

# Vasodilatory mechanisms of unoprostone isopropyl in isolated porcine retinal arterioles

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**Purpose:** To clarify the vasodilatory mechanism of unoprostone isopropyl (UI), we examined its effects on the retinal microvascular diameter to determine the dependence on the endothelium and/or smooth muscle to reveal the signaling mechanisms involved in this vasomotor activity.

**Methods:** Porcine retinal arterioles were isolated, cannulated, and pressurized without flow in vitro. Video microscopic techniques recorded the diametric responses to UI.

**Results:** The retinal arterioles dilated in response to UI in a dose-dependent (100 pM-10  $\mu$ M) manner. The nitric oxide (NO) synthase inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) inhibited UI-induced vasodilation. The large-conductance Ca<sup>2+</sup>-activated K channel (BK<sub>Ca</sub> channel) blocker iberiotoxin also inhibited UI-induced vasodilation. The residual vasodilation after L-NAME was eliminated with co-administration of iberiotoxin.

**Conclusions:** UI elicits dilation of the retinal arterioles mediated by NO release and BK<sub>Ca</sub> channel activation.

Unoprostone isopropyl (UI), a prostaglandin F<sub>2a</sub>-related compound, has been introduced recently to treat glaucoma [1]. This drug is thought to reduce intraocular pressure by increasing conventional or uveoscleral outflow [2]. Recently, UI was also reported to affect the ocular blood flow [3-6] and retinal microcirculation in vivo [6] and in vitro [7]. These reports supported the idea that topical UI may increase ocular blood flow. Because we recently reported that retinal blood flow (RBF) is impaired in patients with type 2 diabetes mellitus with mild and no diabetic retinopathy (DR) [8], topical UI may be useful as a novel treatment of DR by improving retinal circulation in patients with diabetes. However, the underlying mechanisms of the effect of UI on retinal circulation remain unclear. In the current study, we examined the effect of UI on retinal microvascular diameter using an isolated vessel technique to exclude the confounding effects of metabolic, hemodynamic, humoral, and glial/neuronal factors associated with in vivo experiments.

## METHODS

**Animal preparation:** The Animal Care Committee of Asahikawa Medical University approved all animal procedures, which were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes were enucleated immediately from pigs of either sex

(age, 16–24 weeks; weight, 10–15 kg) after they were killed in a local slaughterhouse; the eyes were transported to the laboratory in a moist chamber on ice.

**Isolation and cannulation of microvessels:** The techniques for identifying and isolating retinal microvessels have been described previously [9-13]. Briefly, the isolated retinal arterioles (90–130  $\mu$ m in situ) were cannulated with a pair of glass micropipettes and pressurized to 55 cmH<sub>2</sub>O (~40 mmHg) intraluminal pressure without flow using two independent pressure reservoir systems [14]. The vasomotor activity of the isolated vessels was continuously recorded throughout the experiments via videomicroscopic techniques [9].

**Control experiment:** Cannulated, pressurized arterioles were bathed in physiologic salt solution (PSS) with albumin (0.1%) at 36 to 37 °C to allow development of stable basal tone; after about 30 to 40 min, concentration-dependent vasodilation developed in response to UI (100 pM-10  $\mu$ M). After measurement of the control dose response of UI without drugs, the vessels were washed with PSS to allow redevelopment of the basal tone. In some studies, the vasodilation elicited by UI was re-examined after 30 min to confirm the reproducibility of the response (n=6).

**Mechanistic studies of UI-induced dilation:** In the first series of studies, we examined the role of the endothelium in UI-induced dilation by comparing the responses before and after removal of the endothelium via intraluminal perfusion of the nonionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulfonate (CHAPS; 0.4%) as described previously [13]. We also assessed the role of endothelium-derived relaxing factors (EDRF), i.e., prostaglandins (PG),

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**TABLE 1. RESTING DIAMETERS AND DIAMETRIC RESPONSES OF RETINAL ARTERIOLES TO SNP.**

| Variations           | n  | Resting diameter | Sodium nitroprusside ( $\mu\text{M}$ ) |                |                |                |
|----------------------|----|------------------|--|----------------|----------------|----------------|
|                      |    |                  | 0.1                                    | 1              | 10             | 100            |
| Control              | 20 | 63.5 $\pm$ 2.3   | 4.4 $\pm$ 1.2                          | 15.3 $\pm$ 3.2 | 50.1 $\pm$ 3.4 | 88.3 $\pm$ 2.5 |
| denudation           | 4  | 57.4 $\pm$ 2.4   | 4.1 $\pm$ 2.2                          | 16.3 $\pm$ 3.3 | 52.8 $\pm$ 6.3 | 88.8 $\pm$ 2.2 |
| L-NAME               | 4  | 59.2 $\pm$ 6.5   | 4.2 $\pm$ 2.6                          | 18.5 $\pm$ 5.5 | 51.3 $\pm$ 3.3 | 86.8 $\pm$ 3.3 |
| TEA                  | 4  | 64.6 $\pm$ 5.4   | 5.2 $\pm$ 3.5                          | 16.7 $\pm$ 3.0 | 53.2 $\pm$ 5.8 | 90.2 $\pm$ 4.2 |
| Iberiotoxin          | 4  | 60.5 $\pm$ 4.8   | 5.5 $\pm$ 2.5                          | 16.2 $\pm$ 2.3 | 47.7 $\pm$ 5.2 | 88.7 $\pm$ 2.8 |
| Iberio-toxin +L-NAME | 4  | 58.9 $\pm$ 4.4   | 4.5 $\pm$ 3.4                          | 17.4 $\pm$ 1.8 | 43.5 $\pm$ 3.2 | 84.4 $\pm$ 5.3 |

Data are expressed as the mean percentage of maximal dilation  $\pm$  standard error of the mean. n, number of vessels. There are no significant changes in the resting diameters. Based on two-way ANOVA, the responses to sodium nitroprusside (SNP) are unaffected by any perturbations compared with the control. L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; TEA, tetraethylammonium.

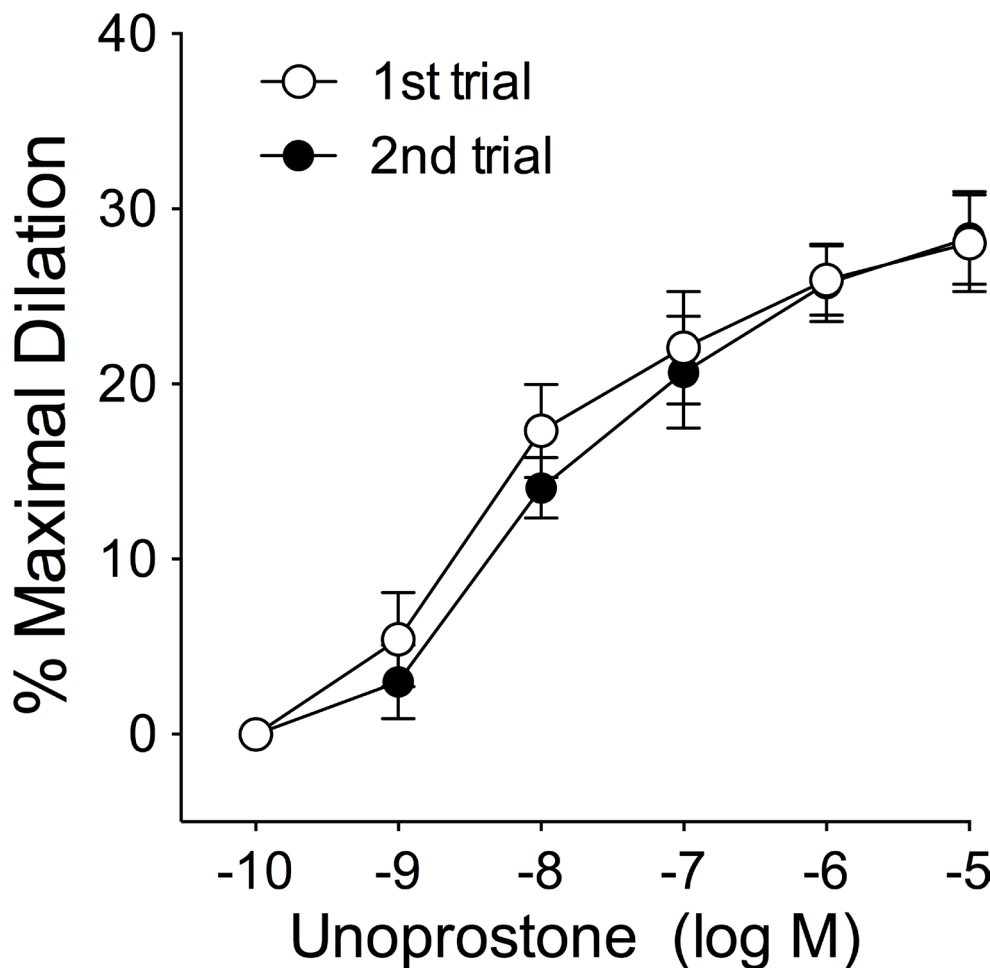


Figure 1. Dilatation as a function of the unoprostone isopropyl concentration. There is no significant difference between the two repeated trials (n=6).

nitric oxide (NO), and cytochrome P450 metabolites, in mediating the vascular response in the presence of known effective concentrations of the specific enzyme inhibitors indomethacin (10  $\mu$ M) [10,15], N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 10  $\mu$ M) [9,10], and sulfaphenazole (10  $\mu$ M) [16], respectively. To study the role of the PGI<sub>2</sub> receptor (IP) on UI-induced dilation, we assessed the arterioles pre-incubated with the IP antagonist CAY10441 (0.1  $\mu$ M) [17]. To study the involvement of the K channels, we examined these pathways by treating the vessels with K channel inhibitors, i.e., the nonselective K channel blocker tetraethylammonium (TEA; 10 mM) [18] and the large-conductance Ca<sup>2+</sup>-activated K channel (BK<sub>ca</sub> channel) blocker iberiotoxin (0.1  $\mu$ M) [12,19-21].

**Response to sodium nitroprusside:** Sodium nitroprusside (SNP; 0.1  $\mu$ M-100  $\mu$ M) was used to probe endothelium-independent NO-mediated vasodilation. The vascular response to SNP was examined in the presence of various interventions (Table 1).

All drugs were administered extraluminally unless otherwise stated. Each pharmacologic inhibitor was incubated with the vessels for at least 30 min.

**Chemicals:** All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except UI and CAY10441, which were provided by R-Teck Ueno, Ltd. (Tokyo, Japan) and Cayman Chemical (Ann Arbor, MI), respectively. L-NAME, TEA, iberiotoxin, and SNP were dissolved in PSS. Indomethacin and sulfaphenazole were dissolved in ethanol. UI was dissolved in dimethyl sulfoxide (DMSO). Subsequent dilutions of these drugs were prepared in PSS. The final concentration of ethanol or DMSO in the vessel bath was 0.1% [10]. Vehicle control studies indicated that this final concentration of solvent had no effect on arteriolar function.

**Data analysis:** At the end of each experiment, the vessels were relaxed in EDTA (1 mM) calcium-free PSS to obtain the maximal diameter at 55 cmH<sub>2</sub>O intraluminal pressure [9,11]. The diametric changes in response to UI and SNP were normalized to the resting diameters and expressed as the percent changes in diameter compared to the maximal

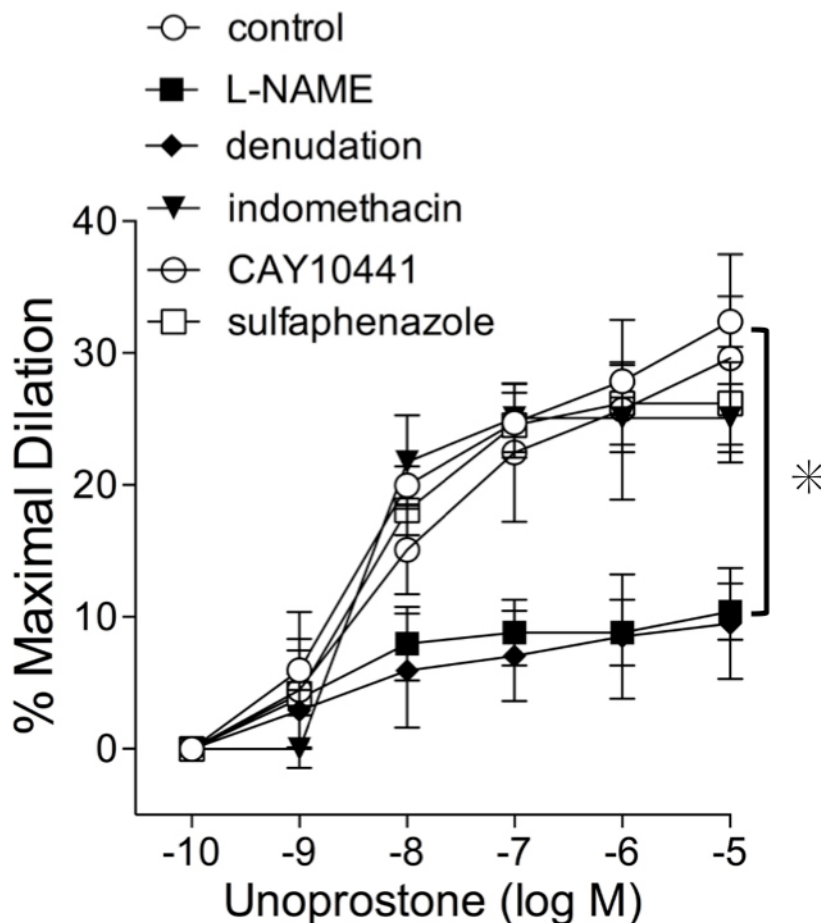


Figure 2. Mechanism of unoprostone isopropyl -mediated endothelium-dependent vasodilation. Role of the endothelium and endothelium-derived relaxing factors in retinal arteriolar dilation in response to unoprostone isopropyl (UI). The effects of endothelium removal, incubation with NG-nitro-L-arginine methyl ester (L-NAME) (10  $\mu$ M, n = 4), indomethacin (10  $\mu$ M, n = 4), sulfaphenazole (10  $\mu$ M, n = 4), and CAY10441 (0.1  $\mu$ M, n = 4). Incubation with L-NAME significantly reduced vasodilation in response to UI, which was comparable to that produced by denudation, but not indomethacin, sulfaphenazole, or CAY10441. \*p<0.05 versus control.

dilation [9,11]. The data are expressed as the mean  $\pm$  standard error of the mean, and *n* represents the number of vessels studied. Statistical comparisons of the changes in resting tone caused by antagonists were performed using the non-parametric Wilcoxon signed-rank test. Two-way ANOVA, followed by the Bonferroni multiple-range test, was used to determine the significance of the difference between control and experimental interventions.  $p < 0.05$  was considered significant.

### RESULTS

**Dilation of retinal arterioles induced by UI:** The basal tone in all vessels ( $n=38$ ) ranged from 53% to 78% (average,  $\sim 59\% \pm 4\%$ ) of their maximal diameters (Table 1). The average resting and maximal vessel diameters were  $57 \pm 6$  and  $94 \pm 5 \mu\text{m}$ , respectively. There were no significant changes in the resting tone after any interventions. UI induced consistent concentration-dependent dilation of the retinal arterioles. The highest concentration (10  $\mu\text{M}$ ) elicited about 30% of the maximal dilation (Figure 1). Further study showed that UI-induced dilation was reproducible and did not deteriorate after repeated applications (Figure 1).

**Role of EDRF:** In the denuded vessels ( $n=4$ ), the UI-induced dilation decreased partly and the response to the highest UI concentration decreased significantly ( $p < 0.01$ ) from 30% to 10% (Figure 2). The NOS inhibitor L-NAME significantly ( $p < 0.001$ ; Figure 2) reduced UI-induced vasodilation, which was comparable to that produced by denudation (L-NAME versus denudation,  $p > 0.05$ ). In contrast, the inhibition of cytochrome P450 epoxygenase and prostaglandins by sulfa-phenazole and indomethacin, respectively, did not affect the vasodilatory responses to UI. Blockage of the IP receptor by CAY10441 did not affect the vasodilatory response to UI (Figure 2).

**Role of K channels:** TEA significantly ( $p < 0.01$ ) inhibited UI-induced vasodilation of the retinal arterioles (Figure 3). In addition, iberiotoxin attenuated UI-induced dilation of the retinal arterioles in a manner similar to that of TEA. The residual vasodilation in the presence of iberiotoxin was almost eliminated with subsequent L-NAME treatment (Figure 4).

**Response to SNP-induced dilation:** Various interventions (denudation, co-incubated with L-NAME, TEA, iberiotoxin, iberiotoxin +L-NAME ) did not affect the SNP-induced

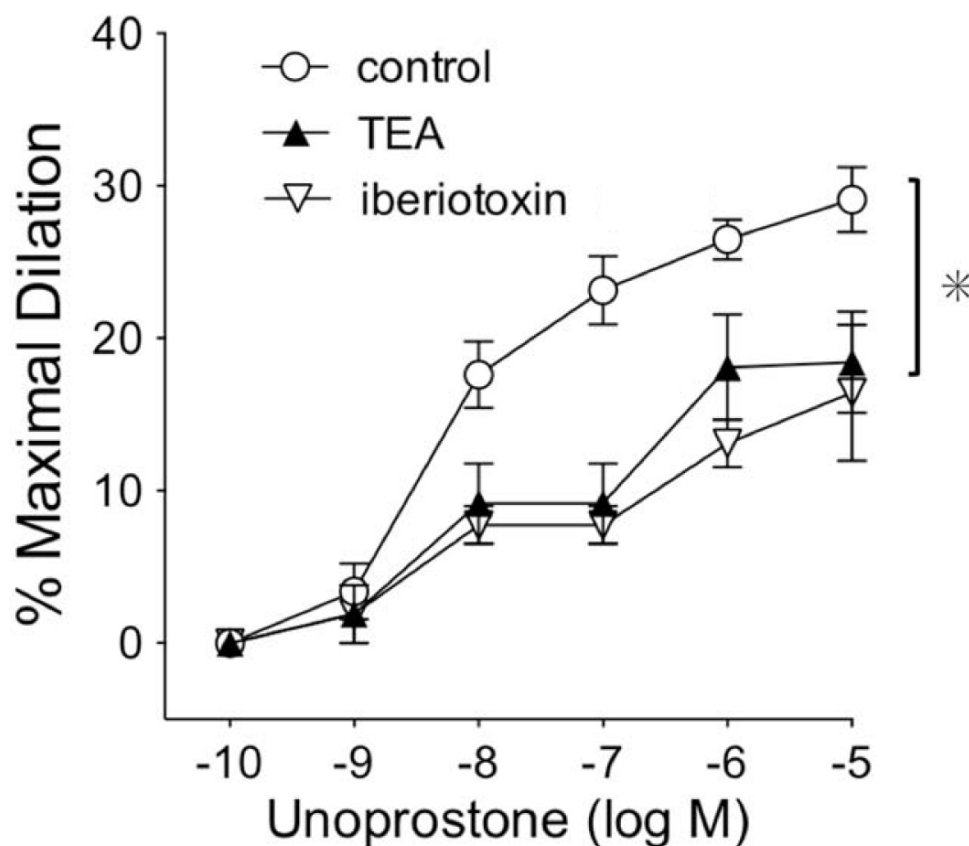


Figure 3. Role of the K channel in retinal arteriolar dilation in response to unoprostone isopropyl (UI). The K channel in retinal arteriolar dilation in response to unoprostone isopropyl (UI), tetraethylammonium (TEA; 10 mM,  $n = 4$ ), and iberiotoxin (0.1  $\mu\text{M}$ ,  $n = 4$ ) reduces vasodilation in response to UI. \* $p < 0.05$  versus control.

dilation of the retinal arterioles ( $88.3 \pm 2.5$  versus  $88.8 \pm 2.2$ ,  $86.8 \pm 3.3$ ,  $90.2 \pm 4.2$ ,  $88.7 \pm 2.8$ ,  $88.7 \pm 2.8$   $\mu\text{m}$ ; Table 1).

Based on two-way analysis of variance, the responses to SNP were unaffected by any perturbations compared with the control.

### DISCUSSION

The current study showed that UI induced concentration-dependent vasodilation of isolated porcine retinal arterioles (Figure 1). These results seem to be comparable to those in the ciliary arteries in rabbits [22,23] and mice [24]. Yu et al. reported that, in the retinal arterioles, UI caused vasodilation only in endothelin-1 (ET-1)-pre-contracted arterioles but did not change the retinal vessel diameter in normal-tone arterioles without ET-1 pre-contraction [7]. Although the authors did not provide a definitive explanation for this discrepancy, the differences between their finding in normal-tone vessels without ET-1 and ours in spontaneous tone vessels without no pre-constrictive drug might be due to differences in experimental protocols with or without flow to the vessel. Indeed, UI also was reported to increase ocular blood flow in vivo [4,5]. These previous in vivo findings seem to be comparable to ours.

It is unlikely that UI can reach the retinal artery after penetrating the cornea or sclera because UI is metabolized to M1 and M2 while penetrating the cornea [25]. However, Watanabe et al. reported that the concentration of  $^3\text{H}$ -UF-021 (UI) increased in various ocular tissues after topical application [26]. In their study, the concentration of  $^3\text{H}$ -UF-021 was 153 ng/g of wet tissue in the retina and choroid and 500 ng/g of wet tissue in the posterior sclera. Because the molecular weight of UI is 424.62 [27], the concentrations of UI (100 pM-10  $\mu\text{M}$ ) used in the present study seem to induce vasodilation in the retinal artery and increase RBF. Further clinical study is warranted to investigate whether topical administration of UI increases RBF in patients with retinal vascular disorders, i.e., DR and retinal vein occlusion.

Although previous studies have reported conflicting results that UI-induced vasodilation is endothelium-dependent [7] or endothelium-independent [22,23], we for the first time observed the inhibitory effects of NOS blockade by L-NAME on UI-induced vasodilation (Figure 2). We also confirmed that removal of the endothelium by CHAPS [13] decreased the unoprostone-induced vasodilation of the retinal arterioles, which is comparable to L-NAME (Figure 2). Taken together, our findings suggested that UI may cause

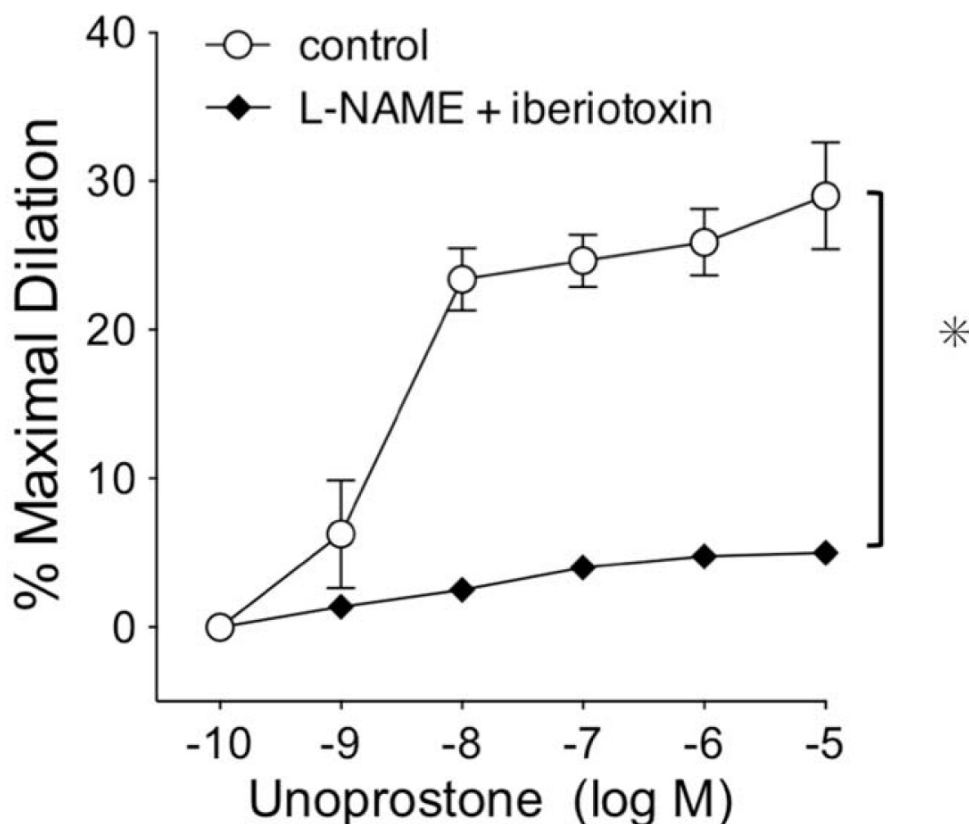


Figure 4. Mechanism of unoprostone isopropyl-mediated vasodilation. Co-incubation with NG-nitro-L-arginine methyl ester (L-NAME; 10  $\mu\text{M}$ ) and iberiotoxin (0.1  $\mu\text{M}$ , n = 4) almost eliminated the retinal arteriolar dilation in response to unoprostone isopropyl. \* $p < 0.05$  versus control.

vasodilation via NO production from the endothelium in the retinal arterioles.

In contrast to L-NAME, the vasodilatory response to UI was unaffected by inhibition of prostaglandin synthesis, i.e., the specific PGI<sub>2</sub> receptor blocker CAY10441 [17] and the cytochrome P450 metabolite by pretreatment with indomethacin and sulfaphenazole (Figure 2). These results indicated that UI-induced vasodilation of the retinal arterioles was mediated primarily via NO derived from the endothelium, independent of other endothelium-derived vasodilators, i.e., prostacycline and the endothelium-derived hyperpolarizing factor.

It is well known that the mechanism of action of UI involves activation of the BK<sub>Ca</sub> channel [27]. The current study is the first to suggest that the BK<sub>Ca</sub> channel is involved in UI-induced vasodilation of the retinal arterioles. Although there are contradictions between our results and other reports [7,22,23], the discrepancies may be due to differences in the experimental protocols or arterial segments, e.g., with or without flow to the vessel, species, and retinal or ciliary arterioles. Further study is needed to clarify the detailed mechanisms.

Previous studies have reported the involvement of various K channels in the vasodilation of the retinal arterioles [9,10,12,18,19,28,29]. The current data showed that the BK<sub>Ca</sub> channel blocker iberiotoxin inhibited UI-induced vasodilation (Figure 3) in the same manner as the nonselective K channel blocker TEA. These results suggested that the BK<sub>Ca</sub> channel, which relaxes the smooth muscle via membrane hyperpolarization [30], may be involved primarily in UI-induced vasodilation of the retinal arterioles. Although the endothelial K<sub>Ca</sub> channel might regulate endothelial NO synthase activity [31], the current finding that the combination of L-NAME and iberiotoxin almost eliminated dilation in response to UI suggested that the BK<sub>Ca</sub> channel may be involved in the endothelium-independent (smooth muscle-dependent) vasodilation of the retinal arterioles in response to UI.

In conclusion, we showed for the first time that UI, a prostaglandin F<sub>2α</sub>-related compound, elicits potent dilation of the retinal arterioles. UI-induced vasodilation may have two components of endothelium-dependent and endothelium-independent pathways, probably via the NO production from the vascular endothelium and activation of the BK<sub>Ca</sub> channel in smooth muscle. Because RBF is impaired in early-stage DR in patients with type 2 diabetes mellitus [8], UI-induced vasodilation may be a novel potential drug for topical treatment of DR.

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