



Ascorbate Blocks Acetylcholine- and Bradykinin-induced Vasodilatation in Retinal Vascular Bed of the Bovine Isolated Perfused Eye

Li-xin XU ^A, Xiao-bo XIA ^B, Aman Shah Abdul Majid ^C, Dan Ji ^B

Abstract

Background The regulation of retinal blood flow is largely dependent on alteration of vascular tone of the retinal arterioles in response to local mechanical and chemical stimuli. Such vasomotor activity can be modulated by the release of vasoactive factors from the endothelium. As most blindness-causing disorders are associated with abnormal retinal blood flow, it is critical to determine the abnormality of microcirculation control involved.

Methods The present study developed a model in isolated bovine retinal vascular bed to study agonist-induced vaso-relaxation. Both acetylcholine and bradykinin were used to stimulate vaso-relaxation and the effects of NOS inhibitor and prostacyclin and ascorbate (Vitamin C) were examined. **Results** Both acetylcholine and bradykinin efficiently stimulated such a non-traditional vaso-relaxation that neither NO nor prostacyclin is involved. Hence this process was linked to the mediator belonging to endothelium-derived hyperpolarizing factor (EDHF). As a concentrated antioxidant in eyes, ascorbate showed capability to block acetylcholine- and bradykinin-induced vasodilatation in bovine retinal arteries. **Conclusion** Acetylcholine and br-

- bradykinin induces EDHF-mediated vaso-relaxation in bovine retinal blood vessels which is blocked by ascorbate. This study provides insight to EDHF-mediated vasomotor response, as well as various eye disorders caused by dysfunctional retinal arteries, like central retinal artery occlusion.

Key Words: vasodilator, acetylcholine, bradykinin, bovine retinal artery

Introduction

Major eye disorders, like glaucoma, diabetic retinopathy, age-dependent macular degeneration, are associated with abnormal retinal blood flow. Glaucoma is the first leading cause of irreversible blindness (Koustenis et al., 2017, Linsenmeier et al., 2017). Based on estimation, there would be 60.5 million people with open angle glaucoma (OAG) and angle closure glaucoma (ACG) in 2010, increasing to 79.6 million by 2020 (Quigley et al., 2006). Patients with normal-tension glaucoma (NTG) had narrower central retinal vessel diameters than did the eyes of normal subjects (Shin et al., 2017). Optical coherence tomography angiography (OCTA) is powerful to diagnose abnormal blood flow in glaucoma eyes, which indicated that glaucoma preferentially affects perfusion in the superficial vascular complex (SVC) in the macula more than the deeper plexuses (Takusa-

Significance | This study showed a model in retinal vascular bed of the bovine isolated perfused eye to study agonist-induced vaso-relaxation.

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gawa et al., 2017). It was reported that blood flow in ophthalmic artery (OA), central retinal artery, and short posterior ciliary artery is all abnormal in primary open-angle glaucoma patients and correlated with the abnormal 24-hour blood pressure (Marjanovic et al., 2016). Diabetes was the seventh leading cause of death in the US in 2010. The prevalence of diabetes among US adults is projected to increase from 14% in 2010 to 21% by 2050, representing a significant burden on the population (Boyle et al., 2010). Diabetic retinopathy (DR), a major microvascular complication of diabetes, is one of the leading causes of vision loss and visual impairment in the working age population worldwide (Tarr et al., 2013). Total retinal blood flow (RBF) is impaired in patients with both non-proliferative DR (NPDR) and proliferative DR (PDR), which could be detected by multiplane en face Doppler optical coherence tomography (OCT) (Lee et al., 2017). In addition, wall shear stress (WSS) could also be altered as the marker of abnormal hemodynamics of diabetic microvasculopathy (Pechauer et al., 2018; Khansari et al., 2017). Hence it is worth to explore more eye diseases and more animal eye disease models where retinal blood flow might be impaired.

The release of endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO) and prostacyclin (PGI₂) in response to stimulations has been well described in various vascular beds in retina (Haefliger et al., 2001; Opatrilova et al., 2018). The rats with experimental early diabetic retinopathy have higher retinal nitric oxide concentration and this concentration is inversely correlated with blood glucose, while nitric oxide is lower than control level in severe diabetic rats (Guthrie et al., 2014). Not only in eye, the increased plasma NO levels are associated with increased severity of diabetic retinopathy indicated with in vivo structural changes in inner segment ellipsoid and pigment epithelium (RPE) (Sharma et al., 2015). The critical role of NO in retinal circulation control is also supported by the observation that neuronal nitric oxide synthase (nNOS) contributes to regulation of the retinal circulation during rest and flicker stimulation in cats (Yoshioka et al., 2015). The soluble guanylyl cyclase/cGMP system plays an important role in the vasodilator response to nitric oxide in most retinal vasculature, but the cyclooxygenase-1/cAMP-mediated pathway could still contribute to the vasodilator effects of NO, for example, prostaglandin I₂/prostanoid IP receptor signaling functions in rat retinal arterioles (Mori et al., 2015). In addition to NO and prostacyclin, more EDRFs are explored in various eye diseases, which would also contribute to the pathogenesis of these diseases.

The anti-oxidation function of ascorbate (vitamin C) in eyes has been widely studied. It profoundly impacts on the vasomotor response in retinal microcirculation. As vasodilators are released from endothelium, l-ascorbic acid 2-phosphate (Asc-2P) extended the proliferation of cultured human corneal endothelial cells (HCECs), partly due to protection against oxidative DNA damage (Shima et al., 2011). It was also demonstrated that ascorbate restored nitric oxide-dependent vasodilatation following its impairment by oxidant stress

in isolated arterial rings (Dudgeon et al., 1998; Fontana et al., 1999). This protective effect might be mediated by the clearance of superoxide anion which destroys nitric oxide (Gryglewski et al., 1986; Rubanyi et al., 1986), or mediated by the elevation of tetrahydrobiopterin, the cofactor of nitric oxide synthase (Heller et al., 2001; Huang et al., 2000). It was further observed that ascorbate in aqueous humor promoted NO production in macrophages by stabilizing tetrahydrobiopterin and increasing intracellular arginine (McKenna et al., 2013). Accordingly, studies about ascorbate in multiple models of retinal microcirculation are required to further understand its effects and regulation.

As various vascular beds within the eyes are under tight vasomotor regulation but utilize different mediators, therefore the present study aimed to determine how vasodilatation is mediated in retinal vascular bed.

2. Materials and methods

Drugs and chemicals

Acetylcholine chloride (Ach), L-NAME (N^G-nitro-L-arginine methylester, MW 270), Bradykinin acetate, Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid, MW 358), papaverine hydrochloride (MW 376), ascorbate (MW 176) and U46619 (9, 11-dideoxy-11 α , 9 α -epoxy-methanoprostaglandin F_{2 α}) were all obtained from Sigma chemical corporation (Poole, UK). All drugs were dissolved and diluted in distilled water except indomethacin (3 \times 10⁻³ M stock solution), which was dissolved in 10ml of Na₂CO₃ (3 \times 10⁻³ M) solution.

Preparation of the bovine isolated arterially perfused eye

The retinal vascular bed of the bovine eye was perfused using constant flow perfusion method of Wilson *et al.* (1993). In brief, Bovine eyes obtained from a local abattoir within 1 h of killing were cannulated so as to perfuse the retinal artery. The ophthalmic artery was dissected free from connective tissue. In some eyes, the ophthalmic artery bifurcates into two long posterior ciliary arteries approximate 2-3 cm proximal to the sclera. In other eyes, the bifurcation occurs close to the sclera. The retinal is a branch of one long posterior ciliary artery and enters the retina via the optic nerve. The perfusion of the very small retinal artery was achieved by cannulating either the long posterior ciliary artery or the ophthalmic artery as appropriate. In the first case, a cannula was inserted into ciliary artery which appeared to be closer to the optic nerve. The other ciliary artery was tied off with thread. In the second case, the bifurcation occurs too close to the optic nerve for safe dissection, so the cannula was inserted into the ophthalmic artery. In this latter situation, thread was tied around whichever ciliary artery appeared to be distal from the optic nerve. In both these situations, the ciliary which was judged to be supplying the

retinal artery was also tied off at a point distal to the optic nerve (usually about 1 cm beyond the nerve).

The retinal vasculature was perfused at 37°C with modified Krebs solution (NaCl 118 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25.0 mM, CaCl₂ 2.5 mM) with 5 % CO₂. Flow was commenced at 0.2- 0.5 ml min⁻¹ and was raised in 2-5 increments to a final constant rate of 0.8- 1.1 ml min⁻¹ over a 10 min period. After the final flow rate was achieved, eyes were perfused for the equilibration period of 10-20 min. Perfusion pressure was measured using Coult Statham P32 ID transducers contact to Grass Polygraph via a side arm located immediately proximal to the inflow cannula. Only eyes that had a basal perfusion pressure of 20-30 mmHg after the equilibration period were used for further study.

Experimental protocols

After the equilibration period, drugs were added either to the Krebs reservoir for continuous infusion, or as bolus doses immediately proximal to the cannula. The first part of the experiments involved constructing cumulative concentration-response curves to the thromboxane A₂-mimetic, U46619. Vasoconstrictor responses to each concentration of U46619 were allowed to stabilize before a higher concentration was added and continuous infusion of U46619 at a concentration of 2x10⁻⁷ M was chosen to achieve a sub-maximal perfusion pressure (-60-180 mmHg). At first, 2 ml L-NAME (N^G-nitro-L-arginine methyl ester) at a concentration of 10⁻² M was added into 200ml Krebs solution. After 10-15 minutes, the pressure of the perfused retinal vasculature started to increase little by little. 30-40 minutes later, the perfusion pressure achieved a sub-maximal value (.160mmHg) around which is suitable for conducting experiments with vasodilators. Once this perfusion pressure was established, vasodilator responses to acetylcholine and bradykinin were assessed by varying bolus doses (3, 10, 30, 100, 300, 1000 pmol of bradykinin and 0.1, 1, 10 nmol of acetylcholine) injected proximal to the cannula with a Hamilton micro-syringe.

The second part of experiments involved adding 1 ml 50 µM ascorbate into 200ml Krebs solution, and then injects 100pmol bradykinin with a Hamilton micro syringe in every 20 minutes during the following 100 minutes. After 100 minutes, acetylcholine and bradykinin were again tested by varying doses (100, 300, 1000 pmol of bradykinin and 0.1, 1, 10 nmol of acetylcholine) with a Hamilton micro-syringe.

The last part of the experiments involved adding 5 ml papaverine hydrochloride at the concentration of 10⁻² M into 100Krebs solution in order to confirm the maximum relaxation which could be achieved in the artery. Responses were measured using baseline which was established by using papaverine (10⁻⁴M) to produce full relaxation of the vasculature at the end of the experiment.

Vasoconstrictor responses are given in mmHg and vasodilator responses are expressed as percentage (%) reduction of U46619-induced perfusion pressure.

Statistical analysis

Results are expressed as the mean ± s.e.mean of *n* separate observations, each from a separate eye, Graphs were drawn and statistical comparisons made (Student's *t*-test, or one-way analysis of variance with Benferroni's post-test, as appropriate) using the computer package Prism (GraphPad, San Diego, USA). A probability (*P*) less than or equal to 0.05 was considered significant. (SF-1)

RESULTS

Acetylcholine and bradykinin induce vasodilatation in retinal arteries of the bovine isolated perfused eye

Acetylcholine and bradykinin were used for retinal microcirculation models for vasodilatation (McNeish et al., 2001), hence the effects of these to stimulators were detected in our samples. The basal perfusion pressure of the retinal vascular bed of the bovine isolated perfused eye preparation was detected to be stable at the constant flow of 0.8-1.1 ml min⁻¹ was 22±2.8 mmHg (n=8). The Thromboxane A₂-mimetic, U46619 (2x10⁻⁷M), produced a concentration-dependent rise in perfusion pressure, as similar as previous reports using it for vasoconstriction (Hou et al., 2004). Under this situation, the addition of acetylcholine (Figure 1A) and bradykinin (Figure 1B) significantly reduced perfusion pressure in samples of retinal vascular bed of the bovine isolated perfused eye, and this effects were dose-dependent (ach: 0/0.1/1/10 nmol; BK: 3/10/30/100/300/1000 pmol).

Nitric oxide is required for neither basal perfusion pressure nor acetylcholine- and bradykinin-induced vasodilatation

To determine if nitric oxide was involved in Ach- and BK-induced vasodilatation, addition of L-NAME to the perfusate had no effect on this basal perfusion pressure (change of -0.75±0.53 mmHg, n=8). After NO treatment and U46619 vasoconstriction. Ach (Figure 2A) and BK (Figure 2B) still induced vasodilatation indicated by the reduction of perfusion pressure, and the reduction of perfusion pressure for each dose of Ach or BK was similar to the reduction when L-NAME was absent.

Ascorbate blocks Acetylcholine-induced vasodilatation

To determine if ascorbate effects on Ach- and BK-induced vasodilatation in samples of retinal vascular bed of the bovine isolated perfused eye, the ascorbate (50µM) was added to the samples. Consistent with the reduction of ROS species, ascorbate treatment significantly attenuated Ach (0.1/1/10 nmol)-induced vasodilatation, and even high concentration of Ach did not change the perfusion

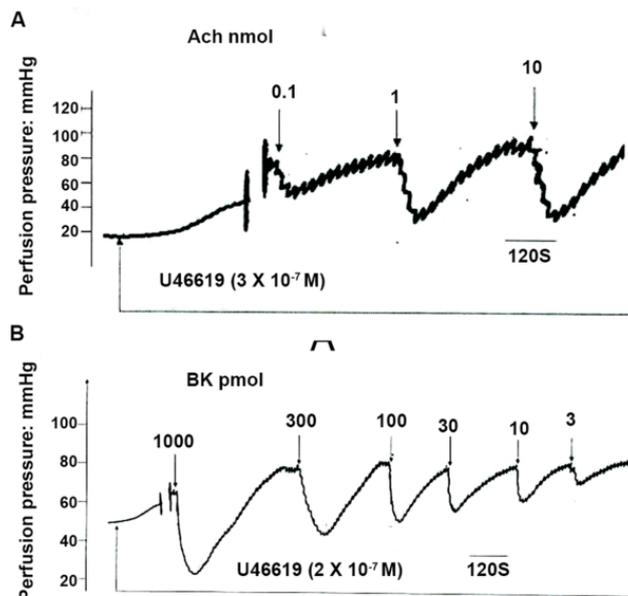


Figure 1. Acetylcholine and bradykinin induce vasodilatation in retinal arteries of the bovine isolated perfused eye. The perfusion pressure was increased using the thromboxane A₂-mimetic, U46619 (3×10^{-7} M), prior to addition of graded doses of Ach.

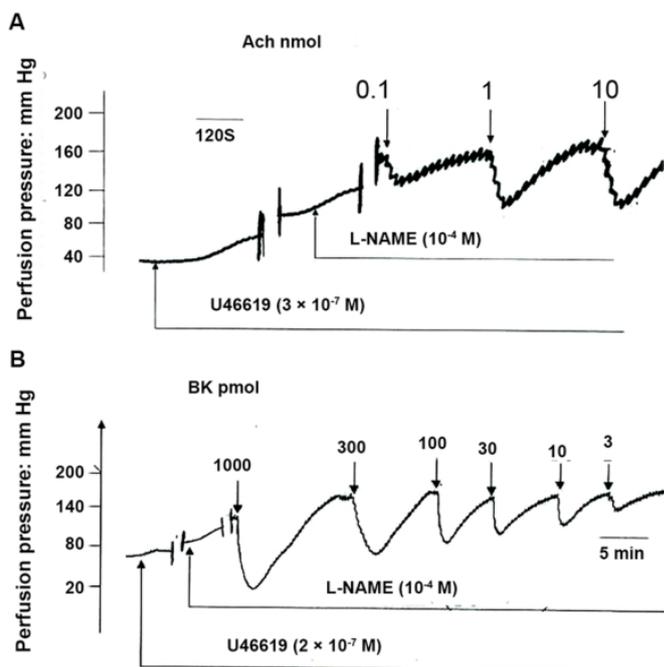


Figure 2. Nitric oxide is required for neither basal perfusion pressure nor acetylcholine- and bradykinin-induced vasodilatation. (A) Representative original trace for Ach. (B) Representative original trace for BK.

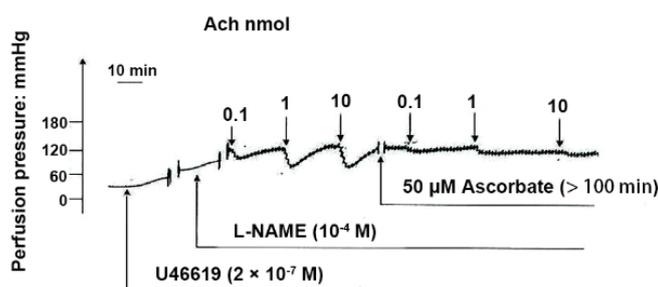


Figure 3. Ascorbate blocks acetylcholine-induced vasodilatation. Representative original trace for Ach.

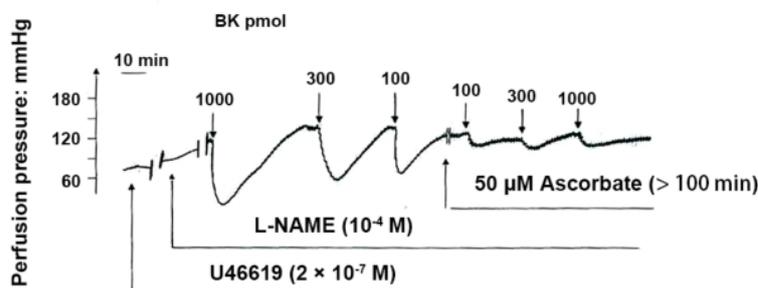


Figure 4. Ascorbate blocks bradykinin-induced vasodilatation. Representative original trace for BK.

pressure in our samples (Figure 3). In the samples without ascorbate treatment, repeat Ach addition still induced vasodilatation.

Ascorbate blocks bradykinin-induced vasodilatation

In addition to the observation for Ach-induced vasodilatation, we also determine the effects of ascorbate on BK-induced vasodilatation. Consistently, ascorbate treatment significantly attenuated BK (100/300/1000 pmol)-induced vasodilatation, and even high concentration of BK did not change the perfusion pressure in our samples (Figure 4), indicating that Ach and BK regulates retinal vasodilatation with same mediators in our model, and ascorbate could block both of the vasodilators. In the samples without ascorbate treatment, repeat Ach addition still induced vasodilatation.

DISCUSSIONS

It was well known that endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO) and prostacyclin (PGI2) plays essential roles in vasomotor control in various vascular beds in retina (Hardy et al., 2000; Mori et al., 2007). Inhibition of inducible nitric oxide synthase has already been shown to be effective in various animal models of diabetic eye disease (Carr et al., 2017). For example, administration of shikonin, a major red naphthoquinone pigment, attenuated diabetes/hypoxia-induced production of inflammatory proteins cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Liao et al., 2017). On the other hand, beraprost sodium, a stable PGI2

analogue, BPS elicits endothelium-dependent and -independent dilation of the retinal arterioles mediated by NO induced by activation of PKA in the endothelium and the KATP channel activation in the vascular smooth muscle, respectively (Ono et al., 2014). According to previous studies (Nelli et al., 2003), we developed the model system in retinal vascular bed of the bovine isolated perfused eye where acetylcholine- and bradykinin-induced vasodilatation could be readily recorded. Interestingly, neither NOS inhibitor (L-NAME) nor cyclooxygenase inhibitor (indomethacin) suppressed the vasodilatation. This is consistent with the ciliary vascular bed of the bovine isolated perfused eye, where vasodilatation induced by acetylcholine or bradykinin was not sensitive to L-NAME (McNeish et al., 2001).

Though the nitric oxide and prostacyclin are traditionally considered as endothelium-derived relaxing factors, some of the endothelium-dependent vasodilations are resistant to the blockade of NO synthase and cyclooxygenase. The nature of these endothelium-derived hyperpolarizing factor (EDHF) is still unknown, but possible candidates include a cytochrome P450 metabolite, an epoxyeicosatrienoic acid an endogenous cannabinoid or potassium ions (Edwards et al., 2000). The endothelium plays an essential role in the dilation of porcine retinal arterioles to histamine via H1- and H2-receptor activation. The EDHF derived from cytochrome P450 contributed in part to this vasodilation via Ca2+ -activated K+ (KCa) channel activation, in addition to the endothelial release of NO and

prostanoids (Otani et al., 2016). It was reported that EDHF is able to relax ocular ciliary artery vascular tone in rats via large-conductance calcium-activated K(+) channel and this ability is injured in spontaneous hypertensive rats (SHR) (Dong et al., 2010). Acetylcholine- and bradykinin-induced vasodilatation in the ciliary vascular bed of the bovine isolated eye was also reported to be independent of nitric oxide and hence might be mediated by an unknown EDHF, (McNeish et al., 2001) which is similarly observed in the present study on bovine retinal arteries.

Therefore, the present study provided opportunity to explore how a EDHF-mediated vasodilatation could be pharmacologically targeted. The anti-oxidation function of ascorbate (vitamin C) in eyes has been widely studied. As ascorbate scavenges superoxide anion, it might be responsible for clearance of the toxic reactive oxygen species (ROS) as the levels of superoxide dismutases are found to be extremely low in eyes. This was observed in patients treated with vitrectomy that the level of ascorbate in the vitreous fluid was significantly reduced and increased oxygen from the retina dramatically induced lens oxidation and protein aggregation in these patients. (Yan et al., 2017). Benefited by the anti-oxidation capacity, ascorbate could block agonist-induced vasodilator responses mediated by EDHP. In the ciliary vascular bed of the bovine isolated perfused eye, EDHF-mediated vasodilator responses induced by acetylcholine or bradykinin were powerfully blocked when ascorbate, reminiscent to the effects of two other reducing agents, N-acetyl-L-cysteine and dithiothreitol (McNeish et al., 2002).

In summary, our present study developed a model in retinal vascular bed of the bovine isolated perfused eye to study agonist-induced vaso-relaxation. In the present study, acetylcholine and bradykinin efficiently stimulated a non-traditional vaso-relaxation that neither NO nor prostacyclin is involved, providing insights to EDHF-mediated vasomotor response. As a concentrated anti-oxidant in eyes, ascorbate (vitamin C) showed capability to block acetylcholine and bradykinin-induced vasodilatation in bovine retinal arteries. However, further studies are warranted to unveil the detail mechanism of this process.

Author Contributions

All authors equally contributed for experimental and data analysis.

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None declared

Competing financial interests

The author(s) declare no competing financial interests.

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