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Apple procyanidins induce hyperpolarization of rat aorta endothelial cells via activation of K^+ channels

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Abstract

Apple procyanidins (AP), one of the polyphenol-rich compounds, showed an endothelial-dependent vasorelaxation in rat aorta, but the mechanisms of beneficial effects are still unclear. The present study was designed to clarify the potential role of AP in rat aorta endothelial cells (RAECs). The treatment of RAECs with AP (1–10 μg/ml) resulted in a dose-dependent hyperpolarization with a maximum effect at 10 μg/ml, and for this reason, AP (10 μg/ml) was used in all the following experiments. AP-induced hyperpolarization was significantly inhibited by pretreatment of nonspecific K^+ inhibitor, tetraethyl ammonium chloride or specific K^+ channel inhibitors, iberiotoxin, glibenclamide, 4-aminopyridine and $BaCl_2$, as well as by high KCl or Ca^{2+}-free solution. AP-induced hyperpolarization was also proved using 64-channel multielectrode dish system that can monitor a direct and real-time change of membrane potential. Furthermore, AP treatment caused a significant increase of nitric oxide (NO) production and cyclic guanosine monophosphate levels via endothelial NO synthase messenger RNA expression. The NO production was inhibited by N^G-monoethyl-L-arginine or Ca^{2+}-free solution and was completely abolished by their combination. Also, AP inhibited endothelial proliferation, while the effect was significantly abolished by N^G-monoethyl-L-arginine or tetraethyl ammonium chloride. These findings suggest that AP induces both hyperpolarization of RAECs via multiple activation of K^+ channels and activation of NO/cyclic guanosine monophosphate pathway via increasing NO production or is responsible for antiangiogenic effect. Diminishment of hyperpolarization as well as NO production of AP in Ca^{2+}-free solution implicated that AP would play a crucial role in promoting Ca^{2+} influx into endothelial cells so as to promote both actions.

Keywords: Apple procyanidins; Hyperpolarization; K^+ channel; NO; Proliferation

1. Introduction

The vascular endothelium and endothelium-released substances play a crucial role in maintaining the balance between vascular relaxation and constriction. When this balance is upset, endothelial dysfunction occurs, causing damage to the arterial wall [1]. This is of great importance because endothelial dysfunction is the initiating step in the pathogenesis of arteriosclerosis promoting morbidity and mortality [2]. However, several epidemiological studies indicate that intake of polyphenol-rich foods, such as fruits and vegetables, and beverages derived from plants, such as cocoa, red wine and tea, (may) represents a great benefit in terms of cardiovascular diseases protection and (may) thereby shows an inverse correlation between polyphenol-enriched diet and risks of cardiovascular disease, such as arteriosclerosis [3–5]. Previous studies have revealed that potential bioactivities contributing to the beneficial effect of polyphenols include inhibition of low-density lipoprotein oxidation [6], platelet activation [7] and expressions of prothrombotic and proatherosclerotic molecules, such as endothelin-1 [8] or vascular endothelial growth factor [9]. In plant polyphenols, the most active fractions have been found in flavan-3-ol–enriched oligomeric condensed tannins fractions, especially dimers and trimers, and procyanidins-condensed tannins oligomers (arising from condensation of flavan-3-ol and flavan-3,4-diol) can protect endothelial cells from lipid oxidation of membrane and cytotoxicity [10,11]. Indeed, the protective effect of these polyphenolic compounds might include the ability to dilate arteries by stimulating rapid formation of nitric oxide (NO), formed by endothelial nitric oxide synthase (eNOS), subsequently leading to an elevated accumulation of cyclic guanosine monophosphate (cGMP), as well as the opening of multiple K^+ channels via endothelium-derived hyperpolarizing factor (EDHF)-mediated responses in endothelial cells [12–14]. In addition, several previous studies have also demonstrated that drugs that cause vasorelaxation by opening K^+ channels and subsequent hyperpolarization may be of clinical value under the condition that pathological vascular dysfunction is caused by membrane depolarization [15]. As mentioned previously, the protective effect of cardiovascular disease by stimulating endothelium-derived relaxing factor, including NO and

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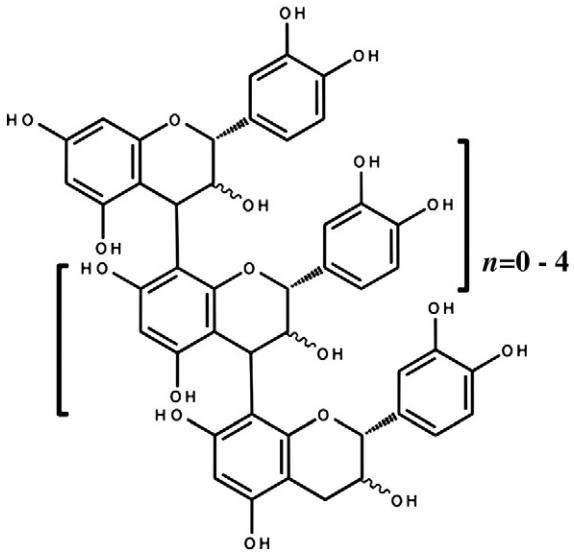

Fig. 1. Structure of major AP. AP used in this study mainly contained oligomers with n=0~4, and their total content was >ca. 45%.

prostacyclin (prostaglandin I_2), and EDHF has been attributable, at least in part, to polyphenols [16], but the mechanisms by which polyphenolic compounds produce their vasorelaxant effects have not yet been established. Furthermore, there are many controversial reports despite the large amount of studies regarding the beneficial effects [17]. In our previous study, it has been demonstrated that apple procyanidins (AP) induced endothelium-dependent vasorelaxation on aorta ring from Sprague-Dawley rats via NO/cGMP pathway in combination with hyperpolarization by multiple K^+ channel activation [18]. However, the molecular mechanisms of AP-induced vasorelaxation on vascular endothelial cells *in vitro* are also still unknown. Accordingly, the objective of the present study was to clarify the endothelium-dependent signaling mechanisms from both the activation of eNOS-derived NO/cGMP level and the hyperpolarization via the activation of K^+ channels induced by AP in rat aorta endothelial cells (RAECs).

2. Materials and methods

2.1. Materials

AP was prepared from apple polyphenol extracts by our previous methods [19]. AP used in this study mainly contained procyanidins ranging from dimers to pentadecamers (Fig. 1) — content in AP: dimers, 13.0%; trimers, 12.3%, tetramers, 8.7%; pentamers, 5.9%, hexamers, 4.9% and >heptamer, 20.9% [18]. Tetraethyl ammonium chloride (TEA), iberiotoxin, glibenclamide, 4-aminopyridine and $BaCl_2$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). N^G-Monomethyl-L-arginine and *O,O′-bis*(2-aminoethyl)ethylene-glycol-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical-reagent grade and used without further purification.

2.2. Cell culture

RAECs were purchased from Cell Applications Inc. (San Diego, CA, USA). RAECs were cultured in rat endothelial cell medium (San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Corporation's GIBCO, Carlsbad, CA, USA). RAECs were cultured in 25-cm^2 flasks (CORNING, Corning, NY, USA) and then incubated at 37°C in 5% CO_2 incubator up to 95% confluence. RAECs of passages 7 were used in all experiments.

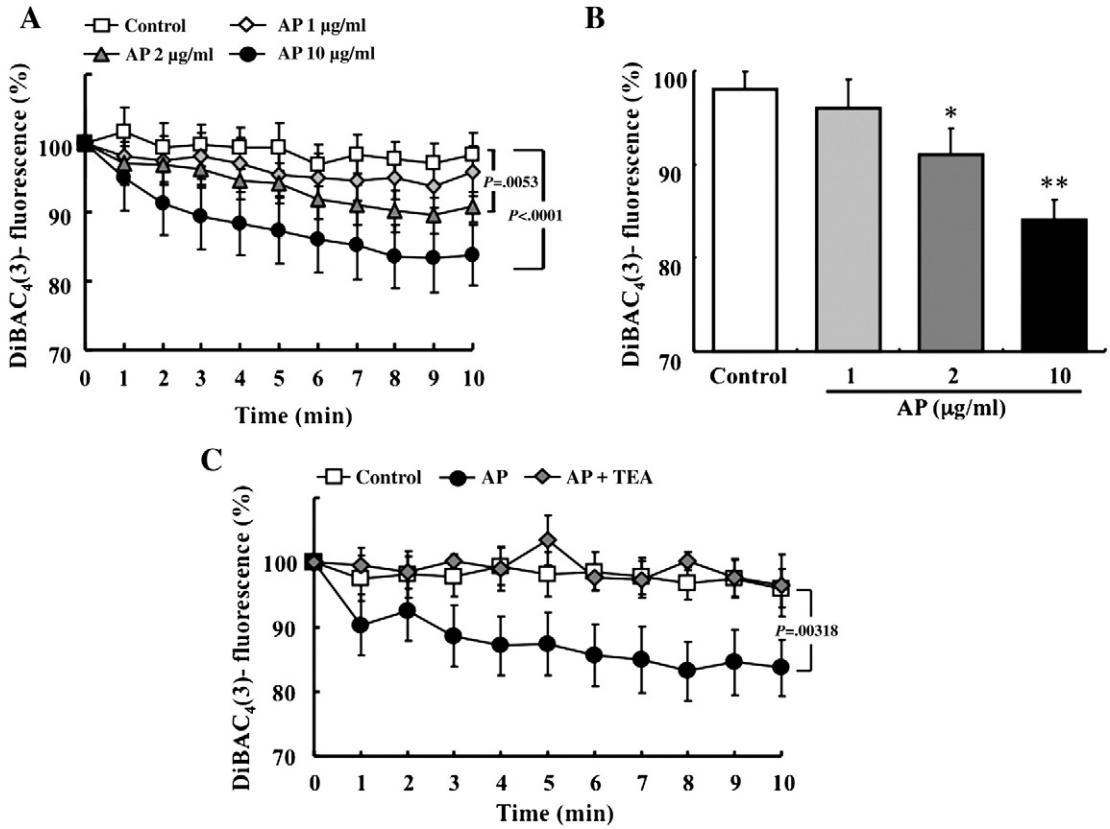

Fig. 2. Change of membrane potential induced by AP in RAECs. (A) Dose-dependent effect of AP (1–10 μg/ml) on the membrane potential. (B) Change of membrane potential induced by AP at 10-min reaction time. (C) Change of AP-induced hyperpolarization in RAECs by pretreatment of nonspecific K^+ channel inhibitor, TEA (1 mM). The results were expressed as mean±S.E.M. (n=4). Significant difference between control and AP groups was evaluated by two-way ANOVA. **P<.01 versus control; *P<.05 versus control by Tukey–Kramer's t test.

2.3. Measurement of membrane potential by bis-(1,3-dibutylbarbituric acid) trimethine oxonol

Changes of membrane potential were analyzed by a membrane potential-sensitive probe *bis*-[1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Dojindo Laboratories]. Briefly, RAECs (1.5×10^4 cells/well) were cultured on 96-well plates (CORNING) with Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation's GIBCO, Grand Island, NY, USA), containing 10% FBS, L-glutamine (2 mM), 1% nonessential amino acids, streptomycin (100 μg/ml), penicillin (100 U/ml), insulin (10 μg/ml) and $NaHCO_3$ (3.7 mg/ml), and then incubated at 37°C in 5% CO_2 incubator up to 95% confluence. The DMEM was changed to FBS-free medium for 24 h to make RAECs quiescent. RAECs were washed twice with assay buffer [HEPES (20 mM), NaCl (120 mM), KCl (2 mM), $CaCl_2$ (2 mM), $MgCl_2H_2O$ (1 mM), glucose (5 mM)] and were then incubated in assay buffer containing $DiBAC_4(3)$ (0.25 μM) for 30 min with or without nonspecific K^+ channel inhibitor, TEA (1 mM) or specific K^+ channel inhibitors, iberiotoxin (0.1 μM), 4-aminopyridine (0.1 mM), $BaCl_2$ (1 mM) or glibenclamide (1 μM), at 37°C in the dark. RAECs were also incubated in assay buffer containing $DiBAC_4(3)$ (0.25 μM) for 30 min in the absence or presence of high concentration of K^+ with KCl (50 mM) or Ca^{2+}-free solution with EGTA (0.1 mM). After these pretreatments, AP (1–10 μg/ml) was treated in each well. Fluorescence was excited at 485 nm, and the emission was detected at 520 nm at intervals of 60 s. The first time point was considered as 100%, and changes of $DiBAC_4(3)$ fluorescence intensity were set in relation to this measurement.

2.4. Measurement of membrane potential by 64-channel multielectrode dish system

Electrophysiological recordings were obtained with a 64-channel multielectrode dish (MED64) system (Alpha MED Sciences, Osaka, Japan). We used an MED-P530A probe with a 300-μm interpolar distance between electrodes, chamber depth of 10 mm and 64 planar microelectrodes in an 8×8 array to determine membrane potential of RAECs. RAECs (1×10^5 cells/probe-dish) were cultured on an MED probe with rat endothelial cell medium containing 10% FBS and were then incubated at 37°C in 5% CO_2 incubator up to 95% confluence. The DMEM was changed to FBS-free medium for 24 h to make RAECs quiescent. An aliquot (1 ml) of AP (10 μg/ml) was injected into a physiological saline solution (PSS) buffer flowed by a peristaltic pump at a flow rate of 1.0 ml/min. After reaching the sample solution onto the RAEC-cultured probe, an AP-induced membrane potential was automatically recorded with a Panasonic amplitude (Panasonic, Tokyo, Japan) in the absence or presence of TEA (1 mM) and was compared with the control group (PSS buffer). The MED64 performer was used for data analysis.

2.5. Real-time reverse transcription polymerase chain reaction for inducible NO synthase and eNOS messenger RNA expression

To obtain enough amounts of RAECs to isolate total RNA for real-time reverse transcription polymerase chain reaction (PCR), RAECs were cultured on a 100×20-mm tissue culture dish (Falcon, South Hackensack, NJ, USA) with DMEM containing 10% FBS and then incubated at 37°C in 5% CO_2 incubator for 6–7 days. The DMEM was changed

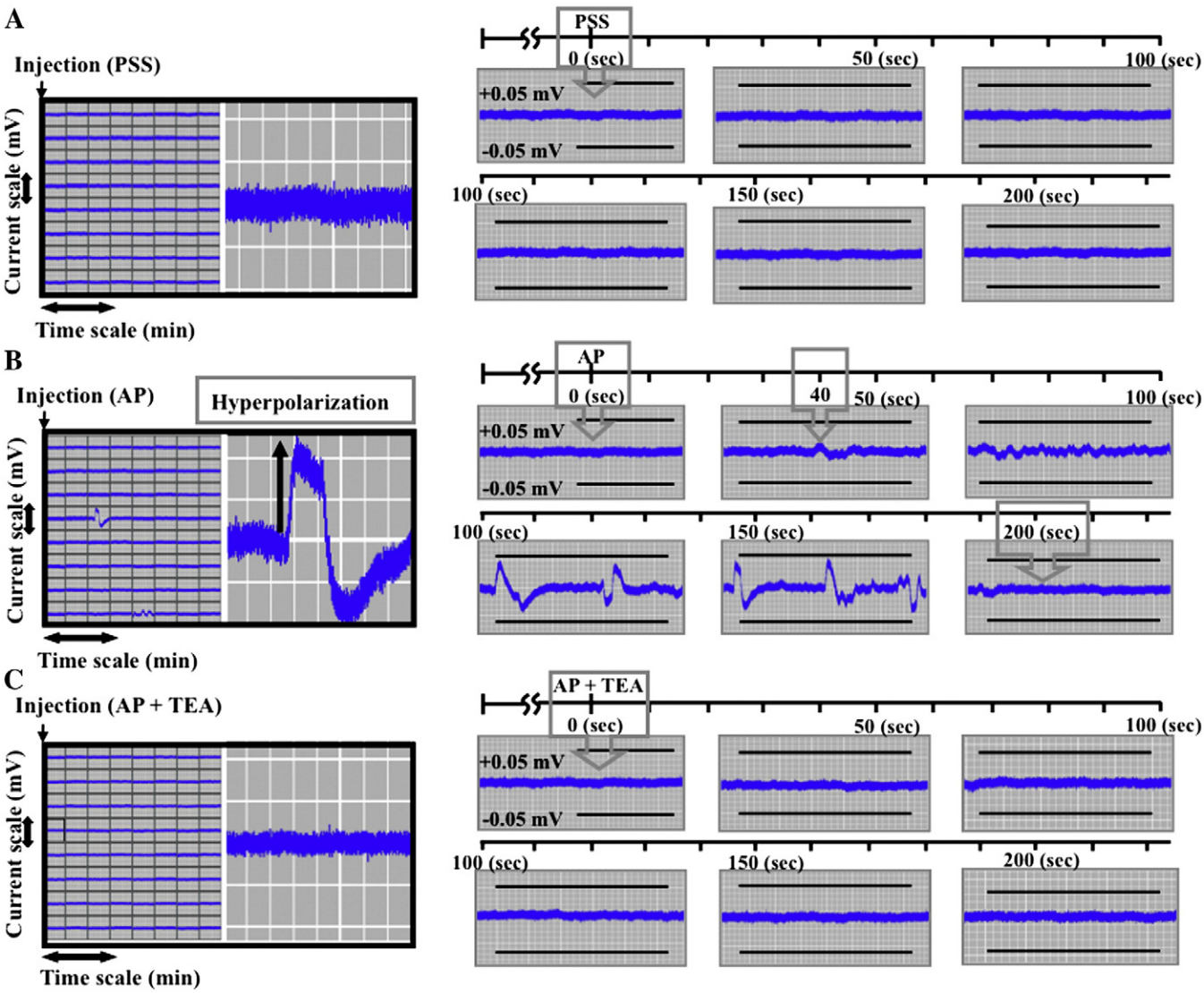

Fig. 3. Effect of nonspecific K^+ channel inhibitor on AP (10 μg/ml)-induced hyperpolarization. Change of AP (10 μg/ml)-induced hyperpolarization by using a MED64 system in the presence of TEA (1 mM). (A) Real-time (0–200 s) change of membrane potential in the control group (PSS buffer). (B) Real-time (0–200 s) change of membrane potential on the AP group. (C) Real-time (0–200 s) change of membrane potential on the AP group with TEA.

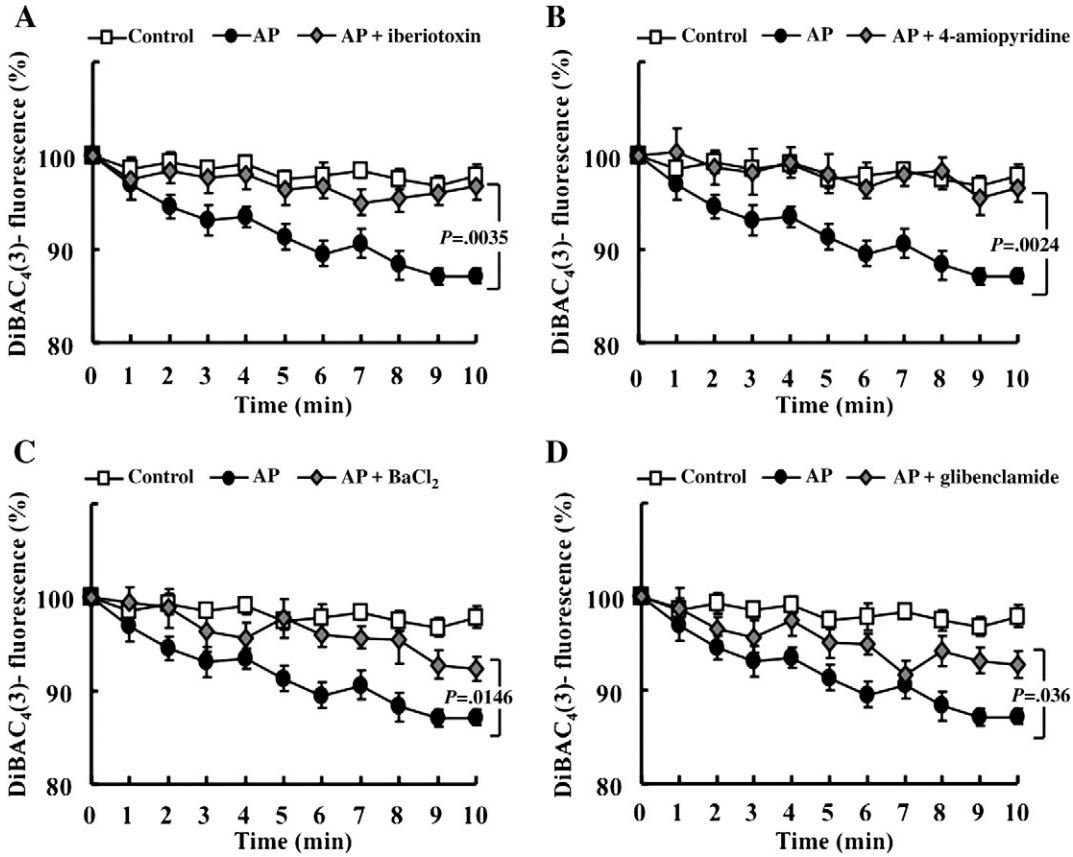

Fig. 4. Change of AP (10 mg/mlg/ml)-induced hyperpolarization in RAECs by pretreatment of specific K^+ channel inhibitors, iberiotoxin (0.1 μM), 4-aminopyridine (0.1 mM), $BaCl_2$ (1 mM) or glibenclamide (1 μM). The results were expressed as mean±S.E.M. (n=8). Significant differences between the absence and the presence of the inhibitor were evaluated by two-way ANOVA.

to FBS-free medium for 24 h. Cultures that were considered more than 95% pure based on morphological examination were used to isolate total RNAs. The total RNAs were isolated from RAECs treated for 10 min with or without AP (10 μg/ml). Before the isolation, the complement DNAs were produced from 1 μg of total cellular RNAs by reverse transcription reagents (Promega Corporation, Madison, WI, USA) as instructed by the manufacturer. Amplification and detection of specific products were performed by an MX 3000 QPCR system (Stratagene Inc., Tokyo, Japan) with the following cycle profile: 1 cycle at 95°C for 30 s and then run for 40 cycles at 95°C for 30 s (denaturation step), at 52°C for 30 s (annealing step) and at 72°C for 1 min (extension phase). At the end of each extension phase, observed fluorescence was used for quantitative analyses. A melting point analysis was carried out by heating the amplicon from 52°C to 95°C, and a characteristic melting point curve was obtained for each product. Sequences of used primers were showed as follow: forward 5′-GATCAATAACCTGAAGCCCG-3′ and reverse 5′-GCCCTTTTTTGCTCCATA GG-3′ for inducible NO synthase (iNOS); forward 5′-CCGCACTTCTGTGCCTTGCTC-3′ and reverse 5′-GCTCGG GTGGATTTGCTGCTCT-3′ for eNOS; forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCAC-CACCCTGTTGCTGTA-3′ for GAPDH [20]. As an internal control, rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used for RNA template normalization. Quantification was reported by proprietary software, as cycle threshold (Ct). The expression of the target genes relative to the housekeeping genes was calculated as the difference between the threshold values for the two groups. All PCR reactions were done in triplicates. PCR reactions were done within the linear range of amplification, and amplification products were separated using a 2% polyacrylamide gel and stained with ethidium bromide. Predicted sizes are 360 bp for eNOS and 578 bp for iNOS.

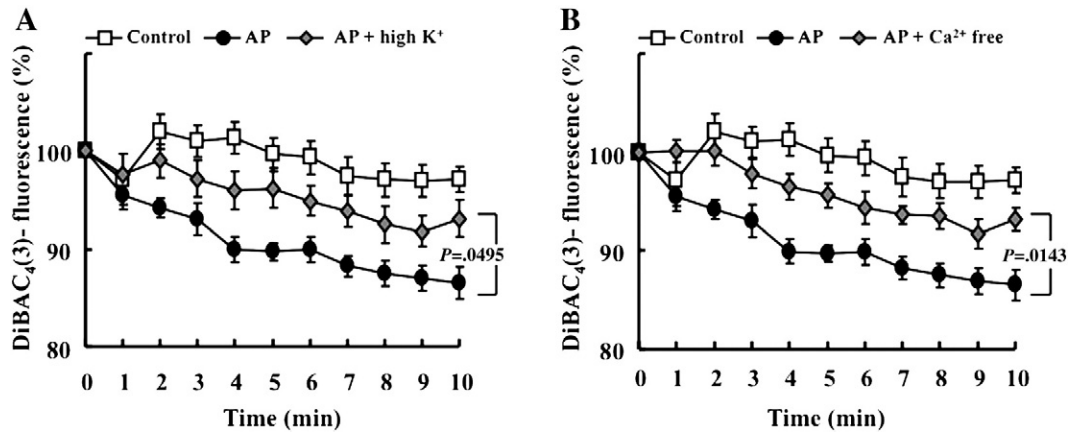

Fig. 5. Change of AP (10 μg/ml)-induced hyperpolarization in RAECs in the presence of high K^+ with 50 mM KCl (A) or Ca^{2+}-free solution with EGTA (0.1 mM; B). The results were expressed as mean±S.E.M. (n=8). Significant differences between the absence and the presence of high K^+ or Ca^{2+}-free solution were evaluated by two-way ANOVA.

2.6. Measurement of NO production in RAECs

The concentration of NO in culture supernatant was determined as nitrite, by the Griess reagent (1% sulfanilamide/0.1% naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4) (Sigma, Japan). RAECs (1.5×10^4 cells/well) were cultured in 96-well plates with DMEM containing 10% FBS, then incubated at 37°C in 5% CO_2 incubator up to 95% confluence. The DMEM was changed to FBS-free medium for 24 h. RAECs were pretreated with L-NMMA (100 μM) or EGTA (2 mM) for 60 min, and then AP (10 μg/ml) was treated in each well for 10 min. Supernatant of cell culture medium was collected and assayed for NO production using the Griess reagent. Culture medium (100 μl) was incubated with Griess reagent (100 μl). The absorbance was measured at 540 nm using a microplate reader (Wallac 1420; Perkin Elmer Life Science, Tokyo, Japan). The absorbance value was then converted to NO amount (pmol/well) from RAECs using standard curves prepared with serial dilutions of $NaNO_2$ standards. In a separated experiment to visualize NO production in RAECs, an NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2DA; Sekisui Medical, Tokyo, Japan), was used. Briefly, RAECs (2×10^5 cells/dish) were cultured in a 35-mm μDish (Ibidi GmbH, Martinsried, Germany) with DMEM containing 10% FBS. The DMEM was changed to FBS-free medium supplemented with L-arginine (100 μM) for 24 h. RAECs were then loaded with DAF-2DA (1 μM) for 60 min at 37°C and rinsed three times with PSS buffer. RAECs were then treated at the time of 1 to 10 min with or without AP (10 μg/ml). Acetylcholine (Ach; 100 μM) was used as positive control. After stimulation, RAECs were fixed by treatment of 4% paraformaldehyde for 5 min at 4°C. Fixed RAECs were visualized by a Nikon confocal microscope with an attached camera (Nikon, Tokyo, Japan) using appropriate filters, with a peak excitation wavelength of 488 nm and a peak emission wavelength of 515 nm.

2.7. Measurement of cGMP

cGMP level in RAECs was determined by a cGMP EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA) as instructed by the manufacturer. Briefly, RAECs (1×10^5 cells/well) were cultured in 6-well plates (CORNING) with DMEM containing 10% FBS. AP (10 μg/ml) was treated in each well for 15 min. After incubation, RAECs were homogenized with HCl (0.1 M) by a microhomogenizer (KINEMATICA AG, Lucerne, Switzerland). The homogenates were centrifuged for 10 min at 1000×*g* at 4°C, and the supernatants were used for cGMP enzyme immunoassay. The amount of cGMP from RAECs was expressed as pmol of cGMP/well. The absorbance was measured at 405 nm by a Wallac 1420-microplate reader.

2.8. Cell proliferation experiment

Proliferation experiment of RAECs by AP was conducted with a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium (WST-8) assay as previous description [21]. Briefly, RAECs (5×10^3 cells/well) were cultured on 96-well plates with DMEM containing 10% FBS, and then incubated at 37°C in 5% CO_2 incubator up to 95% confluence. The DMEM was changed to FBS-free medium for 24 h. RAECs were incubated with AP (10 μg/ml) for 24 h in the absence or presence of L-NMMA (100 μM) or TEA (1 mM) in a volume of 100 μl. After the incubation, 10 μl WST-8-8 solution (Dojindo Laboratories) was added to each well, incubated for 4 h. The absorbance was measured at 450 nm using a Wallac 1420-microplate reader.

2.9. Statistical analysis

Results are expressed as mean±S.E.M. *n* refers to the RAECs preparations used in each experiment. Statistical difference between curves was analyzed using a two-way of variance (ANOVA) followed by post hoc Tukey–Kramer's *t* test. Statistical difference between two groups was analyzed by unpaired Student's *t* test. $P<.05$ was considered to be statistical significant. All analyses were conducted with Stat View J5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of AP on membrane potential of RAECs

AP-induced hyperpolarization in RAECs was evaluated by a membrane potential-sensitive probe $DiBAC_4(3)$ at an AP concentration of 1, 2 or 10 μg/ml. As shown in Fig. 2, the treatment of RAECs with AP resulted in a significant decrease of $DiBAC_4(3)$ fluorescence in an AP concentration-dependent manner; the highest (16%) decrease or much hyperpolarization of RAECs was observed at a concentration of 10 μg/ml for 10 min ($P<.01$ vs. control, $n=4$; Fig. 2B). Though data were not shown, higher concentrations of AP (>20 μg/ml) were responsible for reduced $DIBAC_4(3)$ response on RAECs due to their ability to damage cell or induce cytotoxicity.

3.2. Effect of nonspecific K^+ channel inhibitor on AP-induced hyperpolarization

Effect of nonspecific K^+ channel inhibitor, TEA, on AP-induced hyperpolarization in RAECs was primarily investigated, based on our finding that AP evoked vasorelaxation via K^+ channel activation [18]. As shown in Fig. 2C, pretreatment of RAECs with TEA (1 mM) completely ameliorated AP (10 μg/ml)-induced hyperpolarization up to a similar level of the control group ($P=.00318$ vs. AP group, $n=4$). A direct or real-time monitoring of RAEC-membrane potential with an MED64

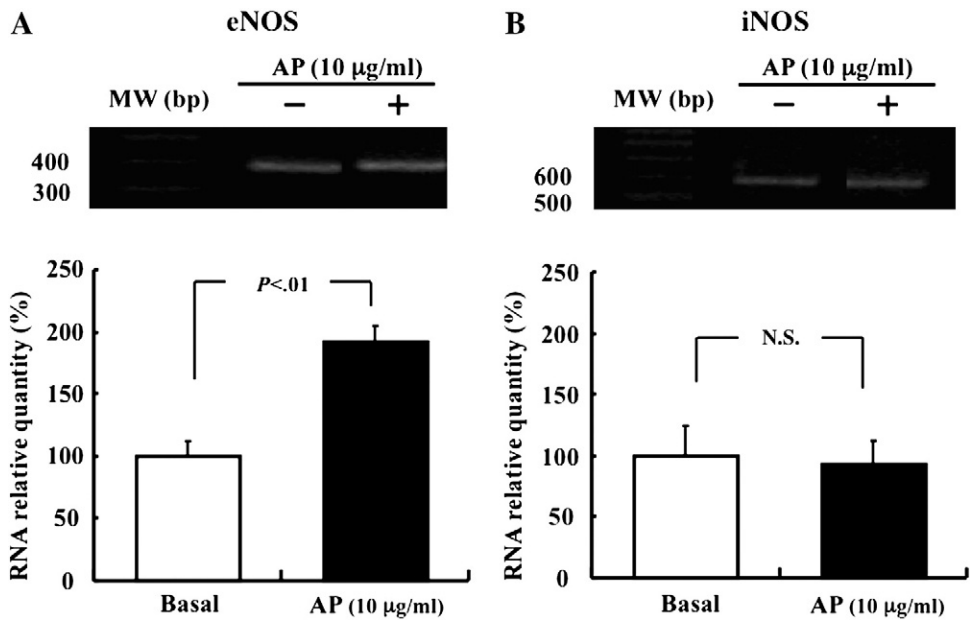

Fig. 6. Changes of eNOS and iNOS mRNA expressions by AP (10 μg/ml) in RAECs. (A) The expression of eNOS mRNA induced by AP at 10 min. (B) The expression of iNOS mRNA induced by AP at 10 min. The results were expressed as mean±S.E.M. ($n=3$). MW indicates molecular weight markers; N.S., no significance at $P>.05$. Significant differences between the basal and AP groups were evaluated by unpaired Student's *t* test.

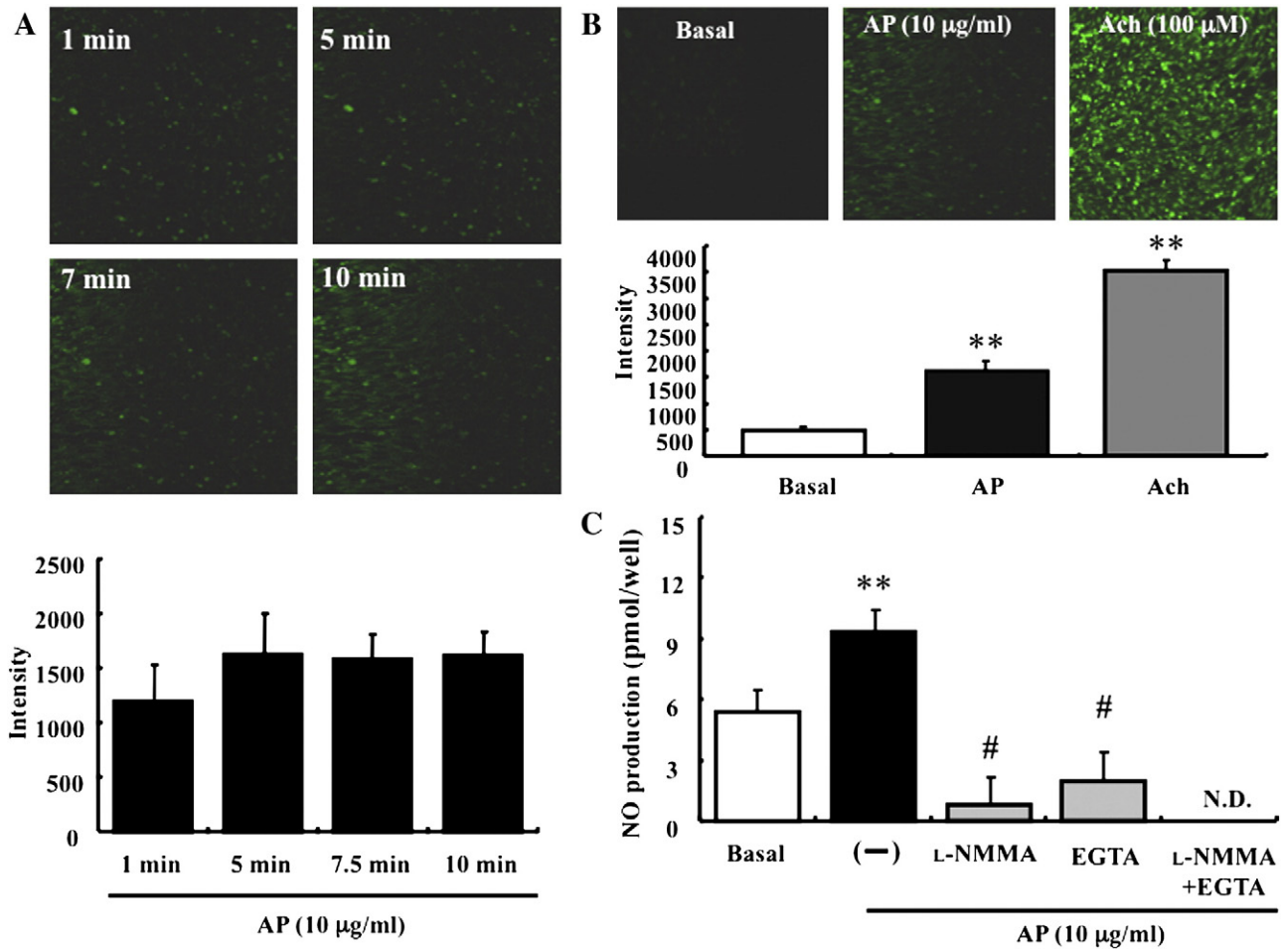

Fig. 7. Effect of AP (10 mg/mlg/ml)-induced NO production in RAECs. Time (1–10 min)–response relationship of AP on NO production (n=3; A) and NO production induced by AP or Ach (100 μM) at 10 min (n=3; B) by using the fluorescence probe DAF-2DA. Ach was used as a positive control. (C) Change of NO production by AP in the pretreatment of 100 μM L-NMMA or 2 mM EGTA and combination of inhibitors (n=4) by using a Griess reagent assay. N.D. means no detection. The results were expressed as mean±S.E.M. **P<.01 versus basal; #P<.05 versus AP group by Tukey–Kramer's t test.

system [22,23] also proved AP (10 μg/ml)-induced hyperpolarization compared with the control group (PSS buffer; Fig. 3A) and the diminishment of the AP power by TEA (Fig. 3B, C). Both observations from separated experiments (Figs. 2 and 3) provided confirmation that AP hyperpolarized an RAEC membrane via activation of K^+ channel.

3.3. Effects of specific K^+ channel inhibitors and high K^+ or Ca^{2+}-free solution on AP-induced hyperpolarization

To make clear the mechanism(s) underlying AP-induced hyperpolarization of RAECs, further $DiBAC_4(3)$ experiments were performed using specific inhibitors for K^+ channel subtypes. AP (10 μg/ml)-induced hyperpolarization was significantly inhibited by the pretreatment of RAECs with iberiotoxin [0.1 μM; a large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) inhibitor; P=.0035 vs. AP group, n=8; Fig. 4A], 4-aminopyridine [0.1 mM; a transient K^+ channel (K_V) inhibitor; P=.0024 vs. AP group, n=8; Fig. 4B], $BaCl_2$ [1 mM; an inward rectifier K^+ channel (K_{ir}) inhibitor; P=.0146 vs. AP group, n=8; Fig. 4C] or glibenclamide [1 μM; an ATP-sensitive K^+ channel (K_{ATP}) inhibitor; P=.036 vs. AP group, n=8; Fig. 4D]. AP (10 μg/ml)-induced hyperpolarization was also diminished in the presence of high K^+ (50 mM KCl; P=.0495 vs. AP group, n=8) or Ca^{2+} -free solution (0.1 mM EGTA; P=.0143 vs. AP group, n=8) as shown in Fig. 5A and B. Altogether AP caused the opening of diverse K^+ channel subtypes including BK_{Ca}, K_v, K_{ir} and K_{ATP},; extracellular Ca^{2+} influx was also required for AP-induced hyperpolarization in RAECs.

3.4. NO level in AP-stimulated RAECs

The messenger RNA (mRNA) expression levels of both eNOS and iNOS in RAECs treated with AP (10 μg/ml) for 10 min were

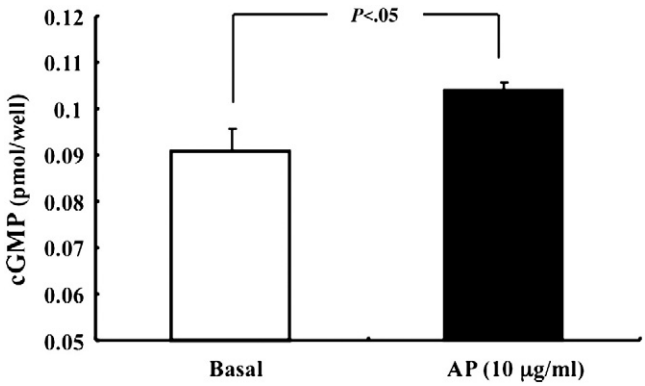

Fig. 8. Change of AP (10 μg/ml)-induced cGMP levels in RAECs. The results were expressed as mean±S.E.M. (n=3). Significant differences between basal and AP group were evaluated by unpaired Student's t test.

determined by a real-time reverse transcription PCR. AP remarkably enhanced the level of eNOS mRNA expression by two-fold compared with basal (P<.01 vs. basal, n=3; Fig. 6A), whereas no increase in the mRNA level of iNOS by AP was observed (n=3; Fig. 6B). A rapid (within 5 min; Fig. 7A) and significant effect of AP on NO production (P<.01 vs. basal, n=3; Fig. 7B), like Ach (100 μM), was revealed by a DAF-2DA confocal microscopy. The AP-stimulated NO production in RAECs (9.4±0.01 pmol/well) compared with basal level (5.4±0.01 pmol/well) was markedly abolished by L-NMMA and/or EGTA (P<.05 vs. AP group, n=4; Fig. 7C), indicating that AP promoted NO production in RAECs through Ca^{2+}-dependent eNOS activation.

3.5. cGMP level in AP-stimulated RAECs

cGMP level in AP-stimulated RAECs was evaluated by a cGMP an enzyme immunoassay (EIA) assay kit. Compared with basal level (0.08±0.004 pmol/well), the addition of AP (10 μg/ml) in RAECs caused a significant (P<.05 vs. basal, n=3; Fig. 8) increase of cGMP levels (0.10±0.001 pmol/well), indicating the involvement of AP in NO/cGMP pathway.

3.6. Antiproliferative action of AP in RAECs

In a 24-h incubation of RAECs with AP (0, 1 and 10 μg/ml), AP (10 μg/ml) significantly inhibited a 10% FBS-induced cell proliferation (P<.01 vs. control, n=6; Fig. 9A). AP (1 μg/ml) failed to show the antiproliferative action. The cell-line study also revealed that the presence of either L-NMMA or TEA significantly ameliorated the AP-induced antiproliferative power (P<.01 vs. AP group, n=6; Fig. 9B), which suggested that AP would regulate RAEC proliferation cascades via NO and/or K^+ channel activation.

4. Discussion

Many medicinal plants used for a range of ailments and disorders contain vegetable tannins as their active principles. Several reports regarding procyanidins have demonstrated their beneficial health-promoting effects in the cardiovascular system by antioxidation [24], antiproliferation [25] and antitumor actions [26]. It was also reported that some beneficial effects were responsible for protective action against vascular endothelium disorders, which revealed a great importance in the maintenance of vessel wall integrity [27–29]. The signaling cascades toward relaxation in endothelium layers are regulated by three main pathways: the prostacyclin (or prostaglandin I_2)-cAMP, NO/cGMP and EDHF-mediated hyperpolarization [30]. The properties of the former two factors, NO and prostaglandin I_2, have been extensively investigated, but the nature and mechanisms of action of the third vasodilator, EDHF, are still controversial. Some researchers suggested that several candidates for EDHF, such as K^+ ions, hydrogen peroxide (H_2O_2) and epoxyeicosatrienoic acids [31], may serve as a backup vasodilator in situations associated with an altered bioavailability of endothelium-derived NO [32]. In our previous report [18], AP evoked a marked vasorelaxation in rat aorta through the opening of multiple K^+ channels in endothelial layers, which suggested that AP may play as an EDHF activator in the layers. It seems likely that plant polyphenols such as the extract of Eucommia bark, a traditional Chinese medicinal herb, or ginsenosides [33] could induce an EDHF-mediated relaxation or hyperpolarization [14,34], while their underlying mechanism(s) toward vasorelaxation remains to be fully understood.

Based on the aforementioned, in the present study, we investigated some approaches to determine the underlying cell signaling cascades in RAECs that may be modulated by AP: (1) membrane potential, (2) eNOS/iNOS mRNA expressions, (3) NO/cGMP levels and (4) antiproliferation in AP-treated RAECs. Primarily, we investigated whether AP can induce a hyperpolarization of RAECs. The results in Fig. 2A and B demonstrated that the treatment of RAECs with AP resulted in a time- and concentration-dependent hyperpolarization, by which vasorelaxation could be achieved. The MED64 system that can perform the real-time monitoring of electric change in cells [22,23] also provided confirmation that AP induced hyperpolarization of RAECs. In contrast, the hyperpolarization was completely abolished by both nonspecific K^+ channel inhibitor, TEA (Fig. 2C), and specific K^+ channel inhibitor, iberiotoxin, 4-aminopyridine, $BaCl_2$ or glibenclamide (Fig. 4). The amelioration of AP-hyperpolarization by TEA was observed in the MED64 system as well (Fig. 3). This indicates that AP-induced hyperpolarization was achieved by its combinational activation of BK_{Ca}, SK_{Ca} and K_{ATP} as well as Ca^{2+}-independent K_V and K_{ir} [18,35]. It has been reported that resveratrol [36] and ginsenosides Rg_3 [33] activated Ca^{2+}-dependent K^+ channels, while they failed to activate Ca^{2+}-independent channels. In contrast, the isoflavone equol revealed the Ca^{2+}-independent action for vasorelaxation in human endothelium cells [37], in agreement with our results. The controversial findings suggested that the mechanism(s) of K^+ channel activation of polyphenols must differ from their structural features, but the relationship between structure of polyphenols and K^+ channel activation still remains unclear due to the structural complexity of AP or condensed EC polymer [38]. In view of these findings, our working group was also able to speculate in endothelial cells that K^+ ions released from the endothelium may function as an EDHF in AP-treated rat arteries as previous other reports [39], since less change of membrane potential was observed in high content of KCl in AP-treated RAECs as well as no response in the presence of TEA by MED64 system. Other studies demonstrated that a small increase in extracellular K^+ dilates blood vessels via the electrogenic Na^+/K^+-ATPase leading to hyperpolarization, in combination with a slight

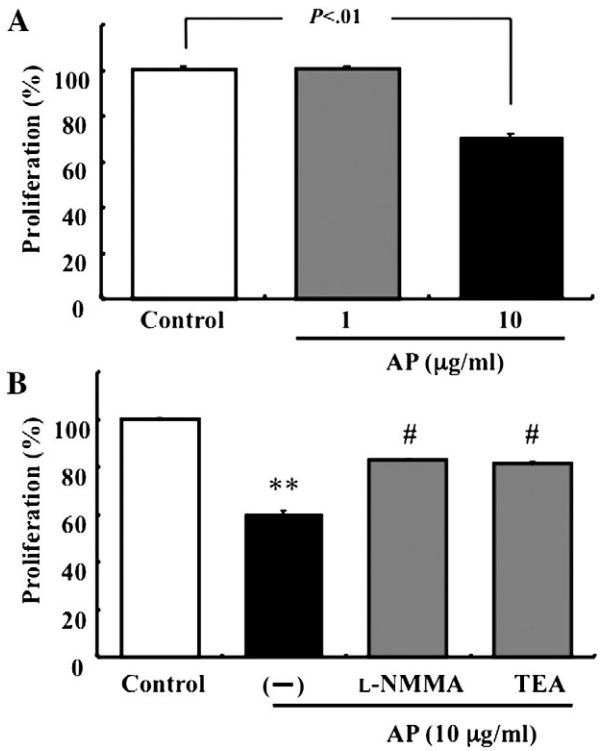

Fig. 9. Antiproliferative effect induced by AP (10 mg/mlg/ml) in RAECs. (A) The effect of dose (1–10 μg/ml)–response relationship of AP on proliferation of RAECs. (B) Change of AP-induced antiproliferative effect by pretreatment of 100 μM L-NMMA or 1 mM TEA. The results were expressed as mean±S.E.M. (n=6). Significant differences between the control and AP groups were evaluated by unpaired Student's t test. **P<.01 versus control; #P<.01 versus AP group by Tukey–Kramer's t test.

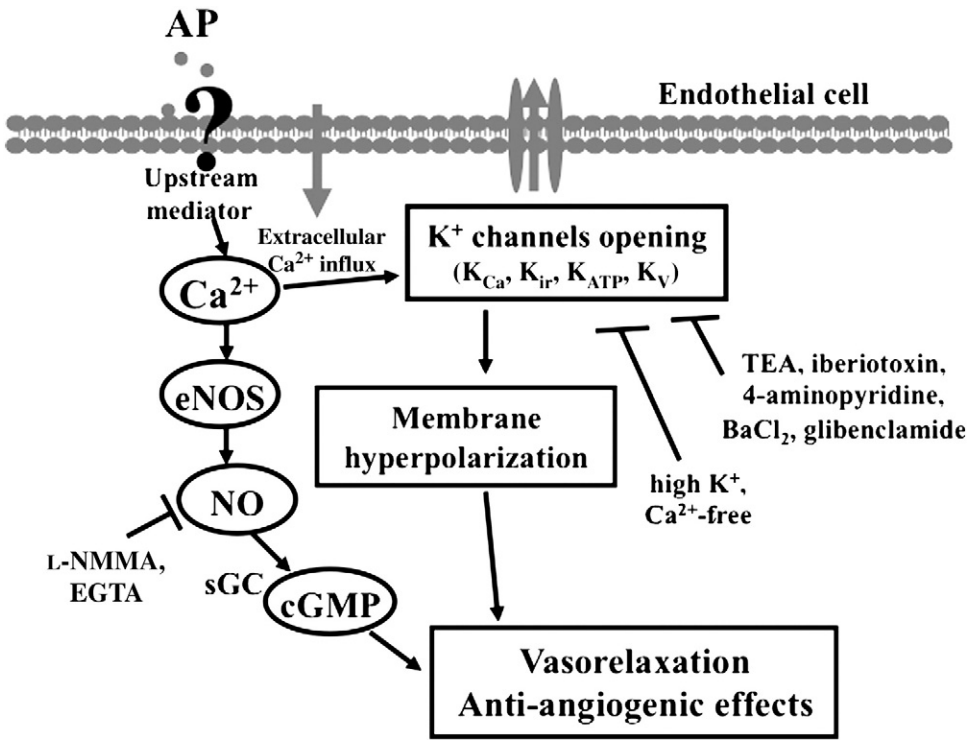

Fig. 10. Proposed mechanism(s) of AP-induced vasorelaxation and antiangiogenic effect in RAECs.

increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [40,41]. In addition, Busse et al. [42] provided the importance that EDHF-mediated responses were initiated by $[Ca^{2+}]_i$ increase, followed by the consequent activations of endothelial BK_{ca}, IK_{ca} and SK_{ca} channels; the efflux of K^+ through the endothelial K_{Ca} channels could then elicit the hyperpolarization by activating K_{ir} channel or electrogenic Na^+/K^+-ATPase. The result that the AP-induced $DiBAC_4(3)$ fluorescence reduction was diminished in Ca^{2+}-free solution (Fig. 5B) also revealed the involvement of AP in $[Ca^{2+}]_i$ increase for hyperpolarization. Taken together, it would be possible that AP may primarily accelerate the influx of Ca^{2+} into RAECs toward the activation of diverse K^+ channels or hyperpolarization. A significant inhibition of AP-induced NO production (Fig. 7C) by EGTA demonstrated that AP was alternatively involved in the activation of NO/cGMP pathway via its increasing $[Ca^{2+}]_i$ action. The promotion of $[Ca^{2+}]_i$ in endothelium cells is achieved by diverse upstream signaling cascades, such as p38 p38 mitogen-activated protein kinase (MAPK), PI3 kinase-Akt pathway, H_2O_2 and epoxyeicosatrienoic acids [43,44]. Within the present experiments, however, we could not focus on the upstream cascades responsible for AP-induced $[Ca^{2+}]_i$ promotion in RAECs, and experiments regarding their mRNA expressions in AP–RAECs are in progress. Enhanced eNOS and changeless iNOS mRNA expressions in AP–RAECs (Fig. 6) allowed us to understand that the antiproliferation of RAECs by AP (Fig. 9) that may lead to vasorelaxation was caused by its EDHF-mediated action, not by its inflammatory or apoptosis action. Other researchers [45,46] have pointed out that quercetin, a flavonoid, inhibited endothelial cell proliferation by inhibiting NAD(P)H oxidases that lead to oxidative stress or in turn by activating NO/cGMP pathway [47]. Since the possibility that AP-induced NO/cGMP production may account for the decrease of NAD(P)H oxidase activity leading oxidative stress would not be excluded from the overall AP action in RAECs.

In conclusion, we first demonstrated that AP plays crucial roles in promoting hyperpolarization via multiple K^+ channel activations as well as increased NO release via promotion of NO/cGMP pathway responsible for antiangiogenic effect by a reduction of cell proliferation in RAECs. AP-induced bimodal actions would be caused by the increasing influx of Ca^{2+} into RAECs (Fig. 10). Further studies must be, however, needed for clarifying which upstream signaling mediator(s) is involved in AP actions using AP or its individuals.

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