), other than prostanoids and NO, in monkey cerebral artery.

Endothelium-dependent relaxation caused by ADP or 2MeSADP have been reported in cerebral artery (12, 18, 19). In the previous studies with dogs and rats, ADP- and 2MeSADP-induced endothelium-dependent dilations were abolished by treatment with L-NA, showing that only NO is involved in the response (12, 18, 19). 'EDHF-type' relaxation in cerebral arterioles was reported in eNOS-deficient mice, but receptor subtype or the involved mediators were not analyzed (20). In human cerebral arteries, ADP-induced relaxation is reportedly dependent on the endothelium, but the underlying mechanism or the involved mediator(s) were not examined (4). In monkey cerebral artery, the natural nucleotides like ATP and ADP elicited partial endothelium-dependent relaxation (10), indicating that it is difficult to distinguish the remaining endothelium-dependent relaxation under inhibition of COX and NOS from the endothelium-independent relaxation. Furthermore, the natural nucleotides are rapidly degraded into de-phosphorylated metabolites (e.g., ATP into ADP, AMP, and adenosine) that may also activate other purinergic receptor subtypes (1, 2). In the present study, 2MeSADP mimicked the natural nucleotide in inducing the endothelium-dependent relaxation, but whether or not natural nucleotides are able to induce EDHF-mediated relaxation in monkey cerebral artery is not known yet. Our results show for the first time that 'EDHF-type' relaxation induced by nucleotides contributes to the control of cerebrovascular tone in the primates. This finding may be of particular importance since unlike

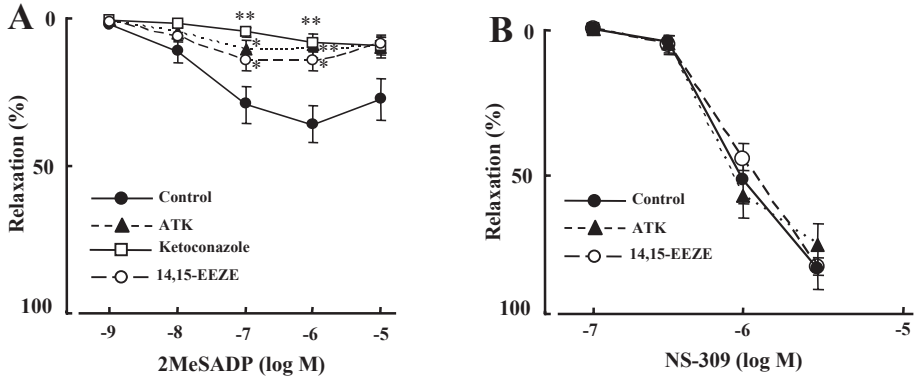


**Fig. 6.** Modifications of concentration–response curve for 2MeSADP (1 nM – 10 $\mu$M, panel A*)* and NS-309 (0.1 – 3 $\mu$M, panel B) in monkey cerebral artery strips with the endothelium (control) by ATK (10 $\mu$M, panels A and B), ketoconazole (10 $\mu$M, panel A), and 14,15-EEZE (1 $\mu$M, panels A and B). Strips were pretreated with indomethacin (10 $\mu$M) plus L-NA (10 $\mu$M) and partially contracted with $PGF_{2\alpha}$. Relaxation induced by papaverine (0.1 mM) was taken as 100% on the ordinate. Vertical bars represent the S.E.M. of 5 – 7 experiments. Significantly different from the control, $*P < 0.05$, $**P < 0.01$.

the periphery, regulation of cerebrovascular tone is preferentially mediated by vasodilating mediators rather than vasoconstricting mediators (8).

$P2Y_1$- and $P2Y_2$-receptor subtypes are reportedly expressed on human vascular endothelial cells (21) and mediate, upon activation, endothelium-dependent relaxation in rat cerebral artery (19). In the present study, treatment with 10 $\mu$M MRS2179, a selective $P2Y_1$-receptor antagonist, blocked the relaxations by 2MeSADP at lower concentrations, but did not inhibit them at higher concentrations. Similar results were obtained in our recent study (12). However, activation of $P2Y_2$, rather than $P2Y_1$, receptors was observed to mediate the 'EDHF-type' relaxation in rat cerebral artery (22). Therefore, we examined the effect of suramin, a non-specific inhibitor of P2Y receptors, to test the possibility of involvement of other P2Y subtypes in the response. When the preparation was treated with suramin in addition to MRS2179, no further inhibition was obtained by suramin. Furthermore, raising the concentration of MRS2179 up to 100 $\mu$M abolished the relaxation induced by 2MeSADP. These results indicate that endothelial $P2Y_1$ receptor solely mediates the relaxation by 2MeSADP in monkey cerebral artery. Involvement of endothelial $P2Y_1$ in the 'EDHF-type' relaxation by ADP has been reported in other blood vessels of different species (23 – 25). On the other hand, treatment with U73122 suppressed the relaxation by 2MeSADP, indicating the involvement of PLC as a membrane-bound effector for $G_q$-coupled P2Y receptor(s). These results show the functional evidence that 2MeSADP elicits 'EDHF-type' relaxation in monkey cerebral artery via stimulation of endothelial $P2Y_1$ receptor and its key effector molecule of PLC.

Nucleotides, as agonists stimulating G protein–coupled receptors, were reported to increase endothelial intracellular calcium concentration and to produce endothelium-derived mediators for relaxation (1, 5). Among these mediators, metabolites of arachidonic acid produced by catalysis of CYP have been shown to mediate 'EDHF-type' relaxation in both animal and human blood vessels (26, 27). In monkey coronary and lingual artery, we previously reported the involvement of arachidonic acid metabolites in agonist-induced 'EDHF-type' relaxation (15, 17). In the present study, we found that ATK markedly suppressed the 2MeSADP-induced relaxation, indicating that $PLA_2$ activation is involved in the 'EDHF-type' relaxation in monkey cerebral artery. Activation of $PLA_2$ has been reported to be involved in 'EDHF-type' relaxation in monkey coronary and lingual arteries (15, 17) as well as cerebral arteries from rat (28) and guinea pig (29). Treatment with ketoconazole inhibited the 'EDHF-type' relaxation induced by 2MeSADP in monkey cerebral artery. Since the specificity of ketoconazole as a CYP inhibitor may be questionable, we examined the effect of 14,15-EEZE, an EETs antagonist on the response, and observed that 14,15-EEZE markedly inhibited the 'EDHF-type' relaxation induced by 2MeSADP. The present study together with our previous investigations suggests the involvement of endothelium-derived CYP products, probably EETs in the 'EDHF-type' relaxation in monkey arteries (15, 17, 30). Since EETs antagonist did not abolish the 2MeSADP-induced relaxation, possible involvement of endothelial mediators other than EETs is not excluded. In the cerebral circulation, exogenous application of all EET isomers showed that all EETs act as vasodilators with different potencies among animal species (31 – 33). The present study suggested that endogenous EETs are involved in the 'EDHF-type'

relaxation in monkey cerebral artery since the EETs antagonist 14,15-EEZE inhibited the relaxation. 14,15-EEZE is not a specific inhibitor for EETs because 14,15-EEZE can antagonize the action of all EETs isomers. In this regard, it remains to be determined which kind of EET isomers is responsible for the endothelium-dependent relaxation in monkey cerebral artery.

Endothelium-derived EETs have been identified as relaxant mediators that are transferred to the underlying vascular smooth muscle where they cause potassium channel activation, hyperpolarization, and relaxation in several blood vessels (34 – 36). In the present study, the combination of charybdotoxin plus apamin but not glibenclamide markedly inhibited the 'EDHF-type' relaxation induced by 2MeSADP showing the involvement of $K_{ca}$ channels, but not $K_{ATP}$ channels, in the response of monkey cerebral artery. The 2MeSADP-induced relaxation was partially sensitive to iberiotoxin, suggesting the contribution of $BK_{ca}$ channels to the response. Similarly 'EDHF-type' relaxation induced by acetylcholine in monkey coronary and lingual arteries was inhibited by the combination of charybdotoxin plus apamin; however, iberiotoxin was ineffective in these arteries (15, 17). Arteries from different regions of the same animal may differ in the mechanism of 'EDHF-type' relaxation as shown in the response of guinea-pig arteries to acetylcholine (29). Of note, nucleotide-induced 'EDHF-type' relaxation in cerebral arteries was shown to be partially sensitive to iberiotoxin in the rat (22). Therefore, differences in the agonist examined, the blood vessel used, and/or other factor(s) may be the underlying reason for this discrepancy. Nevertheless, these results show functional evidence that 2MeSADP elicits 'EDHF-type' relaxation in monkey cerebral artery with the involvement of endothelium-derived EETs and the activation of $K_{ca}$ channels.

In conclusion, our findings indicate that 2MeSADP elicits 'EDHF-type' relaxation via stimulation of endothelial $P2Y_1$ receptor in monkey cerebral artery. Furthermore, $PLA_2$, CYP-derived EETs, and activation of $K_{ca}$ channels appear to be involved in the relaxation. The present study may serve to increase our understanding of endothelial mechanisms regulating cerebrovascular tone by nucleotides in the primates. Further work is needed to explore modifications of cerebral artery response to nucleotides during states of cerebrovascular disorders.

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## References

1 Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev. 2006;58:58–86.
2 Erlinge D, Burnstock G. P2 receptors in cardiovascular regulation and disease. Purinergic Signal. 2008;4:1–20.
3 Potter P, White TD. Release of adenosine 5-triphosphate from synaptosomes from different regions of rat brain. Neuroscience. 1980;5:1351–1356.
4 Hardebo JE, Kahrstrom J, Owman C. P1 and P2 purine receptors in brain circulation. Eur J Pharmacol. 1987;144:343–352.
5 Burnstock G. Purinergic signaling. Br J Pharmacol. 2006;147: S172–S181.
6 Olivecrona GK, Gotberg M, Harnek J, Wang L, Jacobson KA, Erlinge D. Coronary artery reperfusion: the ADP receptor $P2Y_1$ mediates early reactive hyperemia in vivo in pigs. Purinergic Signalling. 2006;1:59–64.
7 Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: recent advances. Pharmacol Rev. 2009;61: 62–97.
8 Toda N, Okamura T. Cerebral vasodilators. Jpn J Pharmacol. 1998;76:349–367.
9 Bellien J, Thuillez C, Joannides R. Contribution of endothelium-derived hyperpolarizing factors to the regulation of vascular tone in humans. Fundam Clin Pharmacol. 2008;22:363–377.
10 Toda N, Kawakami M, Yamazaki M, Okamura T. Comparison of endothelium-dependent responses of monkey cerebral and temporal arteries. Br J Pharmacol. 1991;102:805–810.
11 Ayajiki K, Fujioka H, Torii R, Toda N, Okamura T. Endothelial and neuronal functions in cerebral and temporal arteries from monkeys fed a high-cholesterol diet. J Cardiovasc Pharmacol. 2002;40:456–466.
12 Geddawy A, Shimosato T, Tawa M, Imamura T, Okamura T. Comparison of endothelium-related responses to nucleotides of dog and monkey cerebral arteries. J Pharmacol Sci. 2010;112: 378–381.
13 Toda N, Hatano Y, Hayashi S. Modifications by stretches of the mechanical response of isolated cerebral and extracerebral arteries to vasoactive agents. Pflugers Arch. 1978;374:73–77.
14 Rodríguez-Rodríguez R, Yarova P, Winter P, Dora KA. Desensitization of endothelial $P2Y_1$ receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries. Br J Pharmacol. 2009;158:1609–1620.
15 Fujioka H, Ayajiki K, Shinozaki K, Toda N, Okamura T. Mechanisms underlying endothelium-dependent, nitric oxide- and prostanoid-independent relaxation in monkey and dog coronary arteries. Naunyn Schmiedebergs Arch Pharmacol. 2002;366: 488–495.
16 Sandow SL, Tare M, Coleman HA, Hill CE, Parkington HC. Involvement of myoendothelial gap junctions in the action of endothelium-derived hyperpolarizing factor. Circ Res. 2002;90: 1108–1113.
17 Ayajiki K, Okamura T, Fujioka H, Imaoka S, Funae Y, Toda N. Involvement of CYP3A-derived arachidonic acid metabolite(s) in responses to endothelium-derived $K^+$ channel opening substance in monkey lingual artery. Br J Pharmacol. 1999;128: 802–808.
18 Toda N, Ayajiki K, Okamura T. Cerebroarterial relaxations mediated by nitric oxide derived from endothelium and vasodilator nerve. J Vasc Res. 1993;30:61–67.

19 You J, Johnson TD, Childres WF, Bryan RM Jr. Endothelial-mediated dilations of rat middle cerebral arteries by ATP and ADP. Am J Physiol. 1997;273:H1472–H1477.
20 Faraci FM, Lynch C, Lamping KG. Responses of cerebral arterioles to ADP: eNOS-dependent and eNOS-independent mechanisms. Am J Physiol Heart Circ Physiol. 2004;287:H2871–H2876.
21 Wang L, Karlsson L, Moses S, Hultgårdh-Nilsson A, Andersson M, Borna C, et al. P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol. 2002;40:841–853.
22 You J, Johnson TD, Marelli SP, Mombouli JV, Bryan RM Jr. P2u receptor mediated release of endothelium-derived relaxing factor/nitric oxide and endothelium-derived hyperpolarizing factor from cerebrovascular endothelium in rats. Stroke. 1999;30: 1125–1133.
23 Mistry H, Gitlin JM, Mitchell JA, Hiley CR. Endothelium-dependent relaxation and endothelial hyperpolarization by P2Y receptor agonists in rat-isolated mesenteric artery. Br J Pharmacol. 2003;139:661–671.
24 Malmsjø M, Chu ZM, Croft K, Erlinge D, Edvinsson L, Beilin LJ. P2Y receptor-induced EDHF vasodilatation is of primary importance for the regulation of perfusion pressure in the peripheral circulation of the rat. Acta Physiol Scand. 2002;174: 301–309.
25 Wihlborg AK, Malmsjö M, Eyjolfsson A, Gustafsson R, Jacobson K, Erlinge D. Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. Br J Pharmacol. 2003;138:1451–1458.
26 Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature. 1999;401:493–497.
27 Miura H, Gutterman DD. Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P450 monooxygenase and $Ca^{2+}$-activated $K^+$ channels. Circ Res. 1998;83:501–507.
28 You J, Marrelli SP, Bryan RM Jr. Role of cytoplasmic phospholipase $A_2$ in endothelium-derived hyperpolarizing factor dilations of rat middle cerebral arteries. J Cereb Blood Flow Metab. 2002;10:1239–1247.
29 Dong H, Jiang Y, Cole WC, Triggle CR. Comparison of the pharmacological properties of EDHF-mediated vasorelaxation in guinea-pig cerebral and mesenteric resistance vessels. Br J Pharmacol. 2000;130:1983–1991.
30 Ayajiki K, Fujioka H, Toda N, Okada S, Minamiyama Y, Imaoka S, et al. Mediation of arachidonic acid metabolite(s) produced by endothelial cytochrome P-450 3A4 in monkey arterial relaxation. Hypertens Res. 2003;26:237–243.
31 Iliff JJ, Jia J, Nelson J, Goyagi T, Klaus J, Alkayed NJ. Epoxyeicosanoid signaling in CNS function and disease. Prostaglandins Other Lipid Mediat. 2010;91:68–84.
32 Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. Am J Physiol Heart Circ Physiol. 1992;263:H519–H525.
33 Ellis EF, Police RJ, Yancey L, McKinney JS, Amruthesh SC. Dilation of cerebral arterioles by cytochrome P-450 metabolites of arachidonic acid. Am J Physiol Heart Circ Physiol. 1990; 259:H1171–H1177.
34 Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium derived hyperpolarizing factors. Circ Res. 1996;78:415–423.
35 Gauthier KM, Edwards EM, Falck JR, Reddy DS, Campbell WB. 14, 15-Epoxyeicosatrienoic acid represents a transferable endothelium-dependent relaxing factor in bovine coronary arteries. Hypertension. 2005;45:666–671.
36 Archer SL, Gragasin FS, Wu X, Wang S, McMurtry S, Kim DH, et al. Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle $BK_{ca}$ channels. Circulation. 2003;107:769–776.