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# Chloroquine induces endothelium-dependent nitric oxide-mediated vasodilation in isolated rat aorta

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**Abstract.** Toxic doses of chloroquine induce vasodilation, which contributes to hypotension. In this study, we aimed to investigate the involvement of endothelial nitric oxide in the vasodilatory effect of chloroquine on isolated rat aortas and elucidate the underlying mechanisms. We evaluated the effects of endothelial denudation and several inhibitors, including the nitric oxide synthase inhibitor $N^W$-nitro-L-arginine methyl ester (L-NAME), the non-specific guanylate cyclase (GC) inhibitor methylene blue, the nitric oxide-sensitive GC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the phosphoinositide-3 kinase inhibitor wortmannin, on chloroquine-induced vasodilation in endothelium-intact aortas. The effects of chloroquine and protein phosphatase 2 (PP2) on the phosphorylation of endothelial nitric oxide synthase (eNOS), Src kinase, and caveolin-1 in human umbilical vein endothelial cells were also investigated. Chloroquine-induced vasodilation was more pronounced in endothelium-intact aortas than in endothelium-denuded ones. L-NAME, methylene blue, ODQ, and wortmannin attenuated the vasodilatory effect of chloroquine in endothelium-intact aortas. Chloroquine increased cyclic guanosine monophosphate (cGMP) levels and stimulatory eNOS phosphorylation (Ser1177) while decreasing inhibitory eNOS phosphorylation (Thr495). PP2 inhibited chloroquine-induced phosphorylation of caveolin-1 and Src kinases. These findings suggest that chloroquine-induced vasodilation is mediated by the eNOS-GC-cGMP pathway, with eNOS phosphorylation regulated by caveolin-1 and Src kinase. Methylene blue may alleviate toxic-dose chloroquine-induced vasodilation.

**Key words:** Chloroquine — Bitter taste receptor agonist — Nitric oxide — Endothelium — Vasodilation

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**Highlights**

- Chloroquine causes vasodilation in isolated rat aorta by stimulating the endothelium to produce nitric oxide, which is mediated by the endothelial nitric oxide synthase-guanylate cyclase-cyclic guanosine monophosphate pathway
- Chloroquine-induced phosphorylation of endothelial nitric oxide synthase appears to be mediated by caveolin-1 and Src kinase

## Introduction

Chloroquine and hydroxychloroquine, quinine derivatives with similar cellular mechanisms, are used to treat malaria, rheumatoid arthritis, and systemic lupus erythematosus (Lebin and LeSaint 2020). Chloroquine, an agonist of bitter taste receptors, which are G protein-coupled receptors, lowers systolic blood pressure and reduces increased vascular resistance in the forearm caused by cold while enhancing forearm blood flow (Anigbogu et al. 1993). This finding indicates that the hypotensive effect of chloroquine may be partially attributable to its ability to decrease vascular resistance. Extraoral bitter taste receptors exist in endothelial cells, vascular smooth muscle, and respiratory and genitourinary systems (Lu et al. 2017; Kertesz et al. 2022; Xin and Zhao 2022). The bitter taste receptor agonist chloroquine relaxes the ileal smooth muscle *via* nitric oxide and the activation of large conductance calcium-activated potassium channels (Jing et al. 2013). Bitter taste receptors in the sinonasal cavity stimulated by ligands produce nitric oxide (Yan et al. 2017). Additionally, chloroquine induces relaxation of the rat corpus cavernosum, which appears to be mediated by nitric oxide (Navarro-Dorado et al. 2023). Denatonium benzoate, a bitter taste receptor agonist, induces calcium-dependent nitric oxide production in human bronchiolar cell lines expressing bitter taste receptor and endothelial nitric oxide synthase (Carey et al. 2022). Nitric oxide causes vasodilation through the activation of a pathway involving guanylate cyclase (GC), cyclic guanosine monophosphate (cGMP), and cGMP-dependent protein kinase (Sohn et al. 2004). These findings suggest that bitter taste receptor agonists, such as chloroquine, may promote vasodilation through pathways involving bitter taste receptors, nitric oxide, GC, cGMP, and cGMP-dependent protein kinase (Sohn et al. 2004; Carey et al. 2022). Hydroxychloroquine induces vasodilation, which is mediated by endothelial nitric oxide at low concentrations and calcium channel inhibition at high concentrations (Arslan et al. 2022). It improves impaired endothelial nitric oxide-mediated vasodilation in mice with lupus by reducing the levels of reactive oxygen species (Gómez-Guzmán et al. 2014). Chloroquine induces endothelium-independent vasodilation in the aorta pre-contracted with norepinephrine and potassium chloride (KCl) in the endothelium-denuded aorta (Aziba and Okpako 2003). Moreover, chloroquine induces the relaxation of the intact trachea contracted by acetylcholine and serotonin *via* calcium-activated potassium channels and reduces bronchoconstriction in an allergen-induced asthma model in anesthetized and intubated mice (Deshpande et al. 2010; Sharma et al. 2017). Endothelial nitric oxide plays a crucial role in controlling vascular tone and blood flow (Sohn et al. 2004), but its role in chloroquine-induced vasodilation is unknown. Therefore, in this study, we aimed to investigate the involvement of endothelial nitric oxide in chloroquine-induced vasodilation and its underlying mechanisms. Based on previous research (Aziba and Okpako 2003; Jing et al. 2013; Yan et al. 2017; Carey et al. 2022; Kertesz et al. 2022; Navarro-Dorado et al. 2023), we tested the biological hypothesis that endothelial nitric oxide augments vasodilation induced by the bitter taste receptor agonist, chloroquine.

## Materials and Methods

The Institutional Animal Care and Use Committee of Gyeongsang National University approved the experimental protocol (GNU-210302-R0026). This study was conducted in compliance with the Animal Care and Use guidelines of Gyeongsang National University.

### *Isolation of rat thoracic aorta and isometric tension measurement*

Male Sprague-Dawley rats (body weight: 240–280 g) were obtained from Koatech (Pyeongtaek, Gyeonggi-do, Korea) and anesthetized using 100% carbon dioxide. The procedure for isolating the rat aorta to measure the isometric tension followed the method described by Park et al. (2023). After opening the thoracic cavity, the descending thoracic aorta was removed and placed in Krebs solution, which contains 118 mM sodium chloride, 25 mM sodium bicarbonate, 11 mM glucose, 4.7 mM KCl, 2.4 mM calcium chloride ($CaCl_2$), 1.2 mM magnesium sulfate, and 1.2 mM monopotassium phosphate, with great care. The aorta was immersed in Krebs solution, whereas fat and connective tissues were meticulously excised under a microscope. Subsequently, the aorta was cut into 2.5-mm-long segments. For some segments, the endothelium was removed by rolling the aorta

back and forth using two 25-gauge needles placed in the lumen. These segments were suspended in an organ bath containing Krebs solution at 37°C. A grass isometric transducer (FT-03, Grass Instruments, Quincy, MA, USA) was installed in the organ bath. As described by Klöss et al. (2000), the baseline resting tension (24.5 mN) was maintained for 1.5 h to achieve a stable plateau, while the Krebs solution was replaced with a fresh one. The pH was maintained at 7.4 by aeration with 95% oxygen and 5% carbon dioxide. The integrity of the endothelium in the intact aorta was validated by inducing a sustained contraction with phenylephrine ($10^{-7}$ M), followed by the addition of acetylcholine ($10^{-5}$ M) to the organ bath. An aorta that showed >85% relaxation in response to acetylcholine was considered endothelium-intact. For endothelium-denuded aortas, persistent contractions were induced with phenylephrine ($10^{-8}$ M) followed by acetylcholine ($10^{-5}$ M). A relaxation response of <15% was interpreted as endothelial denudation. Endothelium-intact and endothelium-denuded aortas were cleaned several times with fresh Krebs solution to restore baseline resting tension before initiating the experimental protocols.

*Experimental protocols*

First, chloroquine-induced vasodilation was examined in endothelium-intact and endothelium-denuded aortas to determine the effect of endothelial removal on chloroquine-induced vasodilation. Phenylephrine ($10^{-6}$ M) was used to elicit stable and sustained contractions in the aortas, followed by the cumulative addition of chloroquine ($10^{-6}$–$10^{-3}$ M; therapeutic plasma concentration: $9.625\times10^{-7}$ M, toxic plasma concentration: $1.94\times10^{-6}$ M) to the organ bath to generate chloroquine concentration-response curves (Schulz and Schmoldt 2003).

Second, the effects of different inhibitors that block endothelium-dependent vasodilation, on chloroquine-induced vasodilation in endothelium-intact aortas were investigated. The aortas were treated with several inhibitors alone for 15 min (Sung et al. 2012; Lee et al. 2020): the nitric oxide synthase (NOS) inhibitor $N^W$-nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M), the non-specific GC inhibitor methylene blue ($10^{-6}$ M), the nitric oxide-sensitive GC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, $10^{-5}$ M), the cytochrome P450 epoxygenase inhibitor fluconazole ($10^{-5}$ M), and the phosphoinositide-3 kinase inhibitor wortmannin ($3\times10^{-7}$ M). Following these treatments, phenylephrine ($10^{-6}$ M) was administered to elicit stable and sustained contractions in the endothelium-intact aortas with or without inhibitors. Chloroquine ($10^{-6}$–$10^{-3}$ M) was cumulatively added to the organ bath to induce vasodilation in the presence or absence of various inhibitors.

Third, the effect of chloroquine on KCl-induced contractions in a calcium-free Krebs solution, followed by calcium-induced contractions in isolated endothelium-denuded aortas, was examined. The 60 mM KCl-induced contraction acquired prior to this protocol was used to express the magnitude of the KCl-induced contraction in calcium-free Krebs solution and the subsequent calcium-induced contraction in the presence or absence of chloroquine. After thoroughly rinsing out the 60 mM KCl-induced contraction with fresh Krebs solution to recover the baseline resting tension, the experimental protocol was followed. Chloroquine ($10^{-4}$ M) was applied to endothelium-denuded aortas in a calcium-free Krebs solution for 20 min. Subsequently, KCl (10–60 mM) was cumulatively added to the organ bath with a calcium-free Krebs solution in the presence or absence of chloroquine, followed by the addition of cumulative $CaCl_2$ (1.25–5 mM) to the organ bath.

*Cyclic guanosine monophosphate measurement*

The cGMP levels were assessed in isolated endothelium-intact rat aortas using a method previously described by Park et al. (2023). Measurements were performed using an Abcam cGMP Complete Kit (Cambridge Science Park, Cambridge, England). The descending thoracic aorta, with intact endothelium, was immersed in a Krebs solution within a 10 ml organ bath maintained at 37°C for 60 min, including the drug treatment time. The aortic strips with intact endothelium were treated with chloroquine at a concentration of $10^{-5}$ M for 30 min. Following this treatment, the thoracic aortic strips were rapidly frozen in liquid nitrogen and homogenized in 0.1 M hydrochloric acid. The acidic supernatants were acetylated, and cGMP levels were measured *via* enzyme-linked immunosorbent assay using a cGMP complete kit. The cGMP content in each aortic strip was measured in picomoles *per* milliliter (pmol/ml).

*Cell culture*

Human umbilical vein endothelial cells (HUVECs, C-003-5C) from the American Type Culture Collection (Manassas, VA, USA) were grown in endothelial cell medium (ECM) provided by ScienCell (Carlsbad, CA, USA) according to Park et al. (2023) guidelines. The ECM was enriched with 15% fetal bovine serum (ScienCell), 1% endothelial cell growth supplement (ScienCell), and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin, ScienCell). The cells were maintained in a 5% carbon dioxide humidified incubator at 37°C. Cells from passages 3 to 5 were incubated for an additional 4 h in serum-free ECM before drug treatment.

*Western blot analysis*

The phosphorylation of endothelial NOS (eNOS) at Ser1177 and Thr495, Src kinase (Tyr416), and caveolin-1 (Tyr14) in

HUVECs was determined *via* Western blotting, as described by Park et al. (2023). Cells were treated with chloroquine at a concentration of $10^{-4}$ M for 20 min to evaluate stimulatory eNOS phosphorylation at Ser1177. Cells were treated with chloroquine at a concentration of $10^{-4}$ M for 30 min to assess inhibitory eNOS phosphorylation at Thr495. The expression of phosphorylated Src kinase at Tyr416 and caveolin-1 at Tyr14 was examined by treating the cells with chloroquine ($10^{-4}$ M) alone for 5 min, protein phosphatase 2 (PP2, $5\times10^{-6}$ M) for 30 min, followed by chloroquine ($10^{-4}$ M) for 5 min, or PP2 ($5\times10^{-6}$ M) alone for 35 min. Following treatment, the cells were collected in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Cell lysates were centrifuged at 20,000×*g* for 15 min at 4°C, and the protein concentration in the supernatant was measured using a bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific). After boiling for 10 min, the protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk in tris-buffered saline with 0.5% Tween-20 (TBST) at 25°C for 60 min before being incubated overnight at 4°C with primary antibodies (anti-phospho-eNOS at Thr495 [1:1000], anti-phospho-eNOS at Ser1177 [1:1000], anti-eNOS [1:1000], anti-Src kinase [1:1000], anti-phospho-Src kinase at Tyr416 [1:1000], anti-caveolin-1 [1:2000], anti-phospho-caveolin-1 at Tyr14 [1:1000], and anti-β actin [1:10,000]). The membranes were then rinsed three times with TBST for 10 min each before incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (1:5000) for 60 min at 25°C. Protein bands were detected using the WesternBright™ ECL Western blotting detection kit (Advansta, Menlo Park, CA, USA) and scanned with a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were normalized by total form. β-actin was used as a loading control. Protein quantification was performed using ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MD, USA).

### *Intracellular calcium measurement*

The intracellular calcium levels after chloroquine treatment were determined using a confocal laser microscope (IX70 Fluoview, Olympus, Tokyo, Japan), as previously described by Park et al. (2023). HUVECs were seeded and cultured in confocal cell culture dishes (SPL, Pocheon, Korea). The cells were treated with 2.5 μM Fluo-4 AM (Invitrogen, Waltham, MA, USA) in Hanks Balanced Salt Solution media for 30 min before being rinsed twice with phosphate-buffered saline. To measure intracellular calcium levels, the cells were treated with chloroquine ($10^{-4}$ M). Additionally, some cells were pretreated with L-NAME ($10^{-4}$ M) followed by chloroquine ($10^{-4}$ M) or treated with L-NAME ($10^{-4}$ M) alone. Calcium levels were measured every 2.5 s at excitation and emission wavelengths of 485 and 520 nm, respectively. The intracellular calcium level was determined using Fluo-4 AM fluorescence images. Calcium levels were determined by measuring the fluorescence intensity (F) relative to baseline fluorescence intensity ($F_0$) before drug treatment. The net change in calcium ion concentration was expressed as $(F_{max} - F_0)/F_0$, where $F_{max}$ indicates the maximum calcium level in fluorescence intensity following treatment with chloroquine alone, L-NAME followed by treatment with chloroquine, or L-NAME alone. Intracellular calcium levels were monitored for approximately 6 min.

### *Materials*

All chemicals used in this study were of high purity and commercially available. Chloroquine, methylene blue, phenylephrine, ODQ, acetylcholine, PP2, fluconazole, wortmannin, L-NAME, and anti-β actin antibody were purchased from Sigma-Aldrich (St Louis, MO, USA). PP2, wortmannin, fluconazole, and ODQ were dissolved in dimethyl sulfoxide to a final concentration of 0.1%. Cell Signaling Technology (Beverly, MA, USA) provided antibodies against Src kinase, phospho-Src kinase, phospho-eNOS (Ser 1177 and Thr 495), caveolin-1, and phospho-caveolin-1. BD Biosciences (Franklin, NJ, USA) supplied the anti-eNOS antibody.

### *Statistical analysis*

The primary goal of this study was to investigate the effects of endothelial denudation and various inhibitors on chloroquine-induced vasodilation. The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed. The impact of endothelial denudation and different inhibitors on chloroquine-induced vasodilation, along with the effects of chloroquine on KCl-evoked contractions in calcium-free Krebs solution followed by calcium addition-evoked contractions, were analyzed using two-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test (Prism 5.0 [GraphPad Software Inc., San Diego, CA, USA]). The effects of chloroquine on eNOS phosphorylation and cGMP synthesis were evaluated using paired and unpaired Student's *t*-tests, respectively. The influence of chloroquine and PP2, alone or in combination, on the phosphorylation of caveolin-1 and Src kinase, and the effects of chloroquine and L-NAME, alone or in combination, on the calcium level were determined using a one-way ANOVA, followed by Bonferroni's multiple comparison test. Statistical significance was set at $p < 0.05$.

## Results

*Effect of endothelial denudation and various inhibitors on the vasodilation evoked by chloroquine in isolated endothelium-intact rat aorta*

Vasodilation triggered by chloroquine was significantly greater in the aorta with endothelium than in that without endothelium (Fig. 1A,B). A two-way ANOVA revealed a significant interaction between endothelial denudation and chloroquine concentration ($F[6, 36] = 142.5$, $p < 0.001$). Bonferroni *post-hoc* analysis showed significant reductions in vasodilation at $3\times10^{-5}$ M ($T = 14.5$, $p < 0.001$) and $10^{-4}$ M ($T = 28.59$, $p < 0.001$) chloroquine in denuded aortas compared to intact ones. In endothelium-intact aortas, the NOS inhibitor L-NAME ($10^{-4}$ M) significantly inhibited chloroquine-induced vasodilation (Fig. 2A). A two-way ANOVA revealed a significant interaction between L-NAME and chloroquine ($F[6, 32] = 44.7$, $p < 0.001$), with Bonferroni *post-hoc* analysis confirming reduced vasodilation at $3\times10^{-5}$ M ($T = 14.22$, $p < 0.001$) and $10^{-4}$ M ($T = 12.81$, $p < 0.001$) chloroquine in the presence of L-NAME compared to the control. Similarly, the

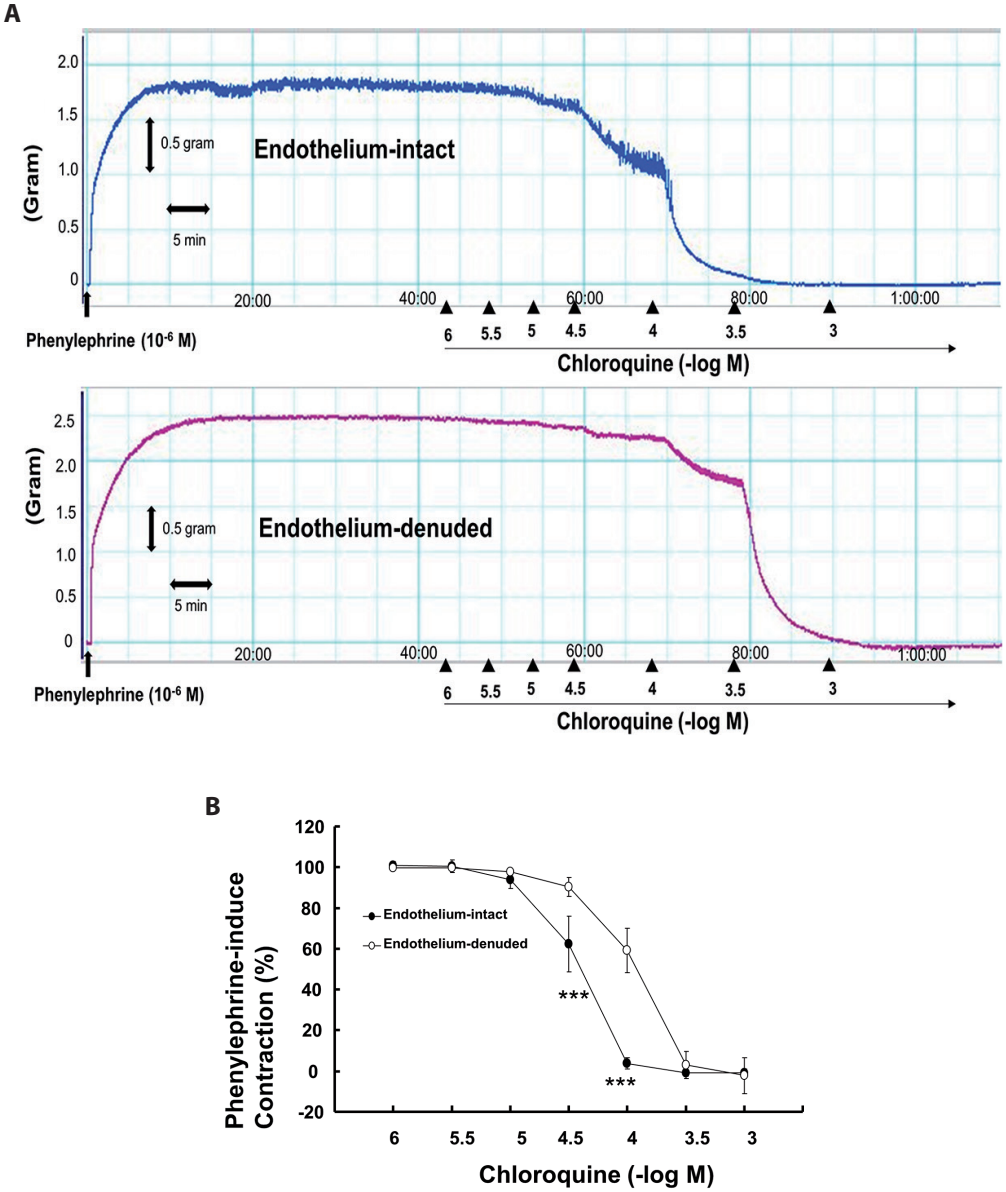


**Figure 1. A.** Original tracing showing chloroquine-induced vasodilation in endothelium-intact and -denuded aortas. **B.** Effect of endothelial denudation on chloroquine-induced vasodilation in isolated aortas. Data are expressed as mean ± SD from $n = 5$ rats, with three to four isolated aortas *per* group from each rat. *n* represents the number of rats from which isolated aortas were obtained. *** $p < 0.001$ *vs.* endothelium-denuded.

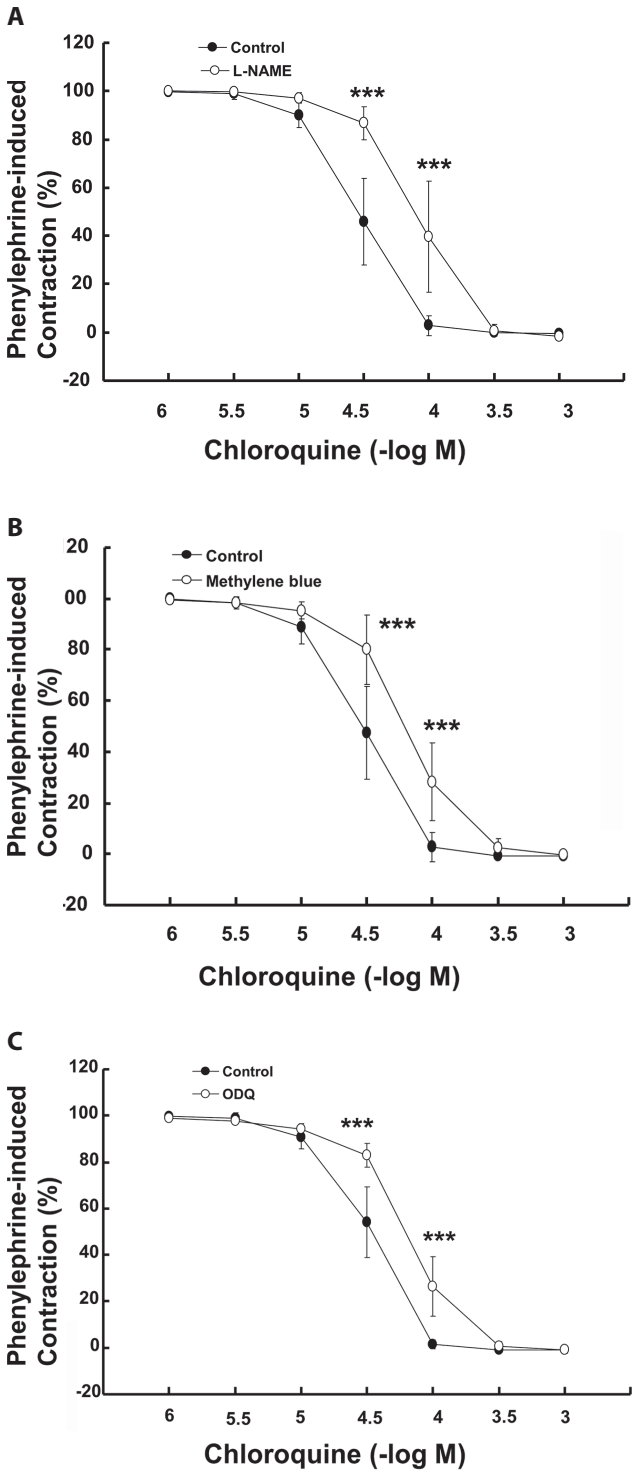


**Figure 2.** Effect of $N^W$-nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M; **A**), methylene blue ($10^{-6}$ M; **B**), and 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, $10^{-5}$ M; **C**) on chloroquine-induced vasodilation in isolated endothelium-intact aorta. Data are presented as mean ± SD from $n = 5$ rats, with two to four isolated aortas *per* group from each rat. *n* represents the number of rats from which isolated aortas were obtained. *** $p < 0.001$ *vs.* control.

non-specific GC inhibitor methylene blue ($10^{-6}$ M) attenuated chloroquine-induced vasodilation in endothelium-intact aortas (Fig. 2B). A two-way ANOVA revealed a significant interaction between methylene blue and chloroquine ($F[6, 31] = 28.93$, $p < 0.001$), with Bonferroni *post-hoc* analysis confirming significantly reduced vasodilation at $3\times10^{-5}$ M ($T = 11.89$, $p < 0.001$) and $10^{-4}$ M ($T = 9.357$, $p < 0.001$) chloroquine in the presence of methylene blue compared to the control. The nitric oxide-sensitive GC inhibitor ODQ ($10^{-5}$ M) also attenuated chloroquine-induced vasodilation in the aorta with endothelium (Fig. 2C). A two-way ANOVA revealed a significant interaction between ODQ and chloroquine ($F[6, 28] = 41.74$, $p < 0.001$), with Bonferroni *post-hoc* analysis confirming reduced vasodilation at $3\times10^{-5}$ M ($T = 14.04$, $p < 0.001$) and $10^{-4}$ M ($T = 10.84$, $p < 0.001$) chloroquine in the presence of ODQ compared to the control. These results suggest that chloroquine-induced endothelium-dependent vasodilation is mediated by NOS and GC. Conversely, the cytochrome P450 epoxygenase inhibitor fluconazole ($10^{-5}$ M) did not affect chloroquine-induced vasodilation in the aorta with endothelium (Fig. 3A). The phosphoinositide-3 kinase inhibitor wortmannin ($3\times10^{-7}$ M) inhibited chloroquine-induced vasodilation in the aorta with the endothelium (Fig. 3B). A two-way ANOVA confirmed a significant interaction between wortmannin and chloroquine ($F[6, 35] = 18.96$, $p < 0.001$), with Bonferroni *post-hoc* tests showing reduced vasodilation at $3\times10^{-5}$ M ($T = 8.676$, $p < 0.001$) and $10^{-4}$ M ($T = 6.591$, $p < 0.001$) in the presence of wortmannin compared to the control.

### *Effect of chloroquine on KCl-evoked contraction in calcium-free Krebs solution and subsequent calcium-evoked contraction in the isolated endothelium-denuded aorta*

Chloroquine at a concentration of $10^{-4}$ M slightly suppressed KCl-induced contractions in calcium-free Krebs solution and significantly attenuated subsequent calcium-evoked contractions (Fig. 4). A two-way ANOVA showed a significant interaction between chloroquine treatment and KCl/$CaCl_2$ administration ($F[9, 25] = 41.76$, $p < 0.001$). Bonferroni *post-hoc* analysis revealed significant reductions in KCI-induced contractions at 50 mM ($T = 3.001$, $p < 0.05$) and 60 mM ($T = 3.521$, $p < 0.01$), as well as in $CaCl_2$-evoked contractions at 1.25 mM ($T = 18.58$, $p < 0.001$), 2.5 mM ($T = 9.814$, $p < 0.001$), 3.75 mM ($T = 5.685$, $p < 0.001$), and 5 mM ($T = 3.785$, $p < 0.01$) in the presence of chloroqine compared to the control.

### *Effect of chloroquine on cGMP in isolated endothelium-intact aorta*

Chloroquine at a concentration of $10^{-5}$ M significantly enhanced cGMP production in isolated aortas with endothe-

lium ($p < 0.01$ compared with the control, Fig. 5; T value and degree of freedom were 4.576 and 10, respectively), suggesting that chloroquine-induced cGMP formation mediates vasodilation through cGMP-dependent protein kinase activation.

*Effects of chloroquine and PP2, alone or in combination, on the phosphorylation of eNOS, caveolin-1, and Src kinase in HUVECs*

Chloroquine at a concentration of $10^{-4}$ M increased the phosphorylation of stimulatory eNOS (Ser1177) in HUVECs ($p < 0.001$ compared with the control; Fig. 6A; T value and degree of freedom were 11.70 and 5, respectively) and decreased the phosphorylation of inhibitory eNOS (Thr495) ($p < 0.01$ compared with the control; Fig. 6B; T value and degree of freedom were 5.609 and 6, respectively). Additionally, chloroquine enhanced caveolin-1 phosphorylation, which was significantly suppressed by the Src kinase inhibitor PP2 ($5\times10^{-6}$ M) (Fig. 6C). A one-way ANOVA indicated significant differences in caveolin-1 phosphorylation among groups ($F[3,24] = 30.56$, $p < 0.001$), with Bonferroni *post-hoc* tests confirming increased caveolin-1 phosphorylation with chloroquine ($T = 5.382$, $p < 0.001$) and reduced caveolin-1 phosphorylation with PP2 and chloroquine treatment ($T = 7.603$, $p < 0.001$). Chloroquine also enhanced Src kinase phosphorylation in HUVECs, which was inhibited by PP2 (Fig. 6D). A one-way ANOVA revealed a significant difference in Src kinase phosphorylation among groups ($F[3,20] = 14.55$, $p < 0.001$), with Bonferroni *post-hoc* analysis confirming increased Src kinase phosphorylation with chloroquine ($T = 4.11$, $p < 0.01$) and reduced Src kinase phosphorylation with PP2 and chloroquine treatment ($T = 3.809$, $p < 0.01$).

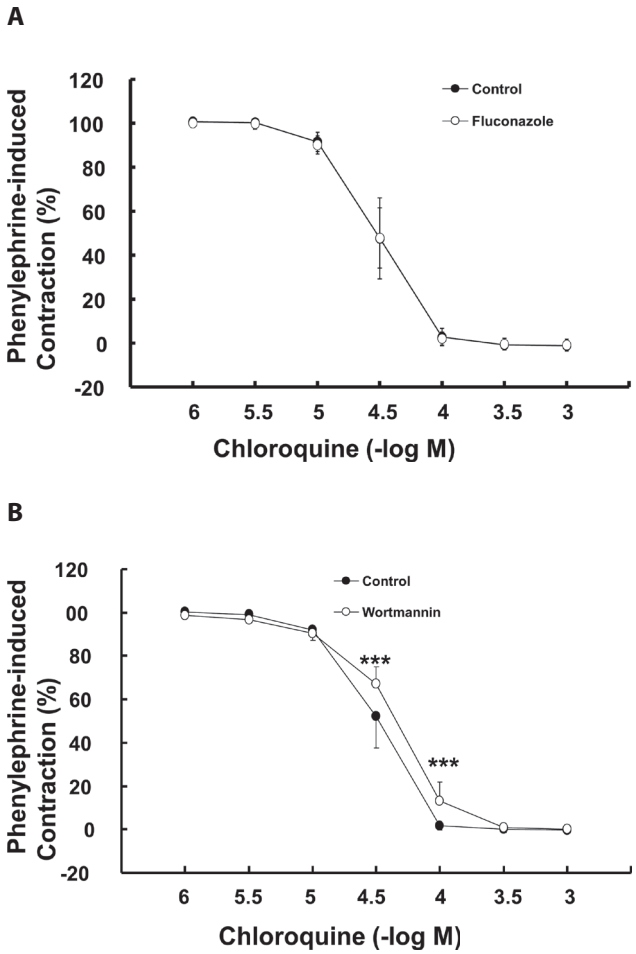


**Figure 3.** Effect of fluconazole ($10^{-5}$ M, **A**) and wortmannin ($3\times10^{-7}$ M, **B**) on chloroquine-induced vasodilation in isolated endothelium-intact aorta. A. Data are shown as mean ± SD from $n = 6$ rats, with four isolated aortas *per* group from each rat. B. Data are shown as mean ± SD from $n = 5$ rats, with three to four isolated aortas *per* group from each rat. $n$ represents the number of rats from which isolated aortas were obtained. *** $p < 0.001$ *vs.* control.

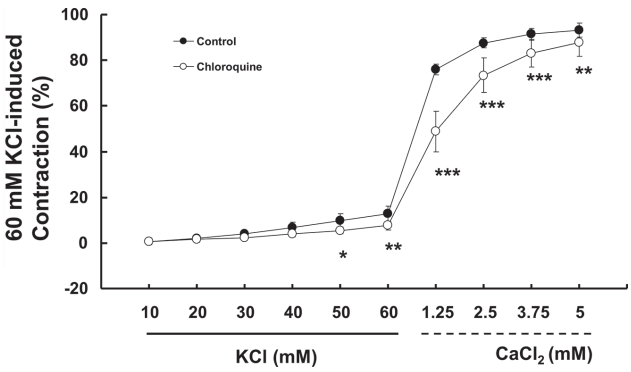


**Figure 4.** Effect of chloroquine ($10^{-4}$ M) on the KCl (10–60 mM)-induced contraction in the calcium-free Krebs solution followed by calcium chloride (1.25–5 mM)-induced contraction of endothelium-denuded aortas. Data are expressed as mean ± SD from $n = 5$ rats, with two to three isolated aortas *per* group from each rat. $n$ represents the number of rats from which isolated aortas were obtained. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control.

*Effects of chloroquine and L-NAME, alone or combined, on the intracellular calcium level in HUVECs*

Chloroquine at a concentration of $10^{-4}$ M elevated calcium levels in HUVECs (Fig. 7). However, pretreatment with the NOS inhibitor L-NAME ($10^{-4}$ M) effectively blocked the increase in endothelial calcium induced by chloroquine ($10^{-4}$ M) (Fig. 7). A one-way ANOVA revealed significant differences in calcium levels among groups ($F[2,105] = 134.5$,

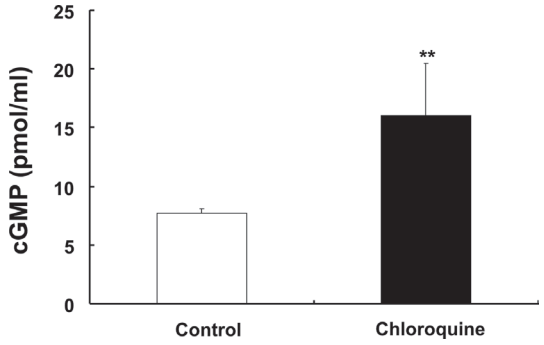


**Figure 5.** Effect of chloroquine ($10^{-5}$ M) on cyclic guanosine monophosphate (cGMP) formation in rat aorta with endothelium. Data are shown as mean ± SD from $n = 4$ rats, with one to two isolated aortas *per* group from each rat. *n* represents the number of rats from which isolated aortas were obtained. ** $p < 0.01$ *vs.* control.

$p < 0.001$). Bonferroni *post-hoc* tests confirmed a significant reduction in calcium levels with L-NAME and chloroquine treatment (T = 14.08, $p < 0.001$).

## Discussion

This study suggests that chloroquine induces endothelial nitric oxide-dependent vasodilation *via* NOS-cGMP. The major findings of this study were as follows: 1) L-NAME, methylene blue and ODQ inhibited chloroquine-induced endothelium-dependent vasodilation; 2) chloroquine increased cGMP formation; 3) chloroquine boosted stimulatory eNOS (Ser1177) phosphorylation while decreasing inhibitory eNOS (Thr495) phosphorylation; and 4) L-NAME pretreatment decreased chloroquine-induced endothelial calcium increase.

Chloroquine caused greater vasodilation in the aorta with the endothelium than in that without the endothelium, implying that its vasodilatory impact depends on the endothelium. eNOS produces nitric oxide from L-arginine and reduced nicotinamide adenine dinucleotide phosphate, which is facilitated by calmodulin in a calcium-dependent manner (Sohn et al. 2004). Nitric oxide diffuses from the endothelium to the vascular smooth muscle, where it activates GC, leading to cGMP production (Sohn et al. 2004). cGMP

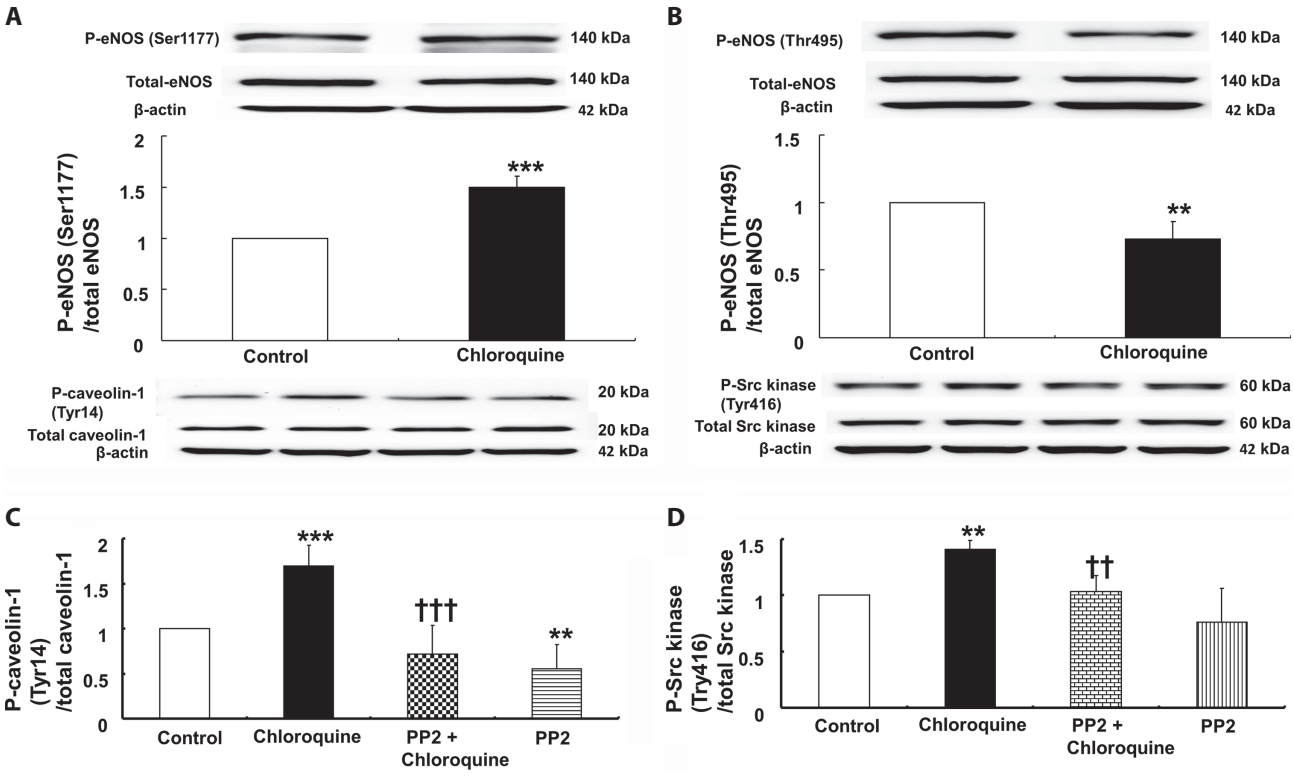


**Figure 6.** Effect of chloroquine ($10^{-4}$ M) on endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 (**A**) and Thr495 (**B**) in human umbilical vein endothelial cells (HUVECs). Data are presented as mean ± SD from $n = 4$ (A) and $n = 5$ (B) independent experiments. *n* represents the number of independent experiments. ** $p < 0.01$, *** $p < 0.001$ *vs.* control. P-eNOS, phosphorylated eNOS. Effects of chloroquine ($10^{-4}$ M) and PP2 ($5\times10^{-6}$ M), alone or in combination, on caveolin-1 (**C**) and Src kinase (**D**) phosphorylation in HUVECs. Data are expressed as mean ± SD from $n = 4$ independent experiments. *n* represents the number of independent experiments. ** $p < 0.01$, *** $p < 0.001$ *vs.* control; †† $p < 0.01$, ††† $p < 0.001$ *vs.* chloroquine alone. P-caveoline-1, phosphorylated caveolin-1; P-Src kinase, phosphorylated Src kinase.

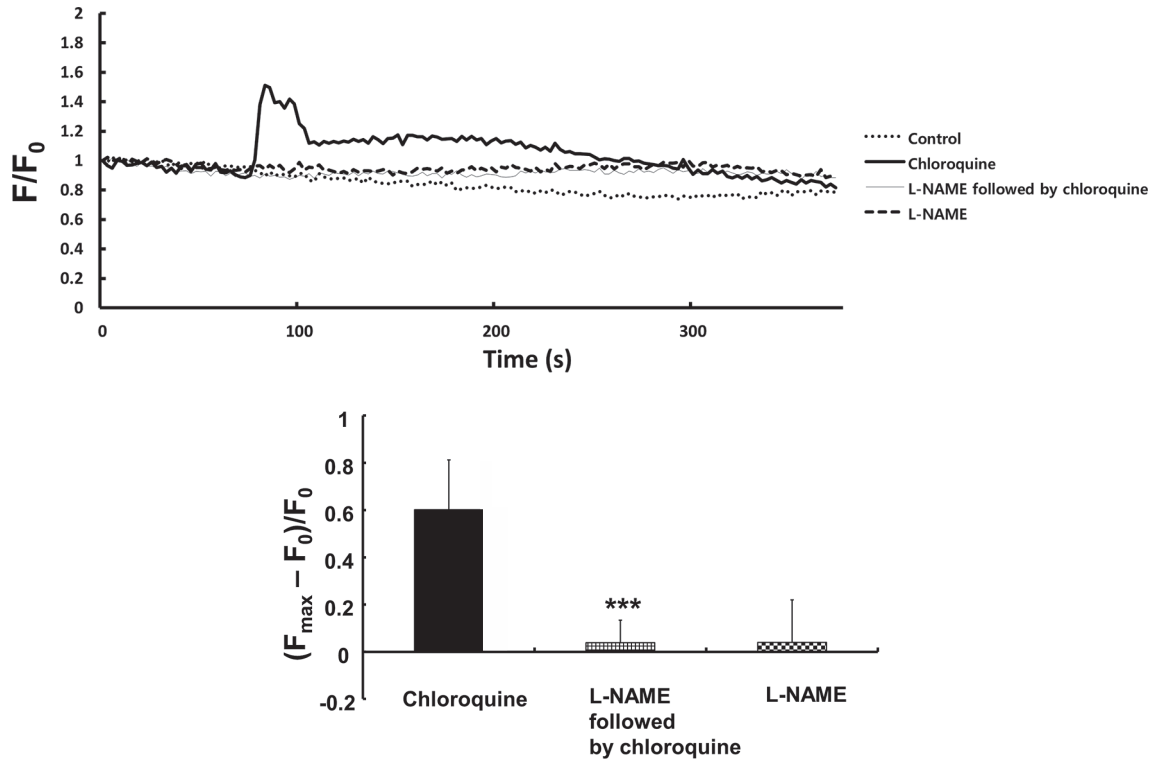


**Figure 7.** Effect of chloroquine ($10^{-4}$ M) alone or $N^{W}$-nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M) followed by chloroquine ($10^{-4}$ M) and L-NAME ($10^{-4}$ M) alone on intracellular calcium level in human umbilical vein endothelial cells. Data are shown as mean ± SD from $n = 6$ independent experiments, with six replicates *per* experiment. $n$ represents the number of independent experiments. *** $p < 0.001$ *vs.* chloroquine alone.

promotes vasodilation by activating cGMP-dependent protein kinases (Sohn et al. 2004). The NOS inhibitor, L-NAME, blocked chloroquine-induced vasodilation, which depends on the endothelium. Inhibitors of GC, such as ODQ and methylene blue, also suppressed chloroquine-induced vasodilation in endothelium-intact aortas. Furthermore, chloroquine increased cGMP production. Collectively, these findings indicate that chloroquine-induced vasodilation occurs through a pathway involving nitric oxide, GC activation, and cGMP synthesis. The contraction induced by agonists like phenylephrine is mitigated by endothelial phosphoinositide-3 kinase-mediated production of nitric oxide in endothelium-intact aorta, underscoring the role of this pathway in attenuating agonist-induced contraction (Budzyn et al. 2005). Thus, as the phosphoinositide-3 kinase inhibitor wortmannin inhibited chloroquine-induced vasodilation in endothelium-intact aorta precontracted with phenylephrine, chloroquine-evoked endothelial nitric oxide-mediated vasodilation seems to be partially associated with the activation of endothelial phosphoinositide-3 kinase. The bitter taste receptor agonist, chloroquine or denatonium, induces relaxation of the ileum and corpus cavernosum, which is partially mediated by nitric oxide (Jing et al. 2013; Navarro-Dorado et al. 2023). Additionally, bitter taste receptors activated by ligands produce nitric oxide in the sinonasal cavity (Yan et al. 2017). Bitter taste receptors are found in endothelial cells of the pulmonary artery (Kertesz et al. 2022). Taking into consideration previous reports (Jing et al. 2013; Yan et al. 2017; Kertesz et al. 2022; Xin and Zhao 2022; Navarro-Dorado et al. 2023), chloroquine-induced activation of the pathway involving NOS-GC-cGMP observed in the current study could be attributed to endothelial bitter taste receptor stimulation by the bitter taste receptor agonist chloroquine in the aorta. Further studies of the upstream cellular signaling pathways involved in chloroquine-evoked nitric oxide-mediated vasodilation are required. One proposed mechanism associated with endothelium-derived hyperpolarizing factor-induced vasodilation is vasodilation generated by arachidonic acid metabolites *via* the cytochrome P450 epoxygenase pathway (Bryan et al. 2005). Therefore, because the cytochrome P450 epoxygenase inhibitor fluconazole did not affect chloroquine-induced vasodilation in endothelium-intact aortas, this finding suggests that chloroquine-induced endothelium-dependent vasodilation is not linked to vasodilation induced by endothelium-derived hyperpolarizing

factors *via* a metabolic pathway involving cytochrome P450 epoxygenase.

Chloroquine inhibits the vasoconstriction induced by norepinephrine and high KCl (60 mM) in endothelium-denuded aortas (Aziba and Okpako 2003), suggesting that chloroquine-induced endothelium-independent vasodilation is mediated by calcium influx inhibition *via* voltage-operated calcium channels (Akata 2007). Consistent with previous findings, chloroquine greatly reduced calcium-induced contraction in calcium-free Krebs solution containing 60 mM KCl (Aziba and Okpako 2003). Considering the findings of previous reports, chloroquine-induced vasodilation, which does not rely on the endothelium, is achieved by inhibiting calcium influx through voltage-gated calcium channels (Aziba and Okpako 2003; Akata 2007).

Phosphorylation of Ser1177 and Thr495 in eNOS activates and inhibits eNOS, leading to increased and decreased nitric oxide production, respectively (Garcia and Sessa 2019). The interaction between caveolin-1 and eNOS negatively regulates eNOS activity and nitric oxide release (Garcia and Sessa 2019). Caveolin scaffolding domains are associated with cellular signal transduction, which involves upstream entities (such as G-protein-coupled receptor and receptor tyrosine kinase) and downstream components (such as eNOS or effector enzymes) (Patel et al. 2008). In agreement with the findings of tension studies, chloroquine ($10^{-4}$ M) increased the phosphorylation of stimulatory eNOS at Ser1177 and reduced the phosphorylation of inhibitory eNOS at Thr495 in HUVECs, leading to enhanced nitric oxide production. Additionally, chloroquine ($10^{-4}$ M) triggered the phosphorylation of caveolin-1 and Src kinase. However, treatment with the Src kinase inhibitor PP2 suppressed chloroquine-induced phosphorylation of caveolin-1 and Src kinase, indicating that Src kinase mediates the phosphorylation of caveolin-1. Taken together with the findings of a previous report (Patel et al. 2008), chloroquine-induced eNOS phosphorylation appears to be mediated by a cellular signaling pathway that includes caveolin-1 and Src kinase. Further studies are needed to examine the upstream cellular signaling pathways involved in chloroquine-induced activation of eNOS, caveolin-1, and Src kinase. Increases in endothelial calcium are required to activate eNOS and the subsequent nitric oxide production (Ogawa et al. 2001; Zuccolo et al. 2017). In agreement with chloroquine-induced eNOS (ser1177) phosphorylation, chloroquine increased the calcium levels in HUVECs. However, pretreatment with L-NAME attenuated the chloroquine-induced calcium increase, suggesting that an increase in endothelial calcium is required for chloroquine to activate eNOS. Acetylcholine induces calcium oscillations and nitric oxide release in brain microvascular endothelial cells (Zuccolo et al. 2017). The source of calcium associated with calcium oscillations induced by acetylcholine is endogenous calcium released from the endoplasmic reticulum and calcium entry through store-operated calcium channels (Zuccolo et al. 2017). Thus, further studies are needed to examine the source of calcium associated with the increase in chloroquine-induced endothelial calcium.

This study has some limitations. First, it utilized the aorta, a conduit vessel, whereas small resistance arterioles predominantly regulate total peripheral vascular resistance and contribute significantly to blood pressure (Rahman and Siddik 2024). Second, the rat aorta was used for isometric tension measurements. HUVECs were used to detect Src kinase, caveolin-1, and eNOS phosphorylation *via* Western blotting. Third, this was an *in vitro* study. However, *in vivo* experiments investigating the effect of L-NAME on the chloroquine-induced decrease in blood pressure are needed to confirm the endothelial nitric oxide-dependent reduction in blood pressure by chloroquine. Despite these limitations, this study is the first to demonstrate that toxic-dose chloroquine (toxic plasma concentration: approximately $1.94\times10^{-6}$ M)-induced vasodilation is mediated partially by endothelial nitric oxide, which contributes to decreased blood pressure (Schulz and Schmoldt 2003). Thus, the non-specific GC inhibitor methylene blue, which is used to treat vasoplegic shock, may alleviate a toxic dose of chloroquine-induced hypotension *via* nitric oxide release (Kofler et al. 2022). Conversely, it can be inferred from this result that the magnitude of toxic-dose chloroquine-induced hypotension is lower in patients with a compromised endothelium, such as those with hypertension, than in those with an intact endothelium. On the other hand, the therapeutic plasma concentration of chloroquine (0.5 μg/ml: corresponding to approximately $9.625\times10^{-7}$ M chloroquine) did not induce vasodilation (Fig. 1B), which may be associated with the non-significant change in blood pressure due to vasodilation.

In conclusion, these findings indicate that chloroquine-induced vasodilation, which is dependent on the endothelium, occurs *via* a pathway that involves NOS-GC-cGMP activation. Additionally, eNOS activation by chloroquine appeared to be mediated by caveolin-1 and Src kinases. Furthermore, chloroquine-induced vasodilation, which is independent of the endothelium, appears to occur through the inhibition of calcium influx *via* voltage-operated calcium channels.

**Conflict of interest.** The authors declared no conflict of interest.

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