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RESEARCH ARTICLE

Enhancing hippocampal blood flow after cerebral ischemia and vasodilating basilar arteries: *in vivo* and *in vitro* neuroprotective effect of antihypertensive DDPH

Li Sun[1, 2], Qin Li[3], Wei-ting Wang[2], Yu-hua Chen[1, *], Lian-jun Guo[2]

1 Department of Neurology, Wuhan Brain Hospital (General Hospital of the Yangtze River Shipping), Wuhan, Hubei Province, China
2 Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei Province, China
3 Department of Pharmacology, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

***Correspondence to:**
Yu-hua Chen, whchyh@163.com.

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Abstract

1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino)-propane hydrochloride (DDPH) is a novel antihypertensive agent based on structural characteristics of mexiletine and verapamine. We investigated the effect of DDPH on vasodilatation and neuroprotection in a rat model of cerebral ischemia *in vivo*, and a rabbit model of isolated basilar arteries *in vitro*. Our results show that DDPH (10 mg/kg) significantly increased hippocampal blood flow *in vivo* in cerebral ischemic rats, and exerted dose-dependent relaxation of isolated basilar arteries contracted by histamine or KCl in the *in vitro* rabbit model. DDPH (3×10^{-5} M) also inhibited histamine-stimulated extracellular calcium influx and intracellular calcium release. Our findings suggest that DDPH has a vasodilative effect both *in vivo* and *in vitro*, which mediates a neuroprotective effect on ischemic nerve tissue.

***Key Words:** nerve regeneration; DDPH; cerebral ischemia; hippocampus; blood flow; isolated basilar artery; dose-response curve; NSFC grant; neural regeneration*

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Introduction

1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) is a new antihypertensive agent based on structural characteristics of mexiletine and verapamine. In preliminary studies, we have demonstrated that DDPH reduces brain ischemia injury *via* an antioxidative effect (Qu et al., 2000, 2003; He et al., 2009), and has a protective effect on neuronal injury caused by acute ischemia in mice and rats (Li et al., 2001; Wang et al., 2001). Pharmacokinetics data shows that DDPH easily crosses the blood-brain barrier and reaches a relatively high concentration in the central nervous system (Wang et al., 2001). DDPH shows neuroprotective potential in ischemic rats following middle cerebral artery occlusion, and significantly reduces infarct volume, ameliorates histopathological damage, and diminishes oxidative stress (Qu et al., 2000; Wang et al., 2001). The hippocampus suffers greater damage from hypoxia and ischemia compared with the cortex. Therefore it is important to further examine the effect of DDPH on hippocampal blood flow after cerebral ischemia. In this study, we investigated the effect of DDPH on isolated basilar arteries, and the underlying mechanism on cerebral vessels.

Materials and Methods

Experimental animals

In total, 54 adult male Sprague-Dawley rats (weighing 200–250 g) and 30 New Zealand white rabbits (aged 7 months, weighing 1.5–2.5 kg, pathogen-free level) were obtained from the Experimental Animal Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei Province, China; license No. SCXK (E) 2010-0007). The animals were housed in cages with free access to water and food, a 12-hour light/dark cycle (07:00 lights off; 19:00 lights on), with controlled temperature (22 ± 1°C) and relative humidity (approximately 60%) conditions. Animals were habituated for 7 days prior to beginning the experiment. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and experiments were approved by the Review Committee for the Care and Use of Laboratory Animals of Tongji Medical College of Huazhong University of Science and Technology (China).

Monitoring hippocampal blood flow in cerebral ischemic rats

Rats were randomly assigned to one of three groups: sham, ischemia, or DDPH. Each group contained six rats. Rats were anesthetized by urethane (1.4 g/kg, intraperitoneal injection) and body temperature maintained at 37–38°C using a constant temperature water cycling system. Animals were mounted in a stereotaxic frame (SN-3; Narishige, Tokyo, Japan), and the skin and fascia were laterally retracted to expose the skull under sterile conditions. The tissue was covered with moist gauze throughout the surgical procedure. A recording electrode was positioned in the hippocampus under stereotactic guidance to record blood flow using a LS-III blood flow meter (Beijing Li Ke High Technology Co., Ltd., Beijing, China). Cerebral ischemia was induced by a combination of right common carotid artery occlusion and hemorrhagic hypotension with mean arterial blood pressure at 40 ± 2 mmHg. DDPH (10 mg/kg, dissolved in saline; Department of Organic Chemistry of China Pharmaceutical University, Nanjing, Jiangsu Province, China) was administrated by intravenous injection to the external jugular vein 30 minutes before establishing cerebral ischemia models. Hippocampal blood flow was monitored before cerebral ischemia, and at 10 and 30 minutes after cerebral ischemia.

Preparation of isolated basilar artery rings

Rabbits were anesthetized and sacrificed. Basilar arteries were carefully dissected from the brain and placed in 4°C Kreb's-Henseleit solution (including NaCl 118.0 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.18 mM, $NaCO_3$ 25 mM, and glucose 5.55 mM; saturated with 95% O_2 and 5% CO_2) (Li et al., 2011). Vessels were cut to 4 mm, fixed to two stainless steel holders and then immediately suspended in a 10 mL organ bath containing oxygenated Kreb's-Henseleit solution at 35°C, gassed with 95% O_2 and 5% CO_2 at pH 7.4. Basilar artery rings were equilibrated for 2 hours at an initial resting tension of 1 g. During this period, Kreb's-Henseleit solution in the bath was replaced every 15 minutes. DDPH at 3×10^{-7} M and 3×10^{-6} M was used (the concentration was defined in the pre-experiment).

DDPH effect on serotonin and histamine dose-response curves in isolated basilar artery rings

Histamine and serotonin (5-HT) induce vasoconstriction by interacting with the corresponding receptors. Histamine and 5-HT dose-response curves reflect the percentage of histamine or 5-HT dose to the maximal contractile response of histamine. DDPH affects histamine and 5-HT dose-response curves, and subsequently the vasoconstrictive effect of histamine. Basilar artery ring contraction was evoked by KCl. Once the contraction reached a plateau, DDPH (3×10^{-7} M and 3×10^{-6} M) was cumulatively added to the bath. Relaxation was expressed as the percentage of decreased maximal tension obtained by KCl-induced contraction. Ranitidine (a histamine receptor blocking agent) was added (1×10^{-4} M; The Third Pharmaceutical Factory of Guangzhou, Guangdong Province, China) before the rings were contracted by histamine (Sigma, St. Louis, CA, USA) to block histamine-2 receptors (Park et al., 2009).

DDPH was added 10 minutes before construction of the histamine dose-response curve. Results were expressed as the percentage of maximum contractile tension to histamine before and after DDPH pretreatment. DDPH or ketanserin (a 5-HT receptor blocking agent, Sigma; Larrauri and Levin, 2010) were added 10 minutes before construction of the 5-HT dose-response curve. Results were expressed as the percentage of maximum contractile tension to 5-HT before and after DDPH pretreatment. E_{max} (maximal effect) and pA_2' (negative logarithm molar concentration of the non-competitive antagonist when excitomotor maximal effect was reduced by half) were calculated.

DDPH effect on the 5-HT dose-response curve with and without calcium in isolated basilar artery rings

The role of Ca^{2+} channels in the vasorelaxant response to DDPH was examined using the previously described experimental protocol (Lam et al., 2008, 2010). Basilar artery rings were equilibrated in Ca^{2+}-free Kreb's-Henseleit solution, and washed three times with 10 minute intervals between each wash. Histamine (3×10^{-5} M) was added to induce contraction and then $CaCl_2$ (2.5 mM) to induce vasoconstriction. When maximum vasoconstriction was achieved, rings were washed and equilibrated for 30 minutes, and subsequently incubated with 3×10^{-5} M DDPH for 15 minutes. The vasoconstrictive effect of histamine and $CaCl_2$ was then repeated and compared against control curves obtained in the absence of these agents. In addition, 4×10^{-8} M nimodipine was applied as a calcium antagonist (Dong et al., 2010).

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed by repeated measures general linear modeling and *t*-tests. $P < 0.05$ was considered to be a significant difference. All data were calculated using Sigma Plot 10.0 software (Systat Software, Inc., San Jose, CA, USA).

Results

DDPH effect on blood flow in rat hippocampus after local cerebral ischemia *in vivo*

Compared with the sham group, blood flow in rat hippocampus significantly decreased 10 minutes after cerebral ischemia ($P < 0.05$), and was significantly lower at 30 minutes compared with 10 minutes after cerebral ischemia ($P < 0.05$). Compared with the ischemia group, blood flow increased after DDPH intervention (10 mg/kg) at 10 and 30 minutes after cerebral ischemia ($P < 0.05$; **Figure 1**).

Vasodilative effect of DDPH on isolated basilar arteries contracted by histamine and KCl

DDPH caused vasorelaxant effects on histamine-contracted isolated basilar artery rings in a dose-dependent manner (**Figure 2A**). The relaxation IC_{50} of DDPH to rings contracted by histamine (3×10^{-5} M) was 1.995×10^{-5} M (**Figure 2B**), and to rings contracted by KCl (80 mM) was 4.677×10^{-6} M (**Figure 2C**).

DDPH effect on the 5-HT dose-response curve in isolated basilar arteries

To examine the vasodilative mechanism of DDPH, we performed several experiments based on contracting isolated basilar artery ring preparations with increasing 5-HT concentrations, with or without DDPH. The 5-HT dose-response curve was significantly shifted to the right in a non-parallel manner by DDPH (3×10^{-7} M and 3×10^{-6} M), with E_{max} decreased ($P < 0.05$; **Figure 3**). The pA_2' value of DDPH was 5.69, and the E_{max} 5-HT dose-response curve decreased by 15.6% and 55.3% in the presence of DDPH (3×10^{-7} M and 3×10^{-6} M, respectively). Ketanserin produced a parallel rightward-shift of the 5-HT dose-response curve without altering the maximal response (data not shown).

DDPH effect on the histamine dose-response curve in isolated basilar arteries

We also examined the effect of contracting isolated ring preparations using increasing histamine concentrations, with or without DDPH. The histamine dose-response curve was significantly shifted to the right in a non-parallel manner by DDPH (5×10^{-6}, 5×10^{-5}, and 5×10^{-4} M) with E_{max} decreased ($P < 0.05$; **Figure 4**). The pA_2' value of DDPH was 4.13.

DDPH effect on histamine-induced contraction with and without calcium

The following studies were performed in Ca^{2+}-free preparations. Priming with 3×10^{-5} M histamine induced transient contraction, and subsequent addition of $CaCl_2$ (2.5 mM) caused stepwise increases in blood vessel tone. DDPH (3×10^{-5} M) inhibited both histamine-stimulated contraction in Ca^{2+}-free solution and contraction elicited by $CaCl_2$ (**Figure 5A**).

In the presence of 3×10^{-5} M DDPH, contraction elicited by histamine in Ca^{2+}-free solution was attenuated by 47.8% ($P < 0.05$), while contraction elicited by $CaCl_2$ was attenuated by 41.0% ($P < 0.05$). In the presence of 4×10^{-8} M DDPH, contraction elicited by histamine in Ca^{2+}-free solution was attenuated by 53.5% ($P < 0.05$), while contraction elicited by $CaCl_2$ was attenuated by 58.0% ($P < 0.05$) (**Figure 5B**).

Discussion

In the present study, DDPH increased hippocampal blood flow in rats following acute brain ischemia, and inhibited histamine-, KCl-, and 5-HT-induced contraction in rabbit basilar artery rings. This vasorelaxant effect on isolated basilar arteries may have been obtained by modifying Ca^{2+}-dependent mechanisms.

The hippocampus is a vulnerable and plastic brain structure that can be injured by various stimuli (Dhikav and Anand, 2011), such as hypoxia and hypoperfusion. Thus, studies examining the effect of DDPH on hippocampal blood flow after cerebral ischemia are of interest. Compared with the ischemia group, blood flow increased in the presence of DDPH (10 mg/kg) at 10 and 30 minutes after cerebral ischemia, demonstrating that hippocampal blood flow increases with DDPH treatment after cerebral ischemia. Hence, further study examining the vasodilative mechanism of DDPH is relevant.

Next, we demonstrated that DDPH is a potent vasodilator of the rabbit basilar artery, the principal vessel supplying the cerebellum, brain stem, and other encephalic regions. The histamine dose-response curve was shifted to the right in the presence of 5×10^{-6}, 5×10^{-5} , and 5×10^{-4} M DDPH, thereby demonstrating relaxation. Maximal contraction induced by histamine was decreased with DDPH treatment. These results suggest that DDPH at 5×10^{-6}, 10^{-5}, and 10^{-4} M inhibited histamine-induced contraction through a non-competitive smooth muscle relaxant mechanism (Ye et al., 1997). In our previous study, we added ranitidine before contracting rings using histamine, to block histamine-2 receptors (Ye et al., 1997). We also confirmed that basilar artery contraction caused by histamine is blocked by treatment with the H1 receptor antagonist, diphenhydramine. Therefore, DDPH at $\geq 5 \times 10^{-6}$ M may possibly interact with H1 receptors and antagonize H1 receptor-mediated responses in basilar artery smooth muscle. Furthermore, the relaxation IC_{50} of DDPH on histamine-contracted rings is 1.995×10^{-5} M, while the relaxation IC_{50} of diphenhydramine and nimodipine are 3.310×10^{-7} and 3.240×10^{-8} M, respectively. Thus, the vasodilative effect of DDPH on histamine-contracted rings is 60 times less than diphenhydramine, and 600 times less than nimodipine.

Our results clearly show that 5-HT-induced contraction is competitively blocked by the 5-HT_{2A} receptor antagonist, ketanserin. Ketanserin produced a parallel rightward-shift of the 5-HT dose-response curve without altering the maximal response. Therefore, DDPH at $\geq 3 \times 10^{-7}$ M may possibly interact with 5-HT_{2A} receptors and antagonize 5-HT_{2A} receptor-mediated responses in basilar artery smooth muscles.

It is reasonable to assume that direct inhibition of Ca^{2+} influx in vascular smooth muscle cells may contribute to the vasorelaxant effect of DDPH. We tested this assumption in basilar artery rings bathed in Ca^{2+}-free buffer and primed with 3×10^{-5} M histamine. Histamine elicited vasoconstriction in Ca^{2+}-free buffer, confirming involvement of intracellular calcium release in contractile responses to histamine. Subsequent $CaCl_2$ addition also elicited vasoconstriction in basilar artery ring preparations. Contraction was attenuated by nimodipine, a typical calcium channel blocker, supporting the role of calcium channels in contractile responses to histamine. Furthermore, DDPH inhibited vasoconstriction induced by histamine in Ca^{2+}-free buffer, indicating that inhibition of intracellular calcium release plays an important role in its vasorelaxant effect. In addition, $CaCl_2$-induced vasoconstriction was ameliorated by DDPH.

In the present study, we have shown that contractile responses to histamine and 5-HT are attenuated by DDPH, evidenced by right-shifted dose-response curves to each contractile agent, and depressed maximal responses to each agonist in the presence of DDPH. Our finding that DDPH relaxed contractions induced by either histamine or KCl, suggests that DDPH has multiple actions, as these two contractile agents induce vascular smooth muscle contraction by two separate mechanisms: histamine-induced contraction is produced by activating histamine receptors on the vascular smooth muscle membrane, leading to mobilization of

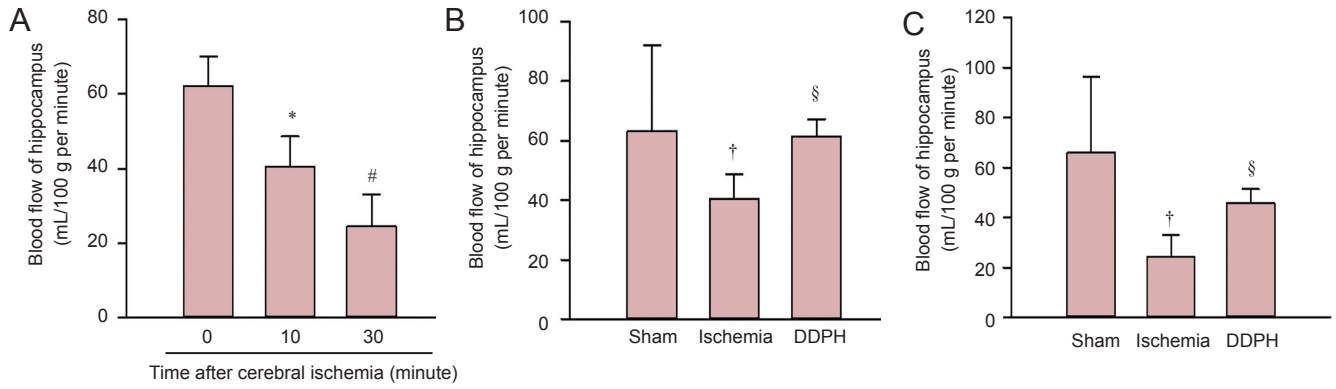

Figure 1 1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) effect on hippocampal blood flow after cerebral ischemia in rats.
(A) Hippocampal blood flow at 10 and 30 minutes after cerebral ischemia. (B) Comparison of hippocampal blood flow between the three groups at 10 minutes after cerebral ischemia. (C) Comparison of hippocampal blood flow between the three groups at 30 minutes after cerebral ischemia. Data are expressed as the mean ± SD ($n = 6$ rats in each group at each time point), and were analyzed by repeated measures general linear modeling and *t*-tests. *$P < 0.05$, *vs.* 0 minute; #$P < 0.05$, *vs.* 10 minutes; †$P < 0.05$, *vs.* sham group; §$P < 0.05$, *vs.* ischemia group.

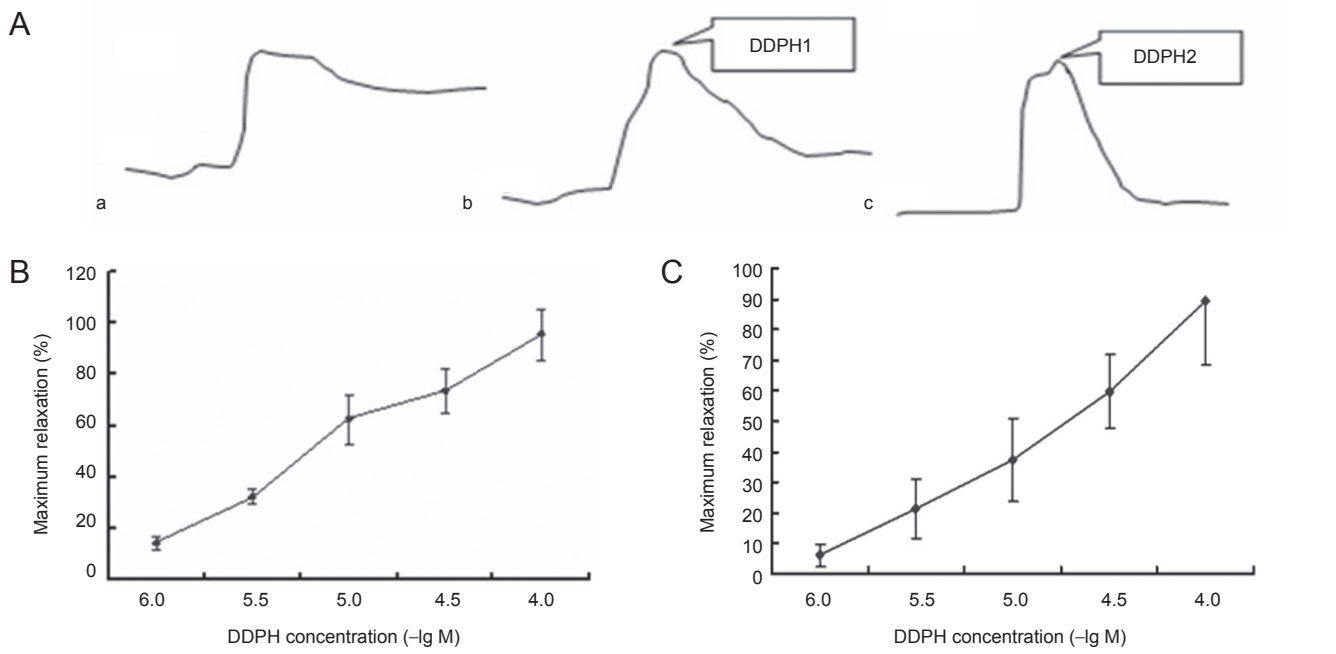

Figure 2 1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) relaxation of isolated basilar artery rings in rabbits.
(A) Original drawings of the DDPH effect on relaxation of isolated basilar artery rings in rabbits. a: Control, b: DDPH 5×10^{-5} M, c: DDPH 1×10^{-4} M. (B) Dose-dependent vasodilative effect of DDPH on isolated rings contracted by histamine. (C) Dose-dependent vasodilative effect of DDPH on isolated rings contracted by KCl. Data are expressed as the mean ± SD ($n = 8$ rabbit isolated basilar artery rings in each group), and were analyzed by repeated measures general linear modeling and *t*-tests.

extracellular and intracellular Ca^{2+} pools, while KCl-induced contraction is produced by membrane depolarization, which induces increased Ca^{2+} influx through voltage-dependent calcium channels (Ebeigbe, 1982). DDPH induced comparable relaxation responses in contractions produced by either agonist, suggesting that DDPH blocks Ca^{2+} influx by intervening in both receptor- and voltage-operated channels.

In conclusion, DDPH has a significant effect on increasing hippocampal blood flow after cerebral ischemia. Furthermore, our *in vitro* study provides evidence for a direct dilatory effect of DDPH on vascular smooth muscle. Thus, our results demonstrate that DDPH can act as an alternative option in treatment of cerebrovascular insufficiency states.

Author contributions: *LS performed the research and wrote the paper. QL provided assistance in writing the paper. WTW performed partial research. YHC and LJG designed the research and revised the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

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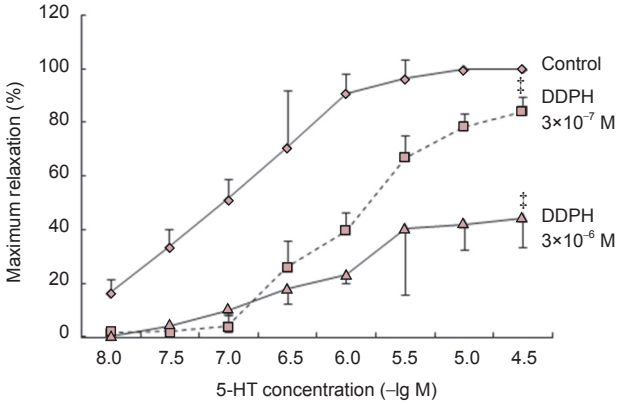

Figure 3 Effect of 1-(2,6-dimethylphenoxy)-2-(3, 4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) concentration on the serotonin (5-HT) dose-response curve in isolated basilar artery rings *in vitro*.

Data are expressed as the mean ± SD (n = 8 rabbit isolated basilar artery rings in each group), and were analyzed by repeated measures general linear modeling and t-tests. ‡$P < 0.05$, *vs.* control group.

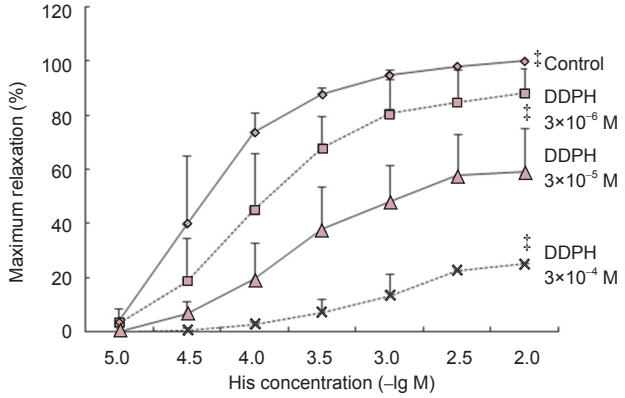

Figure 4 Effect of 1-(2,6-dimethylphenoxy)-2-(3, 4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) concentration on the histamine (His) dose-response curve of isolated basilar artery rings *in vitro*.

Data are expressed as the mean ± SD (n = 8 rabbit isolated basilar artery rings in each group), and were analyzed by repeated measures general linear modeling and t-tests. ‡$P < 0.05$, *vs.* control group.

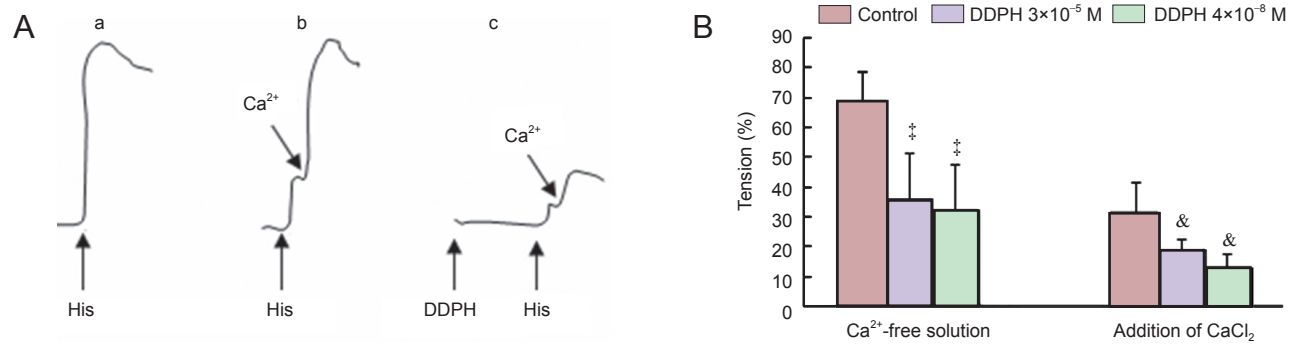

Figure 5 1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) effect on histamine (His)-induced contraction with and without calcium.

(A) DDPH effect on His-induced contraction in Ca^{2+}-free buffer solution and subsequent $CaCl_2$-induced stepwise contraction. a: Normal control (only His), b: Ca^{2+}-free buffer solution + His, then $CaCl_2$ addition; c: pretreatment with DDPH, then repeat b. (B) Basilar artery rings were pre-incubated with 3×10^{-5} M DDPH and 4×10^{-8} M nimodipine for 15 minutes before His-priming, and subsequent $CaCl_2$ treatment. DDPH inhibited His-induced contraction in Ca^{2+}-free buffer solution and subsequent $CaCl_2$-induced stepwise contraction. Data are expressed as the mean ± SD (n = 6 rabbit isolated basilar artery rings in each group), and were analyzed by repeated measures general linear modeling and t-tests. ‡$P < 0.05$, *vs.* control group; &$P < 0.05$, *vs.* Ca^{2+} free solution.

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