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Chloroquine inhibits vasodilation induced by ATP-sensitive potassium channels in isolated rat aorta

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Abstract. This study examined the effect of chloroquine on vasodilation induced by levcromakalim in isolated endothelium-denuded rat aortas and clarified the underlying mechanisms. We examined the effects of chloroquine, hydroxychloroquine, lipid emulsion, reactive oxygen species (ROS) scavenger *N*-acetyl-L-cysteine (NAC), and K_{ATP} channel inhibitor glibenclamide on levcromakalim-induced vasodilation. The effects of chloroquine, hydroxychloroquine, NAC, and levcromakalim on membrane hyperpolarization and ROS production were examined in aortic vascular smooth muscle cells (VSMCs). Chloroquine inhibited levcromakalim-induced vasodilation more than hydroxychloroquine. NAC attenuated chloroquine-mediated inhibition of levcromakalim-induced vasodilation, while lipid emulsion had no effect. Glibenclamide eliminated levcromakalim-induced vasodilation in aortas pretreated with chloroquine. Chloroquine and hydroxychloroquine inhibited levcromakalim-induced membrane hyperpolarization in VSMCs. Chloroquine and hydroxychloroquine both produced ROS, but chloroquine produced more. NAC inhibited chloroquine-induced ROS production in VSMCs. Collectively, these results suggest that, partially through ROS production, chloroquine inhibits levcromakalim-induced vasodilation. In addition, chloroquine-induced K_{ATP} channel-induced vasodilation impairment was not restored by lipid emulsion.

Key words: K_{ATP} channel — Levcromakalim — Vasodilation — Chloroquine — Reactive oxygen species

Abbreviations: H_2DCFDA, 2′,7′-dichlorofluorescin diacetate; K_{ATP} channel, ATP-sensitive potassium channel; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NAC, *N*-acetyl-L-cysteine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VSMCs, vascular smooth muscle cells.

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Introduction

Adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels in the vascular smooth muscle, which are involved in the regulation of blood flow and vascular tone by endogenous vasodilators, are stimulated by pathophysiological conditions including acidosis, hypoxia, and ischemia to induce vasodilation, resulting in the regulation of tissue perfusion (Bradyen 2002). This effect is considered a beneficial and protective response. Chloroquine and hydroxychloroquine, which have similar pharmacological properties, including high lipid solubility, high oral bioavailability, and a large distribution volume, are used for the treatment of rheumatoid arthritis, malaria, and systemic lupus erythematosus (Della Porta et al. 2020). In addition, chloroquine and hydroxychloroquine were reported to inhibit severe acute respiratory syndrome coronavirus 2 in the development of coronavirus disease 2019 in *in vitro* experiments (Liu et al. 2020; Touret et al. 2020). However, follow-up clinical trials using chloroquine and hydroxychloroquine as alternative drugs for the treatment of coronavirus disease 2019 caused cardiac toxicity such as ventricular tachycardia and QT prolongation (Agstam et al. 2021; Tleyjeh et al. 2021). Moreover, chloroquine has a higher toxicity than hydroxychloroquine as the levels at which its plasma concentration ratio (chloroquine to hydroxychloroquine) produces a toxic effect is less than approximately 0.39 (Edwards et al. 1987; Jordan et al. 1999). Furthermore, chloroquine causes hypoglycemia as a side effect by increasing insulin release through the inhibition of K_{ATP} channels in pancreatic beta cells (Davis 1997). Although the properties of K_{ATP} channels are shared between vascular smooth muscle and the pancreas, differences in the properties of K_{ATP} channels exist due to actions induced by modulators such as ATP, sulfonylurea compound, and potassium channel-opening drugs (Yokoshiki et al. 1998). The properties of K_{ATP} channels in vascular smooth muscle and the pancreas are as follows: half of maximum inhibitory concentration by ATP (29 μM Bell-shaped relation in vascular smooth muscle *vs*. 15–40 μM in pancreas), half of maximum inhibitory concentration by tolbutamide on the sulfonylurea (350 μM in vascular smooth muscle *vs*. 3–20 μM in pancreas), and potency of potassium channel opener (pinacidil = cromakalim > diazoxide in vascular smooth muscle *vs*. diazoxide > pinacidil > cromakalim in pancreas) (Yokoshiki et al. 1998).

The production of reactive oxygen species (ROS) including superoxide has been reported to inhibit vasodilation induced by K_{ATP} channels (Kinoshita et al. 2004, 2006; Haba et al. 2009; Santos et al. 2021). Chloroquine has been shown to induce ototoxicity and cardiotoxicity in a rat model of pressure overload hypertrophy through ROS production and consequent oxidative stress (Chaanine et al. 2015; Oliveira et al. 2019). However, to the best of our knowledge, the effect of chloroquine on K_{ATP} channel-mediated vasodilation remains unknown. Lipid emulsions are the recommended treatment for systemic toxicity caused by local anesthetics (Lee and Sohn 2023); they have been reported to ameliorate cardiovascular depression induced by toxic doses of non-local anesthetic drugs with high lipid solubility (Lee et al. 2023). Moreover, lipid emulsions used as adjuvant drugs in supportive treatments have been reported to treat cardiovascular depression caused by toxic doses of chloroquine and hydroxychloroquine (Ten Broeke et al. 2016; Murphy et al. 2018; Bethlehem et al. 2019; Noda et al. 2021). However, the effect of lipid emulsions on the chloroquine-mediated alteration of K_{ATP} channel-induced vasodilation remains to be determined. On the basis of previous reports (Davis 1997; Kinoshita et al. 2004, 2006; Haba et al. 2009; Chaanine et al. 2015; Oliveira et al. 2019; Santos et al. 2021), this study tested the biological hypothesis that chloroquine inhibits K_{ATP} channel-induced vasodilation *via* ROS production. Thus, the goals of this study were to examine the effects of chloroquine and a lipid emulsion, alone or in combination, on vasodilation induced by the K_{ATP} channel agonist levcromakalim in isolated rat aortas and to clarify the underlying mechanism of these effects.

Materials and Methods

The experimental protocol (GNU-210429-R0036) was approved by the Institutional Animal Care and Use Committee of the Gyeongsang National University. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the National Institute of Health.

Preparation of isolated rat aorta and isometric tension measurement

Isolated rat aortas were prepared and isometric tension was measured as described previously (Lee et al. 2021). Male Sprague-Dawley rats (body weight: 230–280 g; Koatech, Pyeongtaek, Gyeonggi-do, Korea) were euthanized with 100% CO_2 supplied to a small hole in the rat cage. The thorax of the rats was opened; the descending thoracic aorta was excised from the thoracic cavity and submerged in Krebs solution containing sodium chloride (118 mM), glucose (11 mM), sodium bicarbonate (25 mM), calcium chloride (2.4 mM), potassium chloride (4.7 mM), monopotassium phosphate (1.2 mM), and magnesium sulfate (1.2 mM). The fat and connective tissue surrounding the isolated rat aorta submerged in Krebs solution were removed under a microscope. Subsequently, the isolated rat aorta was cut into 2.5 mm length segments. The endothelium of all isolated rat

aortas was peeled off by rolling two 25-gauge needles forward and backward, which were inserted into the lumen of the isolated rat aorta. The isolated thoracic aorta was suspended in a Grass isometric transducer (FT-03; Grass Instrument, Quincy, MA, USA) mounted in an organ bath maintained at 37°C. Following a previous report, to reach equilibrium, a baseline resting tension of 24.5 mN was maintained for 90 min (Klöss et al. 2000). Simultaneously, the pre-existing Krebs solution was replaced with a fresh solution every 30 min. We maintained the Krebs solution at pH 7.4 by supplying it with gas containing 5% carbon dioxide and 95% oxygen. Endothelium removal was confirmed using the following method: after the addition of phenylephrine (10^{-7} M) to the organ bath induced stable and persistent contractions, acetylcholine (10^{-5} M) was added to the organ bath with the aorta displaying phenylephrine-evoked contractions. An aorta with less than 15% acetylcholine-induced relaxation was regarded as an endothelium-denuded aorta. Subsequently, fresh Krebs solution was added to the organ bath several times to wash the aorta, which exhibited acetylcholine-induced relaxation from phenylephrine-induced contraction, eventually resulting in the restoration of the baseline resting tension. Next, the following experimental protocols were performed. As the levcromakalim-induced vasodilation is partially mediated by endothelial nitric oxide, all denuded rat aortas used in this experiment were pretreated with the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M) to prevent endothelial nitric oxide release due to the presence of residual endothelium after endothelial denudation (Kinoshita et al. 1999).

Experimental protocols

First, we examined the effect of toxic concentrations of chloroquine (10^{-5}, 3×10^{-5}, and 6×10^{-5} M) and hydroxychloroquine (10^{-5}, 3×10^{-5}, and 6×10^{-5} M) on vasodilation induced by the K_{ATP} channel agonist levcromakalim in isolated endothelium-denuded rat aortas (Schulz and Schmoldt 2003). The isolated rat aortas were pretreated with chloroquine or hydroxychloroquine for 20 min. After phenylephrine (10^{-6} M) induced sustained and stable contraction in the isolated endothelium-denuded rat aorta, levcromakalim (10^{-8} to 10^{-5} M) was cumulatively added to the organ bath to generate levcromakalim-induced vasodilation in the presence or absence of chloroquine or hydroxychloroquine.

Second, to examine the role of ROS and K_{ATP} channel inhibitors in the chloroquine-mediated inhibition of levcromakalim-induced vasodilation, we investigated the effects of the ROS scavenger *N*-acetyl-L-cysteine (NAC, 5×10^{-3} M) and K_{ATP} channel inhibitor glibenclamide (5×10^{-6} M) on chloroquine (6×10^{-5} M)-mediated inhibition of vasodilation induced by levcromakalim in the isolated endothelium-denuded rat aorta (Baik et al. 2016; Lee et al. 2022). The isolated rat aorta was treated with chloroquine alone for 20 min, NAC, and glibenclamide alone for 35 min, or it was pretreated with NAC or glibenclamide for 15 min, followed by chloroquine for 20 min. After phenylephrine (10^{-6} M) produced a sustained and stable contraction, levcromakalim (10^{-8} to 10^{-5} M) was cumulatively added to the organ bath to generate levcromakalim-induced vasodilation in the presence or absence of chloroquine, NAC, and glibenclamide alone or combination treatment with NAC plus chloroquine or glibenclamide plus chloroquine.

Third, to examine whether lipid emulsion attenuates lipid soluble chloroquine-mediated inhibition of levcromakalim-induced vasodilation, the effect of a lipid emulsion (Intralipid; 1%) on chloroquine (6×10^{-5} M)-mediated inhibition of vasodilation induced by levcromakalim was investigated. The endothelium-denuded rat aorta was pretreated with the lipid emulsion (1%) for 15 min, followed by chloroquine (6×10^{-5} M) for 20 min or chloroquine (6×10^{-5} M) alone for 20 min. Then, after phenylephrine (10^{-6} M) produced stable and sustained contraction of the isolated rat aorta, levcromakalim (10^{-8} to 10^{-5} M) was cumulatively added to the organ bath to generate levcromakalim-induced vasodilation in the presence or absence of chloroquine alone or in combination with the lipid emulsion.

Fourth, to examine whether chloroquine-induced inhibition of levcromakalim-mediated vasodilation was specific, the effects of chloroquine (6×10^{-5} M) on vasodilation caused by the calcium channel blocker diltiazem and nitric oxide donor sodium nitroprusside were examined. The aorta was pretreated with chloroquine (6×10^{-5} M) for 20 min. After phenylephrine (10^{-6} M) induced sustained and stable contraction, diltiazem (3×10^{-8} to 3×10^{-4} M), or sodium nitroprusside (10^{-10} to 10^{-7} M) were cumulatively added to the organ bath to induce vasodilation in the presence or absence of chloroquine.

Culture of vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) were separated from the descending thoracic aorta and cultured using Dulbecco's modified Eagle medium supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated fetal bovine serum, as described previously (Baik et al. 2016). Cells at passages 3–5 were used in this study; they were incubated at 37°C in humidified air with 5% carbon dioxide.

Measurement of resting membrane potential in vascular smooth muscle cells

The membrane potential in VSMCs was recorded as previously described (Lee et al. 2020). Briefly, the membrane potential of VSMCs was measured using a whole-cell patch-clamp technique in current-clamp mode ($I = 0$). The

membrane potential was amplified using a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA, USA). The bath solution (pH 7.4) consisted of 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). The pipette solution (pH 7.3) contained 150 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES. The resistance of the pipette tip was 4–6 MΩ. The chemicals were dissolved in the bath solution at the desired concentrations. The experimental data were analyzed using Clampfit (pCLAMP, version 9.2, Axon Instruments).

Measurement of intracellular reactive oxygen species

ROS generation by chloroquine was measured using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) in VSMCs, as described previously (Lee et al. 2022). Cells seeded on a cover glass (at a density of 2×10^5 cells/well in a 6-well culture plate) were incubated at 37°C in a CO_2 incubator overnight and starved overnight with a serum-free medium. Cells were treated with chloroquine (6×10^{-5} M) or hydroxychloroquine (6×10^{-5} M) alone for 10 min, NAC (5×10^{-3} M) alone for 70 min, or NAC (5×10^{-3} M) for 1 h, followed by chloroquine (6×10^{-5} M) for 10 min. The cells were subsequently washed with phosphate-buffered saline (PBS). The cells were incubated with 10^{-5} M H_2DCFDA for 30 min at 37°C and washed twice with PBS. ROS production was photographed using a fluorescence microscope (Nikon Eclipse Ti2; Nikon Co., Tokyo, Japan) and analyzed using the captured cell images.

Chemicals

Chemical substances of the highest purity were used. Chloroquine, hydroxychloroquine, L-NAME, glibenclamide, H_2DCFDA, diltiazem, sodium nitroprusside, NAC, phenylephrine, and acetylcholine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Intralipid (20%) was purchased from Fresenius Kabi AB (Upsala, Sweden). Dulbecco's modified Eagle medium, fetal bovine serum, and penicillin-streptomycin were obtained from Gibco (Life Technologies, Grand Island, NY, USA). Levcromakalim was purchased from Tocris Bioscience (Bristol, United Kingdom). Levcromakalim was dissolved in ethanol (final concentration: 0.19%). All other drugs were dissolved in distilled water.

Statistical analysis

The primary outcome was the effect of chloroquine, hydroxychloroquine, NAC, glibenclamide, and lipid emulsion, alone or in combination, on vasodilation caused by levcromakalim. Normality tests were performed using the Kolmogorov-Smirnov test. The generalized linear mixed effect model (Stata version 14.1, Stat Corp LP, Lakeway Drive College Station, TX, USA) was used for analyzing the effects of chloroquine, hydroxychloroquine, NAC, glibenclamide, and lipid emulsion, alone or in combination, on vasodilation induced by levcromakalim, diltiazem, and sodium nitroprusside (Lavergne et al. 2008). The logarithm of levcromakalim to produce half (50%) the amount of levcromakalim-induced maximal vasodilation (Log ED_{50}) in the presence or absence of chloroquine or hydroxychloroquine was calculated through a non-linear regression by applying levcromakalim dose-response curves to a sigmoidal curve on Prism 5.0 (Graphad Software Inc, San Diego, CA, USA) (Lee et al. 2021). The comparison of Log ED_{50} and maximal levcromakalim-induced vasodilation was performed to examine the effect of chloroquine or hydroxychloroquine on levcromakalim-induced vasodilation. The effects of chloroquine and hydroxychloroquine on the Log ED_{50} of levcromakalim-induced vasodilation were analyzed, respectively, using the Kruskal-Wallis test followed by Dunn's multiple comparison test, and a one-way analysis of variance followed by Bonferroni's test. The Log ED_{50} ratio, which is calculated by dividing the Log ED_{50} of levcromakalim-induced vasodilation in the drug (chloroquine or hydroxychloroquine)-treated group by the Log ED_{50} of levcromakalim-induced vasodilation in the control group, was used to compare the inhibitory potency of chloroquine and hydroxychloroquine on the levcromakalim-induced vasodilation. An unpaired Student's t-test was used to compare the Log ED_{50} ratio between the chloroquine and hydroxychloroquine groups. The effect of chloroquine and hydroxychloroquine on membrane potential evoked by levcromakalim was analyzed using a one-way analysis of variance followed by Bonferroni's test. The effect of chloroquine, hydroxychloroquine, and NAC, alone or in combination, on ROS production was analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Results

Chloroquine (10^{-5}, 3×10^{-5}, and 6×10^{-5} M) inhibited vasodilation caused by levcromakalim (Log ED_{50}: $p < 0.01$, 10^{-5} M chloroquine *vs.* control; $p < 0.001$, 3×10^{-5}, and 6×10^{-5} M chloroquine *vs.* control; Fig. 1A). In addition, hydroxychloroquine (10^{-5}, 3×10^{-5}, and 6×10^{-5} M) also inhibited levcromakalim-induced vasodilation (Log ED_{50}: 10^{-5}, 3×10^{-5}, and 6×10^{-5} M hydroxychloroquine; $p < 0.001$ *vs.* control; Fig. 1B). However, the Log ED_{50} ratio of levcromakalim-induced vasodilation in the chloroquine (10^{-5} M)-treated group was greater than that in the hydroxychloroquine (10^{-5} M)-treated group ($p = 0.001$; Log ED_{50} ratio: 10^{-5} M chloroquine = 1.72 ± 0.32 *vs.* 10^{-5} M hydroxychloroquine = 1.29 ± 0.22), suggesting that chloroquine inhibits levcromakalim-induced vasodilation more than

hydroxychloroquine. In contrast, maximal vasodilation induced by high concentrations of levcromakalim (10^{-5} M) was increased in the chloroquine-treated group compared with the control (6×10^{-5} M chloroquine: $p < 0.001$ *vs*. control; 3×10^{-5} M chloroquine: $p < 0.01$ *vs*. control; Fig. 1A). In addition, a high concentration of hydroxychloroquine (6×10^{-5} M) slightly increased maximal vasodilation induced by high-concentration (10^{-5} M) levcromakalim ($p < 0.05$ *vs*. control; Fig. 1B). Combination treatment with NAC (5×10^{-3} M) and chloroquine (6×10^{-5} M) increased levcromakalim-induced vasodilation compared with chloroquine (6×10^{-5} M) alone ($p < 0.001$ at 10^{-6} and 3×10^{-6} M levcromakalim; $p < 0.05$ at 10^{-5} M levcromakalim; Fig. 2A). Combination treatment with glibenclamide (5×10^{-6} M) and chloroquine (6×10^{-5} M) or glibenclamide (5×10^{-6} M) alone eliminated levcromakalim-induced vasodilation (5×10^{-6} M glibenclamide + 6×10^{-5} M chloroquine or 5×10^{-6} M glibenclamide alone: $p < 0.001$ *vs*. control at 10^{-7} to 10^{-5} M levcromakalim; Fig. 2B). Combination treatment with the lipid emulsion (1%) and chloroquine (6×10^{-5} M) did not significantly alter levcromakalim-induced vasodilation compared with chloroquine alone (Fig. 3). Chloroquine (6×10^{-5} M) increased vasodilation caused by diltiazem ($p < 0.001$ *vs*. control at 10^{-6} to 10^{-4} M diltiazem; Fig. 4A). However, chloroquine (6×10^{-5} M) had no effect on the vasodilation caused by sodium nitroprusside (Fig. 4B).

Levcromakalim (10^{-5} M) caused membrane hyperpolarization in the rat aortic VSMCs ($p < 0.001$ *vs*. control; Fig. 5). Chloroquine (3×10^{-5} M) and hydroxychloroquine (3×10^{-5} M) inhibited membrane hyperpolarization induced by levcromakalim (10^{-5} M) ($p < 0.01$: 3×10^{-5} M chloroquine + levcromakalim *vs*. levcromakalim alone; $p < 0.001$: 3×10^{-5} M hydroxychloroquine + levcromakalim *vs*. levcromakalim alone; Fig. 5).

Chloroquine (6×10^{-5} M) increased ROS production in the rat aortic VSMCs ($p < 0.001$ *vs*. control; Fig. 6A). However, pretreatment with NAC (5×10^{-3} M) inhibited ROS production induced by chloroquine (6×10^{-5} M) in the rat aortic VSMCs ($p < 0.001$ *vs*. chloroquine alone; Fig. 6A). In addition, hydroxychloroquine (6×10^{-5} M) also increased ROS production ($p < 0.001$ *vs*. control; Fig. 6B). Chloroquine (6×10^{-5} M) produced more ROS than hydroxychloroquine (6×10^{-5} M) ($p < 0.001$; Fig. 6B).

Discussion

This is the first study to suggest that chloroquine at toxic doses inhibits levcromakalim-induced vasodilation *via* ROS generation. The major findings of this study are as follows: 1) chloroquine inhibited levcromakalim-induced vasodilation to a greater extent than hydroxychloroquine, 2) the ROS

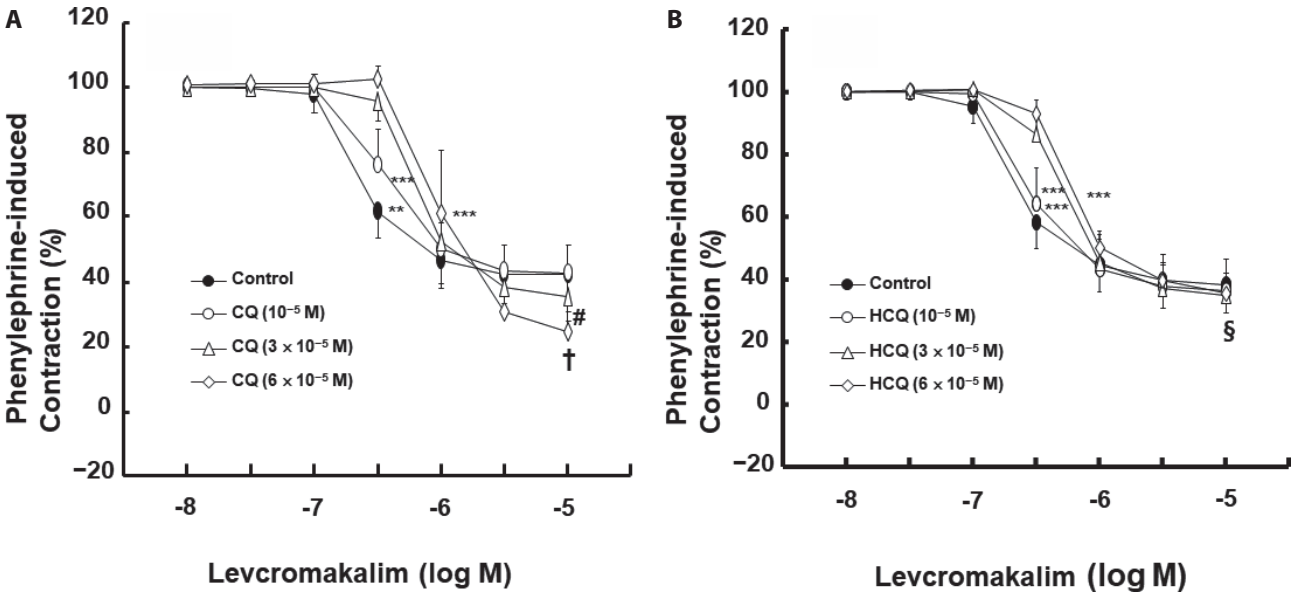

Figure 1. Effect of chloroquine (CQ, **A**) and hydroxychloroquine (HCQ, **B**) on levcromakalim-induced vasodilation in endothelium-denuded rat aortas. n = 13, 12, 7, and 6 for control, 10^{-5} M CQ, 3×10^{-5} M CQ, and 3×10^{-6} M CQ, respectively. n = 12, 12, 5, and 6 for control, 10^{-5} M HCQ, 3×10^{-5} M HCQ, and 3×10^{-6} M HCQ, respectively. Data are shown as the mean ± SD and expressed as the percentage of phenylephrine-induced contractions; n indicates the number of rats. Log ED_{50} of levcromakalim-induced vasodilation: ** $p < 0.01$, *** $p < 0.001$ *vs*. control. Maximal levcromakalim-induced vasodilation: § $p < 0.05$, $^{\#}$ $p < 0.01$, † $p < 0.001$ *vs*. control. Log ED_{50}, the logarithm of levcromakalim to produce half (50%) the amount of levcromakalim-induced maximal vasodilation.

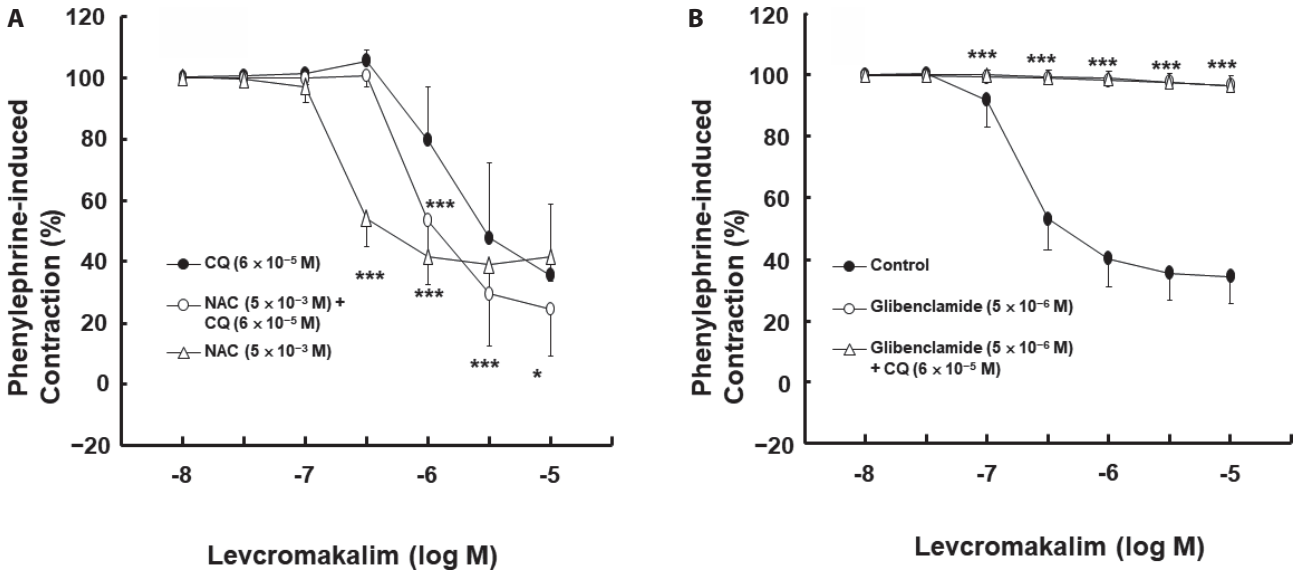

Figure 2. Effect of *N*-acetyl-L-cysteine (NAC, **A**), glibenclamide (**B**), and chloroquine (CQ), alone or in combination, on levcromakalim-induced vasodilation in the endothelium-denuded rat aorta. Data ($n = 6$) are shown as the mean ± SD and expressed as the percentage of phenylephrine-induced contractions; *n* indicates the number of rats. A: * $p < 0.05$, *** $p < 0.001$ *vs*. CQ alone. B: *** $p < 0.001$ *vs*. control.

scavenger NAC attenuated chloroquine-mediated inhibition of vasodilation induced by levcromakalim, and 3) NAC inhibited chloroquine-induced ROS production in vascular smooth muscle cells.

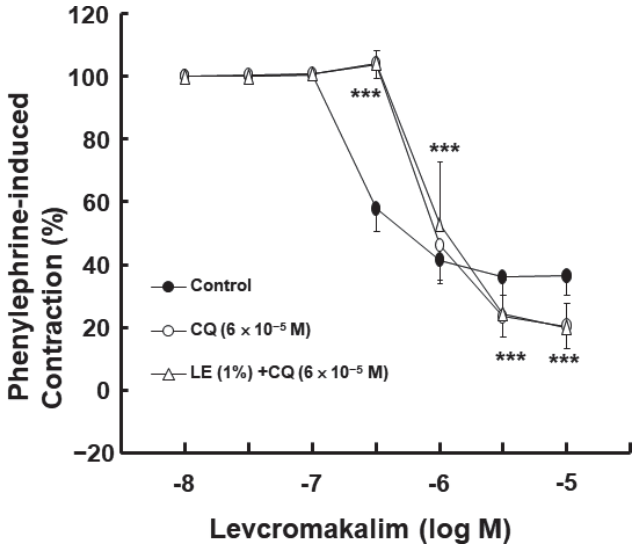

Figure 3. Effect of chloroquine (CQ) alone and in combination with lipid emulsion (LE) on levcromakalim-induced vasodilation in the endothelium-denuded rat aorta. Data ($n = 6$) are shown as the mean ± SD and expressed as the percentage of phenylephrine-induced contractions. *n* indicates the number of rats. *** $p < 0.001$ *vs*. control.

Chloroquine and hydroxychloroquine share certain pharmacologic characteristics; however, chloroquine exerts greater toxicity than hydroxychloroquine (Edwards et al. 1987; Jordan et al. 1999; Della Porta et al. 2020). Consistent with these reports, chloroquine and hydroxychloroquine inhibited vasodilation induced by the K_{ATP} channel agonist levcromakalim (Edwards et al. 1987; Jordan et al. 1999; Della Porta et al. 2020). However, in terms of the magnitude of inhibition of levcromakalim-induced vasodilation, chloroquine (10^{-5} M)-mediated inhibition was greater than hydroxychloroquine (10^{-5} M)-mediated inhibition. Oxidative stress, such as results from the production of superoxide anions (ROS), attenuates levcromakalim-induced vasodilation and membrane hyperpolarization of the artery and aorta (Kinoshita et al. 2004, 2006; Haba et al. 2009). In addition, excess superoxide has been reported to attenuate vasodilation induced by K_{ATP} channel openers in diabetic rats (Liu and Gutterman 2002). Chloroquine induces cardiotoxicity and ototoxicity *via* oxidative stress (Chaanine et al. 2015; Oliveira et al. 2019; Lee et al. 2022). In the present study, NAC attenuated the inhibitory effect of chloroquine on levcromakalim-induced vasodilation, consistent with previous reports (Kinoshita et al. 2004, 2006; Haba et al. 2009; Chaanine et al. 2015; Oliveira et al. 2019; Lee et al. 2022). Moreover, in agreement with the results from the isometric tension measurement, NAC inhibited ROS produced by toxic doses of chloroquine in VSMCs (Schulz and Schmoldt 2003). Collectively, the results of the present study and those of previous reports suggest that toxic doses of

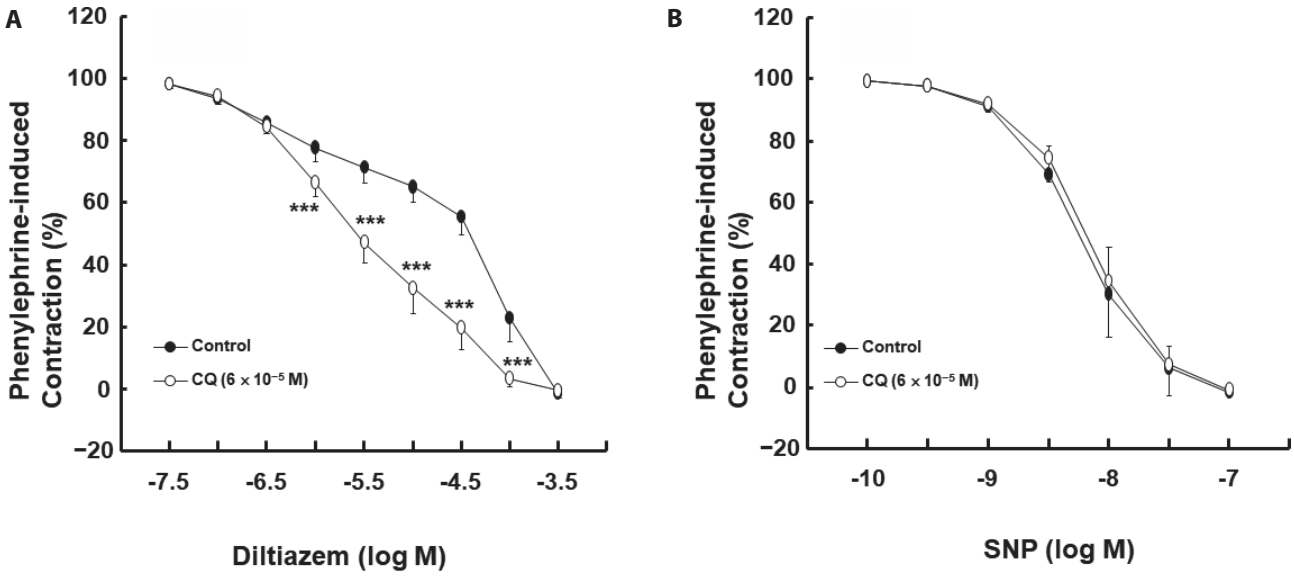

Figure 4. Effect of chloroquine (CQ) on vasodilation induced by diltiazem (**A**) and sodium nitroprusside (SNP, **B**) in the endothelium-denuded rat aorta. Data (n = 5) are shown as the mean ± SD and expressed as the percentage of phenylephrine-induced contractions; n indicates the number of rats. *** $p < 0.001$ *vs*. control.

chloroquine inhibit levcromakalim-induced vasodilation, which is partially mediated by ROS generation (Schulz and Schmoldt 2003; Kinoshita et al. 2004, 2006; Haba et al. 2009; Chaanine et al. 2015; Oliveira et al. 2019; Lee et al. 2022). In addition, as chloroquine-mediated ROS production was higher than that induced by hydroxychloroquine (Fig. 6B), the relatively high levels of inhibition of levcromakalim-induced vasodilation by chloroquine compared with those of hydroxychloroquine may be ascribed to increased ROS production. Further studies are needed to examine the cellular signaling pathways associated with chloroquine-induced ROS production in vascular smooth muscles. The K_{ATP} channel is made up of four inward rectifying potassium channel subunits and four regulatory sulfonylurea receptors (Brayden 2002). Pretreatment with glibenclamide, an inhibitor of the sulfonylurea receptor of K_{ATP} channels, nearly eliminated levcromakalim-induced vasodilation of endothelium-denuded aortas pretreated with or without

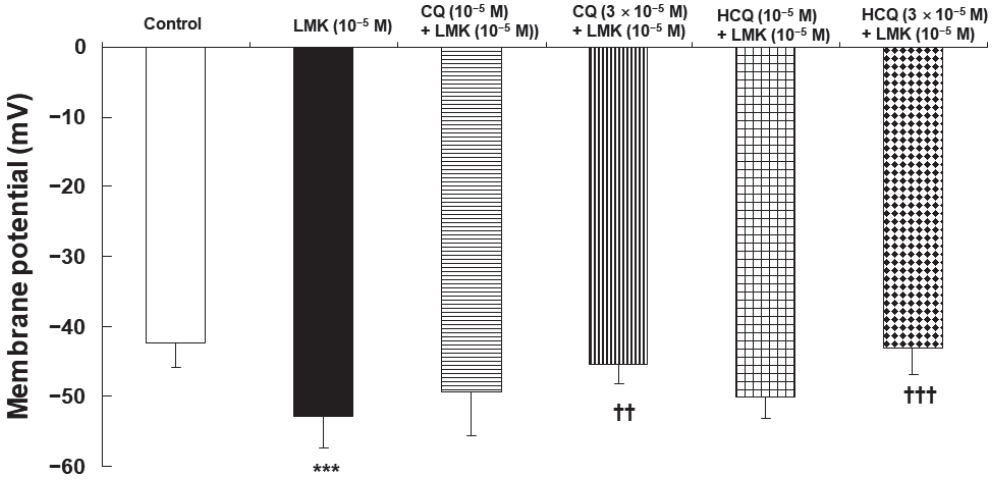

Figure 5. Restoration of membrane potential by high concentrations of chloroquine (CQ) and hydroxychloroquine (HCQ) in levcromakalim (LMK)-induced membrane hyperpolarization of vascular smooth muscle cells. Membrane potentials were recorded within 1 min after chemical treatment under current clamp mode (I = 0). Each bar represents the mean ± SD (n = 7). The values are from three independent experiments. *** $p < 0.001$ *vs*. control, †† $p < 0.01$ and ††† $p < 0.001$ *vs*. LMK alone.

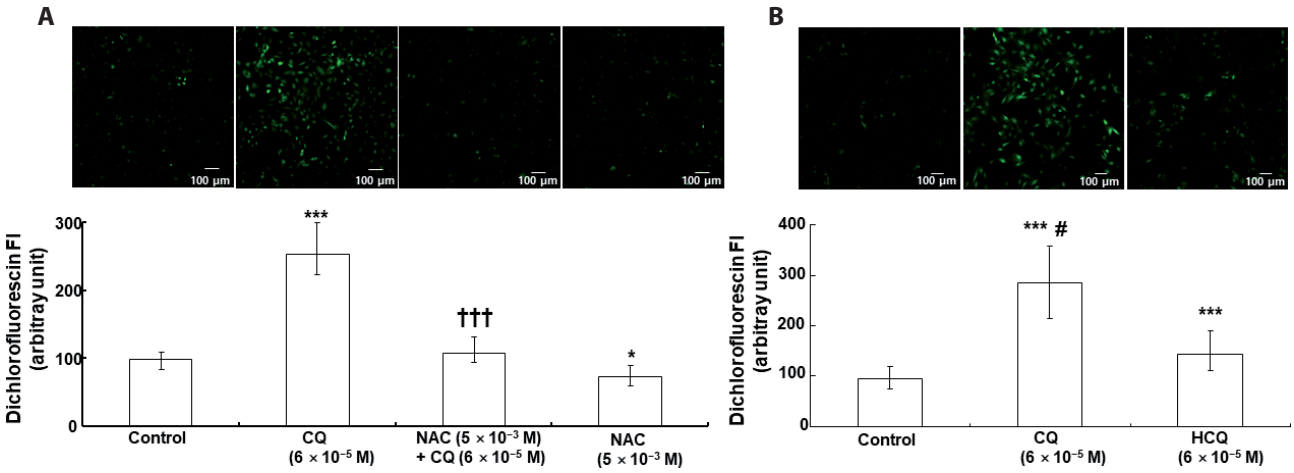

Figure 6. Effect of chloroquine (CQ) and *N*-acetyl-L-cysteine (NAC), alone or combined (**A**), and effect of CQ and hydroxychloroquine (HCQ) (**B**) on the reactive oxygen species (ROS) in the rat aortic vascular smooth muscle cells. Cellular ROS levels were measured using fluorescence microscopy after staining with the fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). Scale bar: 100 μm. Data (n = 3 and 4 for A and B, respectively) are shown as the median ± interquartile range (25 to 75%) and expressed relative to the control values; n indicates the number of independent experiments. A: * $p < 0.05$ and *** $p < 0.001$, *vs.* control. ††† $p < 0.001$, *vs.* CQ alone. B: *** $p < 0.001$, *vs.* control. # $p < 0.001$, *vs.* 6×10^{-5} M HCQ. FI, fluorescence intensity.

chloroquine (Fig. 2B). In addition, levcromakalim-induced membrane hyperpolarization in VSMCs was inhibited by toxic concentrations (3×10^{-5} M) of chloroquine and hydroxychloroquine (Fig. 5). Glibenclamide inhibited membrane hyperpolarization induced by levcromakalim in the vascular smooth muscle (Baik et al. 2016). Thus, chloroquine-mediated inhibition of vasodilation induced by levcromakalim appears to be mediated *via* inhibition of the sulfonylurea receptor in the K_{ATP} channels. However, the antimalarial drug mefloquine was reported to attenuate K_{ATP} channels through its interaction with the Kir6.2 subunit of K_{ATP} channels (Gribble et al. 2000). In addition, NAC nearly eliminated chloroquine-mediated ROS production (Fig. 6A) and attenuated the chloroquine-mediated inhibition of high concentration levcromakalim-induced vasodilation, but did not significantly attenuate chloroquine-mediated inhibition of low concentration (3×10^{-7} M) levcromakalim-induced vasodilation (Fig. 2A). Considering previous reports, mechanisms other than ROS production, such as the interaction with Kir6.2, may contribute to the chloroquine-mediated inhibition of levcromakalim-induced vasodilation (Gribble et al. 2000). Thus, further research is needed to examine these mechanisms, which may be partially associated with chloroquine-mediated inhibition of levcromakalim-induced vasodilation. Chloroquine increased vasodilation induced by the calcium channel blocker diltiazem and exerted no effect on vasodilation induced by the nitric oxide donor sodium nitroprusside; therefore, chloroquine-mediated inhibition of vasodilation induced by levcromakalim appears to be specific. Vasodilation induced by low concentrations of levcromakalim was inhibited by toxic doses of chloroquine and hydroxychloroquine; however, vasodilation induced by high concentrations of levcromakalim (10^{-5} M) was increased by toxic doses of chloroquine (3×10^{-5} and 6×10^{-5} M) or hydroxychloroquine (6×10^{-5} M) (Fig. 1). In addition, a toxic dose of chloroquine (10^{-3} M) has been reported to cause vasodilation of aorta precontracted with 140 mM KCl *via* the inhibition of voltage-dependent L-type calcium channels (Sai et al. 2014). Considering the current results and previous reports, as a toxic concentration of chloroquine (6×10^{-5} M) increased diltiazem-induced vasodilation (Fig. 4A), chloroquine (6×10^{-5} M)-mediated augmentation of vasodilation induced by high-concentration (10^{-5} M) levcromakalim may be associated with the further inhibition of voltage-dependent calcium channels by levcromakalim (10^{-5} M)-induced hyperpolarization (Sai et al. 2014; Jackson and Boerman 2018). Further studies are needed for examining the mechanisms underlying the augmenting effect of chloroquine and hydroxychloroquine on vasodilation induced by high-concentration levcromakalim.

When used in the treatment of systemic toxicity caused by local anesthetics, lipid emulsions absorb highly lipid-soluble drugs (log P > 2) (Lee and Sohn 2023). Furthermore, lipid emulsions have been reported to alleviate cardiovascular depression induced by toxic doses of chloroquine or hydroxychloroquine (Ten Broeke et al. 2016; Murphy et al. 2018; Bethlehem et al. 2019; Noda et al. 2021). In addition, lipid emulsions attenuate chloroquine-induced cardiac toxicity in cardiomyoblasts (Lee et al. 2022). On the basis of clinical case reports and previous studies,

we expected that lipid emulsions would attenuate the inhibitory effect of chloroquine, which is highly lipid-soluble (log P = 4.63), on levcromakalim-induced vasodilation *via* lipid emulsion-mediated absorption of chloroquine. However, the lipid emulsion used in the present study had no effect on chloroquine-mediated inhibition of vasodilation induced by levcromakalim. Thus, we surmise that lipid emulsions may have ameliorated chloroquine- and hydroxychloroquine-induced intractable cardiovascular depression, reported in the previous case reports, by attenuating cardiotoxicity caused by toxic doses of chloroquine or hydroxychloroquine (Ten Broeke et al. 2016; Murphy et al. 2018; Bethlehem et al. 2019; Noda et al. 2021). The present study has some limitations. Although organ blood flow is controlled by small resistance arterioles, the aorta, a large conduit artery, was used in the present study (Clifford 2011). Lipid emulsions are administered for cardiovascular collapse after drug toxicity; however, in the present study, it was administered as a pretreatment before treatment with a toxic dose of chloroquine. Despite these limitations, as ROS scavengers including NAC may attenuate the chloroquine-induced inhibitory effect on beneficial K_{ATP} channel-induced vasodilation, treatment of chloroquine toxicity with ROS scavengers may contribute to the preservation of beneficial K_{ATP} channel-induced vasodilation (Brayden 2002).

In conclusion, a toxic dose of chloroquine inhibited K_{ATP} channel-induced vasodilation in endothelium-denuded rat aorta, partially through ROS generation. These results suggest that lipid emulsions do not restore K_{ATP} channel-induced vasodilation inhibited by a toxic dose of chloroquine.

Conflicts of interest. There are no potential conflicts of interest relevant to this article.

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