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Theophylline-induced endothelium-dependent vasodilation is mediated by increased nitric oxide release and phosphodiesterase inhibition in rat aorta

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Abstract. This study aimed to examine the endothelial dependence of vasodilation induced by the phosphodiesterase inhibitor theophylline in isolated rat thoracic aortas and elucidate the underlying mechanism, with emphasis on endothelial nitric oxide (NO). The effects of various inhibitors and endothelial denudation on theophylline-induced vasodilation, and the effect of theophylline on vasodilation induced by NO donor sodium nitroprusside, cyclic guanosine monophosphate (cGMP) analog bromo-cGMP, and β-agonist isoproterenol in endothelium-denuded aorta were examined. The effects of theophylline and sodium nitroprusside on cGMP formation were also examined. We examined the effect of theophylline on endothelial nitric oxide synthase (eNOS) phosphorylation and intracellular calcium levels. Theophylline-induced vasodilation was greater in endothelium-intact aortas than that in endothelium-denuded aortas. The NOS inhibitor, N^W-nitro-L-arginine methyl ester; non-specific guanylate cyclase (GC) inhibitor, methylene blue; and NO-sensitive GC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one inhibited theophylline-induced vasodilation in endothelium-intact aortas. Theophylline increased the vasodilation induced by sodium nitroprusside, bromo-cGMP, and isoproterenol. Theophylline increased cGMP formation in endothelium-intact aortas, and sodium nitroprusside-induced cGMP formation in endothelium-denuded aortas. Moreover, theophylline increased stimulatory eNOS (Ser1177) phosphorylation and endothelial calcium levels, but decreased the phosphorylation of inhibitory eNOS (Thr495). These results suggested that theophylline-induced endothelium-dependent vasodilation was mediated by increased endothelial NO release and phosphodiesterase inhibition.

Key words: Theophylline — Nitric oxide — Endothelium — Phosphodiesterase — Vasodilation

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Highlights

- Theophylline-induced endothelium-dependent vasodilation is mediated by increased endothelial nitric oxide (NO) and phosphodiesterase inhibition.
- In terms of physiological significance, theophylline-induced endothelial NO-mediated vasodilation may aggravate hypotension caused by theophylline toxicity.

Introduction

Theophylline, a non-selective phosphodiesterase (PDE) and adenosine receptor inhibitor, is used to treat bronchial asthma and chronic obstructive pulmonary disease (Barnes 2013; Bondarev et al. 2022; Akbaş and Güneş 2023). The low safety margin of theophylline results in frequent side effects, including cardiac arrhythmia and hypotension (Kearney et al. 1985; Barnes 2013; Journey and Bentley 2023). Theophylline toxicity increases endogenous catecholamine levels, leading to positive inotropic and chronotropic effects but hypotension, which is mediated by β2-adrenoceptor-mediated vasodilation (Kearney et al. 1985). Endothelial nitric oxide (NO) produces cyclic guanosine monophosphate (cGMP) by activating guanylate cyclase (GC), which subsequently induces vasodilation *via* cGMP-dependent protein kinase activation (Sohn et al. 2004). PDE hydrolyzes cGMP to 5'-GMP, whereas PDE inhibitors, such as theophylline, increase cGMP formation, which may lead to increased vasodilation (Murad 2006). Thus, theophylline-induced PDE inhibition may exacerbate vasodilation by inhibiting cGMP and cyclic adenosine monophosphate (cAMP) degradation of blood vessels, aggravating hypotension observed in patients with theophylline toxicity (Kearney et al. 1985).

Theophylline increases blood flow in the rat ear, which is reversed by the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (Sanae and Hayashi 1998). The xanthine derivative 7-[2-[4-(m-chlorophenyl) piperazinyl]-ethyl]-1,3-dimethyl-xanthine) (MCPT) produces endothelium-dependent vasodilation, which is presumably mediated by endothelial NO and PDE inhibition (Lo et al. 2005). In addition, the selective PDE inhibitor, M&B 22948, induces endothelium-dependent vasodilation by increasing the formation of spontaneously released NO-induced cGMP due to PDE inhibition (Martin et al. 1986). Moreover, the PDE inhibitor (isobutylmethylxanthine)-induced vasodilation of brain arterioles involves increased cGMP and cAMP levels, which are dependent on endothelial NO (Rosenblum et al. 1993). In summary, PDE inhibitors induce vasodilation and increase blood flow, mediated by endothelial NO (Martin et al. 1986; Rosenblum et al. 1993; Sanae and Hayashi 1998; Lo et al. 2005). Moreover, recently, a toxic dose of theophylline-induced distributive shock, which is unresponsive to supportive treatments, has been treated with lipid emulsion, suggesting its potential in inhibiting NO-mediated vasodilation caused by a toxic dose of theophylline (Ok et al. 2015; Chandrasekaran et al. 2020; Lee and Sohn 2023). However, theophylline-induced vasodilation in endothelium-intact aortas pre-contracted with norepinephrine is not inhibited by NOS inhibitor N^G-nitro-L-arginine methyl ester (Marukawa et al. 1994). In addition, theophylline-induced vasodilation is independent of NO release in human arteries pre-contracted with U46619 (Vroom et al. 1996). Taking into consideration these previous reports, the role of endothelial NO on the phosphodiesterase inhibitor theophylline-induced vasodilation is unclear. Based on previous reports, we tested the biological hypothesis that theophylline-induced endothelium-dependent vasodilation involves increased endothelial NO release and PDE inhibition. Thus, this study aimed to examine whether theophylline-induced vasodilation was endothelial NO-dependent and to elucidate the underlying mechanism, with a particular focus on endothelial NO.

Materials and Methods

The Institutional Animal Care and Use Committee of the Gyeongsang National University approved the experimental protocol (GNU-211217-R0106). All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Preparation of isolated rat aorta and isometric tension measurement

The experiment involved 48 rats. These rats were housed in cages with controlled conditions of a temperature of 22-24°C, humidity of 30-70%, and a light-dark cycle of 12 h. They were given access to sterilized water and a standard chow diet *ad libitum*. Male Sprague-Dawley rats (body weight: 220–300 g, Koatech, Pyeongtaek, Gyeonggi-do, Republic of Korea) were anesthetized with 100% carbon dioxide supplied through a small opening in the rat cage. Rat aorta isolation and isometric tension measurements were performed, as previously described (Lee et al. 2021). The thoracic cavity was opened, the descending thoracic aorta was extracted from the thoracic cavity, and was placed in Krebs solution containing sodium chloride (118 mM), sodium bicarbonate (25 mM), glucose (11 mM), potassium chloride (4.7 mM),

calcium chloride (2.4 mM), magnesium sulfate (1.2 mM), and monopotassium phosphate (1.2 mM). The fat and connective tissues surrounding these aortas were eliminated under a microscope. The isolated rat thoracic aortas were sliced into 2.5 mm-length segments. The endothelia of some thoracic aortas were removed by rolling the isolated rat aortas backward and forward using two 25-gauge needles inserted into the lumen of the aorta. The isolated descending thoracic aortas were suspended in a Grass isometric transducer (FT-03, Grass Instrument, Quincy, MA, USA) of an organ bath maintained at 37°C. According to a previous report, a baseline resting tension of 2.5 g was maintained for 90 min to achieve equilibrium (Klöss et al. 2000) and the pre-existing Krebs solution was replaced with fresh Krebs solution every 30 min. The Krebs solution was aerated with gas, which included 95% oxygen and 5% carbon dioxide, to maintain its pH at 7.4. The endothelial integrity of the endothelium-intact aorta was confirmed as follows. After 10^{-7} M phenylephrine induced sustained and stable contractions in the endothelium-intact thoracic aorta, acetylcholine (10^{-5} M) was added to the organ bath, and >80% acetylcholine-induced relaxation from phenylephrine-induced contraction was regarded as an endothelium-intact thoracic aorta. Further, endothelial denudation was verified as follows. After phenylephrine (10^{-8} M) induced stable and sustained contractions, acetylcholine (10^{-5} M) was added to the organ bath, and <15% acetylcholine-induced relaxation from phenylephrine-induced contractions was regarded as endothelium-denuded thoracic aorta. The thoracic aortas showing acetylcholine-induced relaxation from phenylephrine-induced contraction in endothelium-intact and endothelium-denuded thoracic aortas were washed repeatedly with fresh Krebs solution to reach baseline resting tension.

Experimental protocols

First, the effect of endothelial denudation on theophylline-induced vasodilation was examined to determine whether theophylline-induced vasodilation is endothelium-dependent. After inducing sustained and stable contractions in endothelium-intact and endothelium-denuded thoracic aortas using phenylephrine (10^{-6} M), theophylline (10^{-7} to 3×10^{-4} M) was cumulatively added to the organ bath to generate theophylline-induced vasodilation.

Second, the effects of cellular signaling pathway inhibitors in endothelium-intact thoracic aortas were examined to verify whether theophylline-induced vasodilation was associated with endothelial NO-mediated vasodilation. After endothelium-intact rat thoracic aortas were pretreated with the NOS inhibitor, N^W-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M); non-specific GC inhibitor, methylene blue (10^{-6} M); and NO-sensitive GC inhibitor, 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, 10^{-5} M) alone for 20 min, phenylephrine (10^{-6} M) induced sustained and stable contractions (Lee et al. 2021). Theophylline (10^{-7} to 3×10^{-4} M) was cumulatively added to the organ bath to induce vasodilation in endothelium-intact thoracic aortas with or without the inhibitors (L-NAME, methylene blue, and ODQ).

Third, the effects of theophylline on vasodilation induced by the NO donor-induced cGMP-mediated vasodilator (sodium nitroprusside), β-agonist-induced cAMP-mediated vasodilator (isoproterenol), and cGMP analog (8-bromoguanosine 3',5'-cyclic monophosphate sodium salt (bromo-cGMP)) were examined to determine whether PDE inhibitor theophylline augments vasodilation induced by these compounds. The endothelium-denuded thoracic aortas were treated with theophylline (10^{-4} M) for 20 min. After phenylephrine (10^{-6} M) treatment produced sustained and stable contractions, sodium nitroprusside (10^{-10} to 10^{-7} M) or bromo-cGMP (10^{-10} to 3×10^{-5} M) was cumulatively added to the organ bath to produce vasodilation in the endothelium-denuded thoracic aorta in the presence or absence of theophylline; isoproterenol at a concentration of 10^{-3} M was added to the organ bath to produce a similar effect.

Fourth, the effects of potassium chloride (KCl)- and phenylephrine-induced contractions on theophylline-induced vasodilation in endothelium-denuded rat thoracic aortas were examined to compare the magnitude of theophylline-induced vasodilation. After phenylephrine (10^{-6} M) or 60 mM KCl produced sustained and stable contractions in the endothelium-denuded thoracic aorta, theophylline (10^{-7} to 3×10^{-4} M) was cumulatively added to the organ bath to produce theophylline-induced vasodilation.

Cyclic guanosine monophosphate (cGMP) measurement

Isolated rat thoracic aortas were used to determine cGMP levels, as described previously (Park et al. 2023). cGMP Complete Kit (Abcam, Cambridge Science Park, Cambridge, England) was used to measure the cGMP levels. The endothelium-intact and -denuded descending thoracic aortas were placed in Krebs solution contained in a 10 ml organ bath for a total of 60 min at 37°C, which included the drug treatment time. The descending thoracic aorta with the endothelium was treated with theophylline (3×10^{-4} M) alone for 5 min. The endothelium-denuded thoracic aorta was treated with sodium nitroprusside (10^{-8} M) alone for 5 min or with theophylline (3×10^{-4} M) for 10 min, followed by sodium nitroprusside (10^{-8} M) for 5 min. Subsequently, the treated thoracic aortas were frozen using liquid nitrogen and homogenized in 0.1 M hydrochloride. Acidic supernatants were acetylated, and an enzyme-linked immunosorbent assay with the cGMP Complete kit was used to measure

cGMP levels. The cGMP concentration obtained from each thoracic aorta was expressed in pmol/ml.

Cell culture

Human umbilical vein endothelial cells (HUVECs, C-0003-5C, American Type Culture Collection, Manassas, VA, USA) were kept in endothelial cell medium (ECM) (ScienCell, Carlsbad, CA, USA) containing 15% fetal bovine serum (ScienCell), 100 units/ml penicillin, 1% endothelial cell growth supplement (ScienCell), and 100 µg/ml streptomycin (ScienCell), as described previously (Park et al. 2023). Cells at passage 3–5 were incubated for 4 h in the ECM without serum prior to drug pretreatment.

Western blot analysis

Endothelial NOS (eNOS; Ser1177 and Thr495) expression in HUVECs was examined using Western blotting, as described previously (Park et al. 2023). The cells were pretreated with theophylline (3×10^{-4} M) for 5, 10, 30, or 60 min to determine eNOS (Ser1177 and Thr495) phosphorylation. The HUVECs were harvested in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) containing phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific, Rockfield, IL, USA). The lysates were centrifuged (20,000 × *g*) at 4°C for 15 min, and the protein content of the supernatant was determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific). After boiling for 10 min, the samples containing 30-µg protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked using 5% skimmed milk in tris-buffered saline with 0.5% Tween-20 (TBST) for 60 min at 25°C and incubated with primary antibodies (anti-phospho-eNOS at Ser1177 (1:1,000), anti-phospho-eNOS at Thr495 (1:1,000), anti-eNOS (1:1,000), and anti-β actin (1:10,000)) overnight at 4°C. The membrane was then washed for 10 min thrice with TBST before being treated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G diluted to 1:5,000 at 25°C for 60 min. Protein bands were stained using the Westernbright^{TM} ECL Western blotting detection kit (Advansta, Menlo Park, CA, USA) and a ChemiDoc^{TM} Touch Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to image the signaling of protein bands. Proteins were quantified using the ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MD, USA).

Measurement of intracellular calcium

Intracellular calcium was measured using a confocal laser microscope (IX70 Fluoview, Olympus, Tokyo, Japan), as described previously (Park et al. 2023). HUVECs were seeded, cultured in confocal cell culture dishes (SPL; Pocheon, Republic of Korea), incubated with Fluo-4 AM (2.5×10^{-6} M, Invitrogen, Waltham, MA, USA) in Hanks' balanced salt solution medium for 30 min, and washed twice with phosphate-buffered saline solution. HUVECs were treated with theophylline (10^{-4} M) and intracellular calcium levels were measured *per* 2.5 s at the emission and excitation wavelengths of 520 and 485 nm, respectively. Images expressed with Fluo-4 AM were used to analyze intracellular calcium levels, which were calculated as follows: fluorescence intensity (F) divided by the baseline fluorescence intensity (F_0) prior to drug treatment. The net change in the calcium level is shown as $(F_{max} - F_0)/F_0$, where F_{max} is the maximum calcium level based on fluorescence intensity after treatment with theophylline. Intracellular calcium levels were measured for approximately 6 min.

Materials

All chemicals used in the study were commercially available at high purity. Theophylline, L-NAME, methylene blue, ODQ, sodium nitroprusside, isoproterenol, bromo-cGMP, phenylephrine, and acetylcholine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-eNOS (Ser1177 and Thr495) and anti-eNOS antibodies were purchased from Cell Signaling Technology and BD Biosciences (Franklin, NJ, USA), respectively. ODQ was dissolved in dimethyl sulfoxide (DMSO; final DMSO concentration in the organ bath: 0.1%).

Statistical analyses

The primary outcome of this study was the effect of endothelial denudation and various drugs on theophylline-induced vasodilation, and the effect of theophylline on vasodilation induced by sodium nitroprusside, isoproterenol, and bromo-cGMP. The effects of endothelial denudation, various inhibitors, and vasoconstrictors (phenylephrine and KCl) on theophylline-induced vasodilation and the effects of theophylline on vasodilation induced by sodium nitroprusside and bromo-cGMP were analyzed using a linear mixed effect model (Stata version 14.2, StataCorp LLC, Lakeway Drive, College Station, TX, USA) (Lavergne et al. 2008). The effect of theophylline on isoproterenol-induced vasodilation was analyzed using the unpaired Student's *t*-test (Prism 5.0, GraphPad Software,Inc., San Diego, CA, USA). The effect of theophylline on eNOS phosphorylation was analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni's test. The effects of sodium nitroprusside and theophylline, alone or in combination, on cGMP formation were analyzed using one-way ANOVA, followed by Bonferroni's test or the unpaired Student's *t*-test. The effects of theophylline on intracellular calcium levels were analyzed using an unpaired Student's *t*-test.

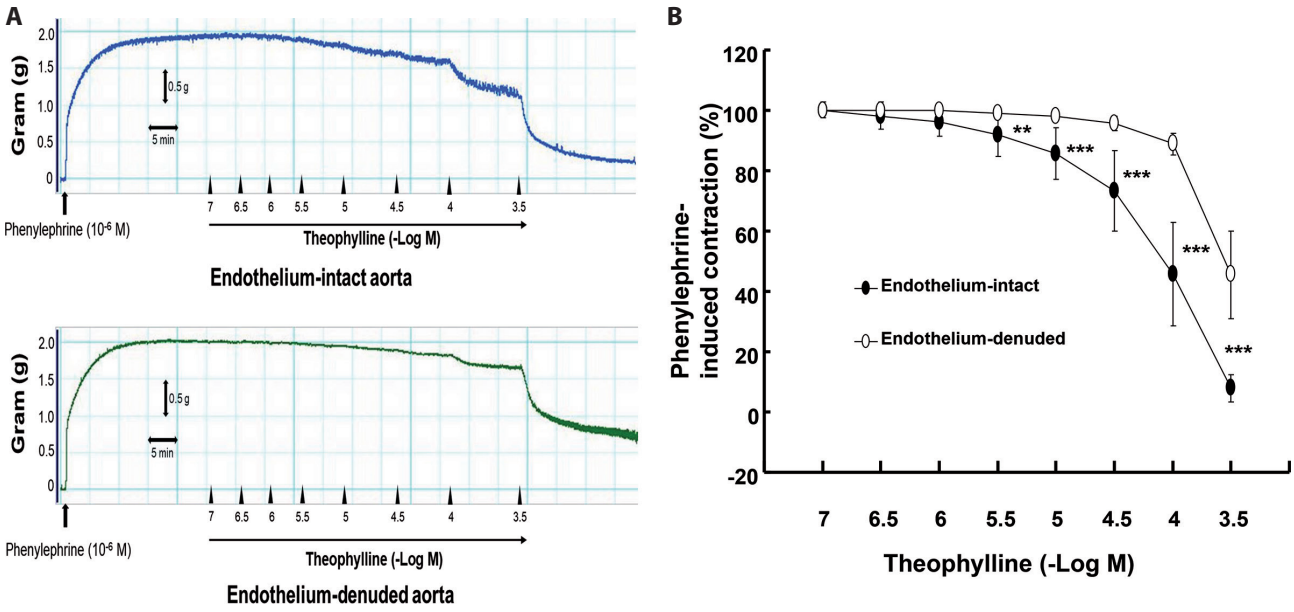

Figure 1. A. Original tracing showing theophylline (10^{-7} to 3×10^{-4} M)-induced vasodilation in endothelium-intact and endothelium-denuded rat aortas precontracted with phenylephrine (10^{-6} M). **B.** Effect of endothelial denudation on theophylline-induced vasodilation in isolated rat aortas precontracted with phenylephrine (10^{-6} M). Data ($n = 5$) are shown as the mean ± SD and expressed as the percentage of phenylephrine-induced contractions; n indicates the number of rats used to isolate the aortas. ** $p < 0.01$ and *** $p < 0.001$ *vs.* endothelium-denuded.

Results

Theophylline-induced vasodilation was more pronounced in endothelium-intact aortas than in endothelium-denuded aortas (Fig. 1A,B; $p < 0.01$ *vs.* control at 3×10^{-6} to 3×10^{-4} M theophylline). NOS inhibitor L-NAME (10^{-4} M) inhibited theophylline-induced vasodilation (Fig. 2A; $p < 0.01$ *vs.* control at 10^{-5} to 3×10^{-4} M theophylline). In addition, non-specific GC inhibitor methylene blue (10^{-6} M), and NO-sensitive GC inhibitor ODQ (10^{-5} M) attenuated theophylline-induced vasodilation in endothelium-intact aortas (Fig. 2B,C; methylene blue: $p < 0.05$ *vs.* control at 3×10^{-6} M theophylline; methylene

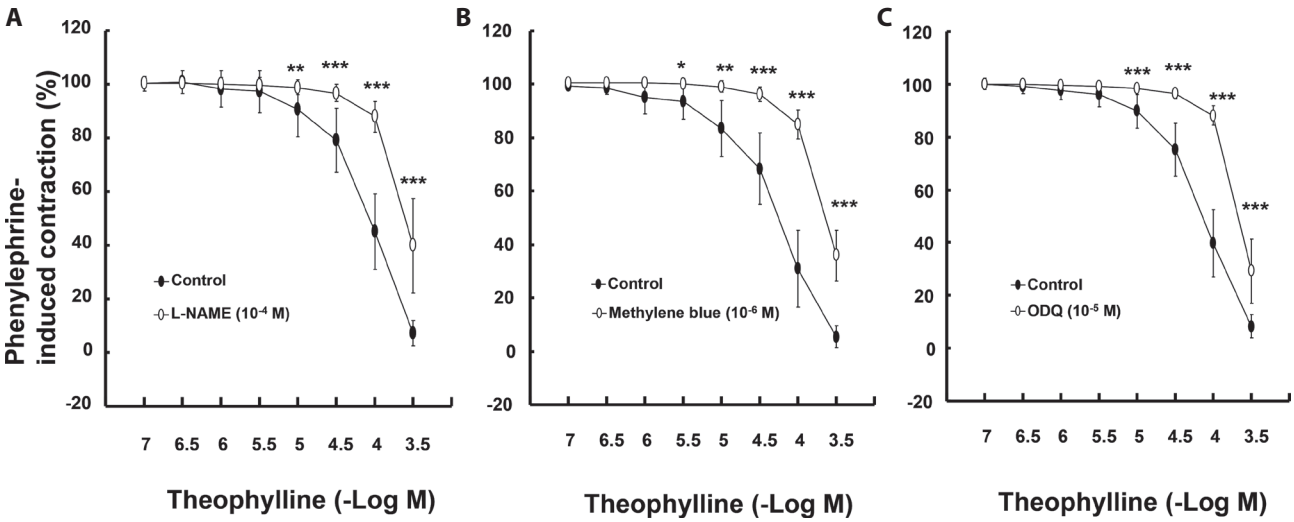

Figure 2. Effect of N^W-nitro-L-arginine methyl (L-NAME, **A**), methylene blue (**B**), and 1H-[1,2,4]oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, **C**) on theophylline-induced vasodilation in isolated endothelium-intact rat aortas precontracted with phenylephrine (10^{-6} M). Data ($n = 5$) are shown as mean ± SD and expressed as the percentage of phenylephrine-induced contraction; n indicates the number of rats used to isolate the aortas. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *vs.* control.

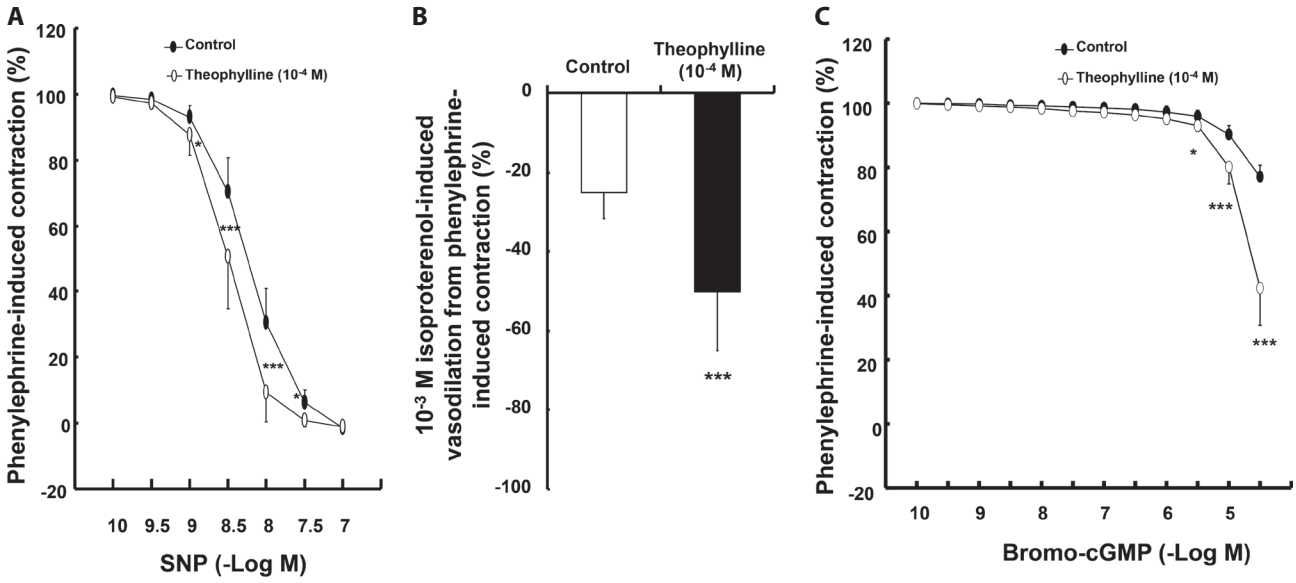

Figure 3. Effect of theophylline on the vasodilation induced by sodium nitroprusside (SNP, **A**), isoproterenol (10^{-3} M, **B**), and 8-bromoguanosine 3',5'-cyclic monophosphate sodium salt (bromo-cGMP, **C**) in isolated endothelium-denuded rat aortas precontracted with phenylephrine (10^{-6} M). Data (n = 5) are shown as mean ± SD and expressed as the percentage of phenylephrine-induced contraction (A, C) and as the percentage of relaxation from phenylephrine-induced contraction (B); n indicates the number of rats used to isolate the aortas. * $p < 0.05$ and *** $p < 0.001$ *vs.* control.

blue and ODQ: $p < 0.01$ *vs.* control at 10^{-5} to 3×10^{-4} M theophylline). Theophylline (10^{-4} M) increased NO donor sodium nitroprusside- and β-agonist isoproterenol (10^{-3} M)-induced vasodilation in endothelium-denuded aortas (Fig. 3A; $p < 0.05$ *vs.* control at 10^{-9} to 3×10^{-8} M sodium nitroprusside) (Fig. 3B; $p < 0.001$ *vs.* control). Theophylline also increased cGMP analog bromo-cGMP-induced vasodilation in the endothelium-denuded aortas (Fig. 3C; $p < 0.05$ *vs.* control at 3×10^{-6} to 3×10^{-5} M bromo-cGMP). Theophylline-induced vasodilation was greater in phenylephrine (10^{-6} M)-induced contractions than in KCl (60 mM)-induced contractions of the endothelium-denuded aorta (Fig. 4; $p < 0.001$ at 10^{-4} and 3×10^{-4} M theophylline).

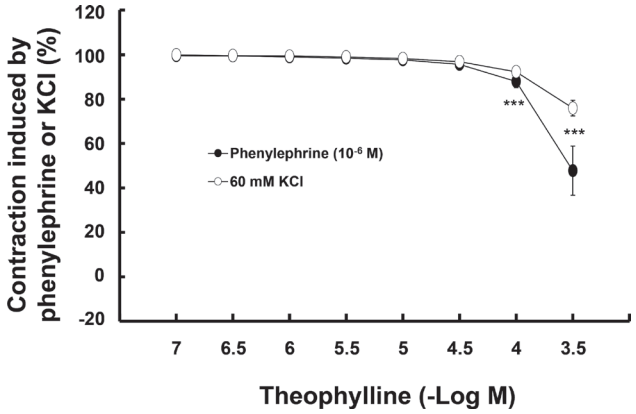

Figure 4. Theophylline concentration-response curves in endothelium-denuded aortas precontracted with phenylephrine (10^{-6} M) or isotonic 60 mM potassium chloride (KCl). Data (n = 5) are shown as mean ± SD and expressed as the percentage of contraction induced by phenylephrine or KCl; n indicates the number of rats used to isolate the aortas. *** $p < 0.001$ *vs.* 60 mM KCl.

Theophylline (3×10^{-4} M) increased cGMP formation in endothelium-intact aortas (Fig. 5A; $p < 0.001$ *vs.* control). Sodium nitroprusside (10^{-8} M) increased cGMP formation in endothelium-denuded aortas (Fig. 5B; $p < 0.05$ *vs.* control). In addition, theophylline (3×10^{-4} M) further increased sodium nitroprusside (10^{-8} M)-induced cGMP formation in endothelium-denuded aortas (Fig. 5B; $p < 0.001$ *vs.* sodium nitroprusside alone).

Theophylline (3×10^{-4} M) increased stimulatory eNOS (Ser1177) phosphorylation (Fig. 6A; $p < 0.01$ *vs.* control at 10, 30, and 60 min). However, it decreased inhibitory eNOS (Thr495) phosphorylation in HUVECs (Fig. 6A; $p < 0.001$ *vs.* control at 30 and 60 min).

In addition, theophylline (10^{-4} M) increased intracellular calcium levels in HUVECs (Fig. 6B; $p < 0.001$ *vs.* control).

Discussion

This is the first study to demonstrate that theophylline-induced endothelium-dependent vasodilation is mediated by increased endothelial NO release and PDE inhibition.

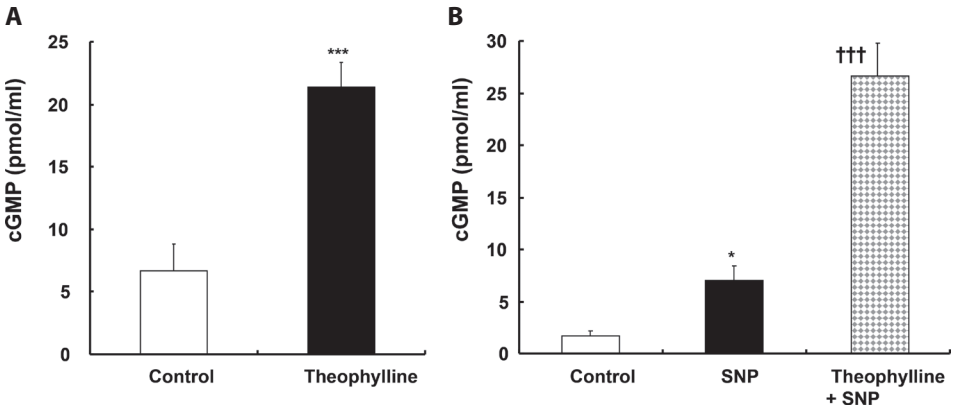

Figure 5. A. Effect of theophylline (3×10^{-4} M) on the cyclic guanosine monophosphate (cGMP) formation in isolated endothelium-intact rat aorta. *** $p < 0.001$ *vs.* control. **B.** Effect of theophylline (3×10^{-4} M) on sodium nitroprusside (SNP, 10^{-8} M)-induced cGMP formation in isolated endothelium-denuded rat aortas. Data ($n = 4$) are shown as mean ± SD; n indicates the number of rats. * $p < 0.05$ *vs.* control. ††† $p < 0.001$ *vs.* SNP alone.

The major findings of this study are as follows: 1) L-NAME, methylene blue, and ODQ inhibited theophylline-induced vasodilation in endothelium-intact aortas; 2) theophylline increased the vasodilation induced by sodium nitroprusside and bromo-cGMP in endothelium-denuded aortas; 3) theophylline increased cGMP formation in the endothelium-intact aorta; and 4) theophylline increased eNOS phosphorylation (Ser1177) and intracellular calcium levels in HUVECs.

Endothelial NO is produced from L-arginine by calcium-dependent eNOS, which is dependent on nicotinamide adenine dinucleotide phosphate (Sohn et al. 2004; Murad 2006). NO activates GC in vascular smooth muscle, which leads to cGMP formation (Sohn et al. 2004; Murad 2006). The increased cGMP levels induce vasodilation *via* activation of cGMP-dependent protein kinases (Sohn et al. 2004). PDE degrades cGMP to 5'-GMP, whereas PDE inhibitors produce increased amounts of cGMP due to the inhibi-

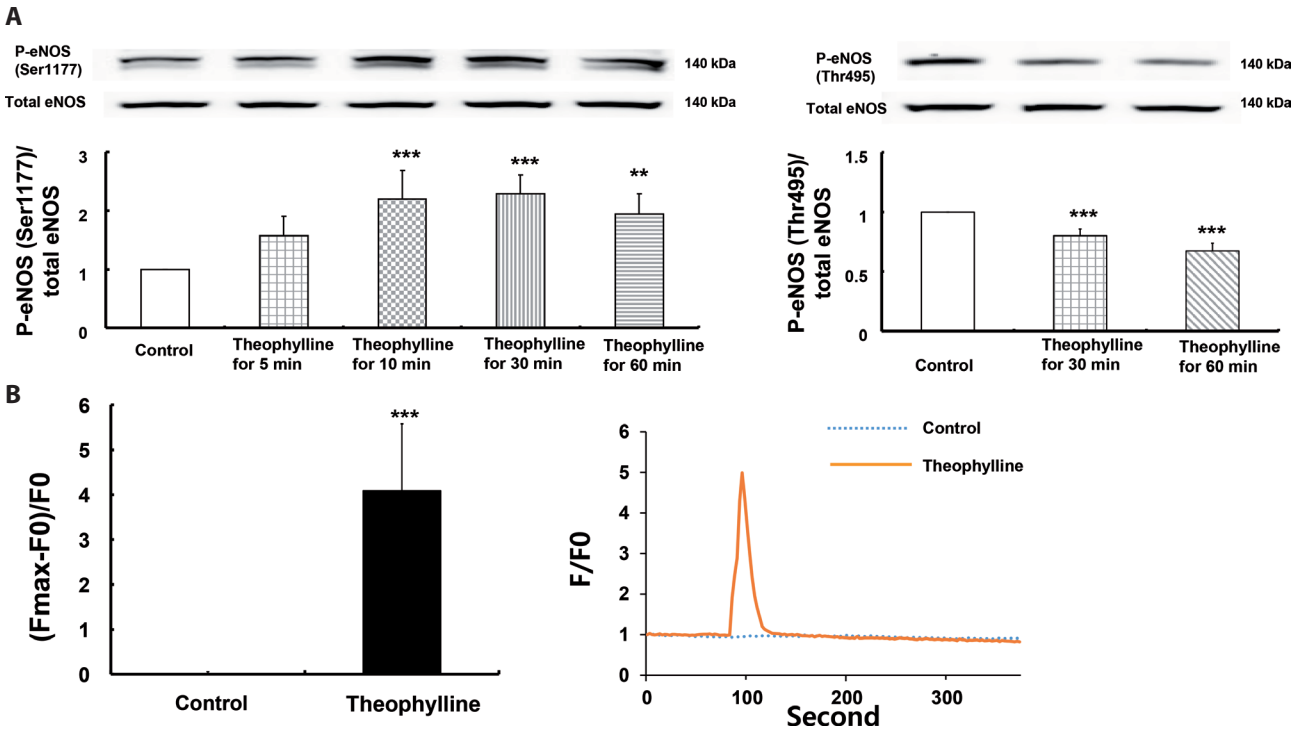

Figure 6. A. Effect of theophylline (3×10^{-4} M) on the endothelial nitric oxide synthase (eNOS, Ser1177 and Thr495) phosphorylation in human umbilical vein endothelial cells (HUVECs). Data ($n = 4$) are shown as mean ± SD; n indicates the number of independent experiments. P-eNOS, phosphorylated eNOS. ** $p < 0.01$ and *** $p < 0.001$ *vs.* control. **B.** Effect of theophylline (10^{-4} M) on calcium levels in HUVECs. Data ($n = 3$) are shown as mean ± SD; n indicates the number of independent experiments. *** $p < 0.001$ *vs.* control.

tion of cGMP degradation, leading to vasodilation (Murad 2006). Xanthine derivative MCPT-induced vasodilation of the endothelium-intact aorta is inhibited by L-NAME, methylene blue, and ODQ (Lo et al. 2005). Consistent with this report (Lo et al. 2005), in the current study, endothelium-dependent theophylline-induced vasodilation was inhibited by the NOS inhibitor L-NAME, non-specific GC inhibitor methylene blue, and NO-sensitive GC inhibitor ODQ, suggesting that theophylline-induced endothelium-dependent vasodilation is mediated by pathways involving eNOS and GC. *In vivo* experiments have revealed that increase of blood flow induced by theophylline in the rat ear without affecting heart rate and blood pressure, and PDE inhibitor (isobutylmethylxanthine)-induced endothelium-dependent vasodilation of pial arterioles are inhibited by an NOS inhibitor (Rosenblum et al. 1993; Sanae and Hayashi 1998). These *in vivo* studies have suggested that PDE inhibitors, such as theophylline and isobutylmethylxanthine, induce endothelium-dependent NO-mediated flow increases and vasodilation, respectively (Rosenblum et al. 1993; Sanae and Hayashi 1998). However, previous study reported that endothelial denudation and NOS inhibitors do not alter theophylline-induced vasodilation in isolated rat aortas (Murakawa et al. 1994). We surmise that this discrepancy may be due to the different preconstrictors used to produce contractions in isolated rat aortas before the addition of theophylline. Phenylephrine used in this experiment produces relatively well-sustained and stable contractions, whereas norepinephrine used in previous experiment may induce less sustained contractions, which may lead to misinterpreting the spontaneous attenuation of norepinephrine-induced contraction as theophylline-induced vasodilation of isolated rat aortas (Murakawa et al. 1994). The PDE inhibitor, M&B 22948, induces endothelium-dependent NO-mediated vasodilation, which is augmented by the attenuated degradation of cGMP induced by the basal release of endothelial NO (Martin et al. 1986). In the current study, theophylline increased NO donor sodium nitroprusside-induced cGMP formation in the endothelium-denuded aorta, and vasodilation induced by sodium nitroprusside and cGMP analog bromo-cGMP in the endothelium-denuded aorta. The decreased cGMP degradation due to the PDE inhibitor, theophylline, contributed to this increased response. Isoproterenol induces vasodilation through a pathway involving adenylate cyclase and cAMP in endothelium-denuded rat aortas (Tanaka and Tsuchida 1998). Thus, theophylline-induced augmentation of isoproterenol-induced vasodilation is due to the inhibited degradation of isoproterenol-induced cAMP formation by theophylline. Similar to the abovementioned results, the xanthine derivative MCPT increased vasodilation induced by sodium nitroprusside and isoproterenol in the endothelium-denuded aorta, suggesting that MCPT-induced inhibition of PDE, which hydrolyzes cGMP and cAMP, contributed to this increased vasodilation through increased cGMP- and cAMP-dependent protein kinase activation, respectively (Lo et al. 2005). In the current study, the increased theophylline-induced vasodilation of the endothelium-intact aorta compared to endothelium-denuded aorta could be attributed to the following two possible factors: first, the augmentation of theophylline-induced increased NO release-mediated vasodilation by theophylline-induced inhibition of PDE, and second, spontaneously released NO-mediated vasodilation augmented by the theophylline-induced inhibition of PDE. Considering the factors described below, endothelium-dependent NO-mediated vasodilation induced by theophylline is presumably mediated mainly by increased endothelial NO release and partially by PDE inhibition. First, increased stimulatory eNOS (Ser1177) phosphorylation or decreased inhibitory eNOS (Thr495) phosphorylation induces endothelial NO production, and the xanthine derivative MCPT increases eNOS expression in HUVECs (Fleming and Busse 2003; Lo et al. 2005). Consistent with previous reports (Fleming and Busse 2003; Lo et al. 2005), theophylline increased eNOS (Ser1177) phosphorylation in HUVECs and decreased inhibitory eNOS (Thr495) phosphorylation. In addition, theophylline increased endothelial calcium levels in HUVECs, which is associated with calcium-dependent eNOS activation. These phenomena may contribute to an increased endothelial NO production. Secondly, theophylline is a nonselective PDE inhibitor used for chronic obstructive pulmonary disease, whereas PDE 5 is mainly involved in vascular tone regulation (Cesarini et al. 2020; Bondarev et al. 2022). Thus, theophylline has a relatively low affinity for PDE 5. MCPT also increases cGMP levels in human platelets (Lo et al. 2005). Similar to this report, theophylline increased cGMP formation in endothelium-intact aortas and sodium nitroprusside-induced cGMP formation in endothelium-denuded aortas (Lo et al. 2005). Further studies are needed to examine the detailed upstream cellular signaling pathways associated with theophylline-induced NO production and their interaction with theophylline-induced PDE inhibition.

The vasodilator bradykinin, which acts on endothelial receptors, increases endothelial calcium levels, and induces vasodilation by activating calcium-dependent eNOS (Ogawa et al. 2001). Consistent with these reports (Ogawa et al. 2001; Lo et al. 2005), theophylline-induced calcium increase in HUVECs appears to activate eNOS, contributing to vasodilation through endothelial NO production.

KCl-induced contraction is mediated mainly by calcium influx *via* voltage-operated calcium channels, whereas contractions induced by agonists, such as phenylephrine and norepinephrine, are mediated by calcium influx through receptor-operated calcium channels and calcium sensitization mechanisms involving Rho-kinase and protein kinase C (Akata 2007). Thus, as norepinephrine, which is released from the sympathetic nerve ending, induces

vasoconstriction and theophylline induced greater vasodilation in phenylephrine-induced contraction than that in KCl-induced contraction in the endothelium-denuded aorta (Rongen et al. 1996), theophylline may induce vasodilation by inhibiting calcium sensitization or calcium influx through receptor-operated calcium channels of vascular smooth muscle *in vivo*. In the present study, pretreatment with L-NAME, methylene blue, and ODQ did not inhibit theophylline-induced vasodilation completely in the endothelium-intact rat thoracic aorta. Additionally, theophylline induced vasodilation in the endothelium-denuded rat thoracic aorta, which is endothelium-independent. The increased cAMP formation, resulting from PDE inhibition, activates cAMP-dependent protein kinase (Akata 2007). The activation leads to vasodilation by activating potassium channels, inhibiting calcium influx, and inhibiting myosin light chain kinase (Akata 2007). Similar to the findings of a previous report by Lo et al. (2005), theophylline increased isoproterenol-induced vasodilation in the endothelium-denuded aorta in the present study. The finding suggests that theophylline-induced increased cAMP formation enhances the vasodilation effects of the β-agonist, isoproterenol. Considering the results of the present study and previous reports, the remaining theophylline-induced vasodilation, which is resistant to inhibitors of the cellular signaling pathway for NO-induced vasodilation, may be suggested to be associated with theophylline-induced activation of cAMP-dependent protein kinase (Lo et al. 2005; Akata 2007).

The limitations of this study are as follows. First, small arterioles mainly contribute to blood pressure by regulating peripheral vascular resistance, whereas this study used aortas, which are conduit vessels (Clifford 2011). Second, isometric tension measurements were performed using isolated rat aortas, whereas Western blotting and calcium measurements were performed using HUVECs. Despite these limitations, toxic doses of theophylline ($>10^{-4}$ M) not only cause hypotension *via* β2-adrenoceptor-mediated vasodilation, but also aggravate hypotension *via* increased NO-induced vasodilation due to an increased NO release and PDE inhibition (Woodcock et al. 1983; Kearney et al. 1985; Schulz and Schmoldt 2003). In addition, toxic doses of theophylline may produce severe hypotension in patients with intact endothelial integrity compared to patients with compromised endothelium. A possible direct underlying mechanism responsible for lipid emulsion resuscitation as an adjuvant drug in drug toxicity-induced cardiovascular collapse includes inhibited NO release (Lee and Sohn 2023). Thus, although theophylline is water-soluble (Log P: –0.02), lipid emulsion-induced stabilization of intractable distributive shock caused by theophylline toxicity observed in the previous case report may be due to lipid emulsion-mediated inhibition of endothelial NO production caused by toxic doses of theophylline (Chandrasekaran et al. 2020; Lee and Sohn 2023). Based on these results, methylene blue, which is used to alleviate vasoplegic shock, may be effective in treating hypotension caused by theophylline toxicity (Jang et al. 2013).

In conclusion, these results suggested that theophylline-induced endothelium-dependent vasodilation is mediated by increased endothelial NO production, which is presumably augmented by PDE inhibition.

Conflict of interest. The authors of this study declare no conflict of interest.

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