Requisite Roles of $A_{2\alpha}$ Receptors, Nitric Oxide, and $K_{ATP}$ Channels in Retinal Arteriolar Dilation in Response to Adenosine

Travis W. Hein, Zhaoxu Yuan, Robert H. Rosa, Jr, and Lib Kuo

PURPOSE. Adenosine is a potent vasodilator of retinal microvessels and is implicated to be a major regulator of retinal blood flow during metabolic stress. However, the receptor subtypes and the underlying signaling mechanism responsible for the dilation of retinal microvessels in response to adenosine remain unclear. In the present study, the roles of specific adenosine receptor subtypes, nitric oxide (NO), and adenosine triphosphate (ATP)-sensitive $K^+$ ($K_{ATP}$) channels in adenosine-induced dilation of retinal arterioles in vitro were examined.

METHODS. Porcine second-order retinal arterioles (40 – 70 μm in internal diameter) were isolated, cannulated, and pressurized to 55 cmH$_2$O luminal pressure without flow. Diameter changes in response to agonists were recorded by using videomicroscopic techniques.

RESULTS. All vessels exhibited basal tone and dilated dose dependently in reaction to adenosine, N$^\text{6}$-cyclopentyladenosine (an adenosine $A_1$ receptor agonist), and 2$\text{\prime}$-[6-(carboxyethyl)phenethylamino]-5$\text{\prime}$-$N$-$ethylcarboxamidoadenosine (CGS21680; an adenosine $A_2\alpha$ receptor agonist). These responses were not altered by the selective adenosine $A_2\beta$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine, but were significantly attenuated by the selective adenosine $A_2\beta$ receptor antagonist 4-(2$\text{\prime}$-[7-amino-2$\text{\prime}$-furyl][1,2,4]triazolo[2,3-$a$][1,3,5]triazin-5-ylamino)ethyl)phenol. Blockade of NO synthase, but not of cyclooxygenase or cytochrome P-450 epoxygenase, significantly attenuated the vasodilations in response to adenosine and CGS21680. The residual vasodilative reactions to both agonists was nearly abolished by the $K_{ATP}$ channel inhibitor glibenclamide.

CONCLUSIONS. These data suggest that adenosine evokes retinal arteriolar dilation via activation of $A_2\beta$ receptors and subsequent production of NO and opening of $K_{ATP}$ channels. A better understanding of the fundamental signaling pathways responsible for adenosine-induced dilation of retinal arterioles may help shed light on the possible mechanisms contributing to retinal ischemia. (Invest Ophthalmol Vis Sci. 2005;46:2113–2119) DOI:10.1167/iovs.04-1438

The microvascular network, especially the arteriolar vessels, plays the central role in regulating blood flow to underlying tissue for proper nutrition and function. Retinal blood flow is closely regulated to meet the metabolic demands of the retinal tissue. Because the retinal circulation lacks autonomic innervation, modulation of retinal vascular tone is vitally dependent on local control mechanisms such as metabolic regulation. In particular, the purine metabolite adenosine has been implicated to be a major vasodilator that mediates autoregulatory adjustments in retinal blood flow. The direct evidence for adenosine’s vasodilative action has been provided in isolated large retinal arteries and small retinal arterioles. Although small retinal arterioles have the largest capacity for regulating retinal blood flow, the underlying mechanisms involved in the dilation of these vessels by adenosine remain unclear.

In microvascular beds that are highly regulated by local metabolism, such as those in the skeletal muscle, coronary and cerebral circulations, the adenosine $A_1$ and $A_2$ receptors have been shown to mediate the arteriolar dilation in response to adenosine. Several lines of evidence have shown that endothelial release of nitric oxide (NO) plays a role, at least in part, in the arterial dilation after adenosine receptor activation in some organ systems. In addition, adenosine receptor-mediated activation of $K^+$ channels has been shown to contribute to the vasodilative response in various vascular beds, including the retina. Specifically, the activation of ATP-sensitive potassium ($K_{ATP}$) channels appears to be involved in retinal arteriolar dilation by exposure to adenosine in vivo and in vitro. However, the specific adenosine receptor subtype responsible for this $K_{ATP}$ channel-mediated response remains unknown. The possible role of NO or other endothelium-derived vasodilators, such as prostaglandins and cytochrome P-450 metabolites, in the arteriolar response has also not been investigated. Since hemodynamic changes are known to influence vascular function and to produce confounding effects on vasomotor responses to agonists in vivo, we elucidated the relative roles of receptor subtypes, endothelium-derived vasodilators, and $K_{ATP}$ channels in the adenosine-induced dilation of porcine retinal arterioles in an isolated vessel preparation in a defined environment.

METHODS

Animal Preparation

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Scott & White Institutional Animal Care and Use Committee. Pigs (8 – 12 weeks old of either sex; 7 – 10 kg) purchased from Barfield Farms (Rogers, TX) were sedated with tiletamine plus zolazepam (4.4 mg/kg, intramuscularly) and xylazine (2.2 mg/kg, intramuscularly), anesthetized with pentobarbital sodium (30 mg/kg, intravenously), intubated, and ventilated with room air. Heparin (1000 U/kg) was administered into the marginal ear vein to prevent clotting, and the eyes were enucleated and immediately placed in a moist chamber on ice.

Isolation and Cannulation of Microvessels

The lens and vitreous body were removed carefully under a dissection microscope. The eye cup was placed in a cooled dissection chamber...
intraluminal pressure. The image was taken through the video port of reservoirs pressurized the vessel to 55 cmH2O intraluminal pressure.

Cannulated arterioles were bathed in PSS-albumin at 36°C to 37°C, to allow development of basal tone (45-µm internal diameter) at 55 cmH2O intraluminal pressure. The image was taken through the video port of an inverted microscope. Magnification, ×20.

Figure 1. An isolated retinal arteriole cannulated with glass micropipettes and secured with ophthalmic sutures. The vessel was transferred to the stage of an inverted microscope and was allowed to develop resting basal tone (45-µm internal diameter) at 55 cmH2O intraluminal pressure. The image was taken through the video port of an inverted microscope. Magnification, ×20.

(−8°C) containing a physiological salt solution (PSS; in mM: NaCl 145.0, KCl 4.7, CaCl2 2.0, MgSO4 1.17, NaH2PO4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and MOPS 3.0 [(N-morpholino)propanesulfonic acid]) with 1% albumin (USB, Cleveland, OH). Single second-order retinal arterioles (in the range of 40–70 µm in internal diameter, 0.6–1.0 mm in length) were carefully dissected with a pair of Dumont microdissection forceps (Fine Science Tools, Foster City, CA) with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY). After careful removal of any remaining neural/connective tissues, the arteriole was then transferred for cannulation to a Lucite vessel chamber containing PSS-albumin solution equilibrated with room air at ambient temperature. One end of the arteriole was cannulated with a glass micropipette (tip outer diameter of 30–40 µm) filled with PSS-albumin solution, and the outside of the arteriole was securely tied to the pipette with an 11-0 ophthalmic suture (Alcon, Fort Worth, TX). The other end of the vessel was cannulated with a second micropipette and also secured with a suture. After cannulation, the vessel and pipettes were transferred to the stage of an inverted microscope (model CKX41; Olympus) coupled to a video camera (Sony DXC-190; Labtek, Campbell, CA) and video micrometer (Cardiovascular Research Institute, Texas A&M University System Health Science Center, College Station, TX) for continuous measurement of the internal diameter throughout the experiment (Fig. 1). The micropipettes were connected to independent pressure reservoirs. Adjusting the height of the reservoirs pressurized the vessel to 55 cmH2O intraluminal pressure without flow. This level of pressure was used based on pressure ranges that have been documented in retinal arterioles in vivo18 and in the isolated, perfused retinal microcirculation.19 Preparations with visible side branches and leaks were excluded from further study.

Experimental Protocols

Cannulated arterioles were bathed in PSS-albumin at 36°C to 37°C, to allow development of basal tone (−30–40 minutes), the dose-dependent vasodilations after addition of the natural ligand adenosine (0.1 nM to 100 µM), the A1 receptor agonist N6-cyclopentyladenosine (CPA; 0.1 nM to 10 µM), and the A2A receptor agonist 2-p-[p(2-carboxyethyl)phenylethyl]amino-5'-N-ethylcarboxamidoadenosine (CGS21680; 0.1 nM to 10 µM; Tocris Cookson, Ellisville, MO) were independently recorded. The vessels were exposed to each concentration of agonist for 3 to 5 minutes until the vessel with PSS-albumin for at least 30 minutes. The reproducibility of the response was confirmed in our pilot studies.

To elucidate the possible signaling mechanisms involved in the retinal arteriolar dilation induced by adenosine and its receptor agonists, the following series of experiments were performed. The contribution of KATP channels to the adenosine-receptor-mediated vasodilation was examined before and after incubation of isolated arterioles with the specific inhibitor glibenclamide (5 µM). The involvement of prostaglandins, NO, and cytochrome P-450 metabolites in mediating the vascular responses was assessed before and after incubation of vessels with known effective concentrations of the specific inhibitors indomethacin (10 µM),22,25 A46-nitro-l-arginine methyl ester, (t-NAME, 10 µM),10,13 and sulfaphenazole (1 µM),24 respectively. In a separate series of experiments, we studied the effect of glibenclamide (5 µM), in the presence of t-NAME (10 µM), on adenosine- and CGS21680-induced vasodilations. To confirm the efficacy of glibenclamide and t-NAME, vasodilations induced by the KATP channel opener pinacidil0,18 and NO-mediated agonist bradykinin8 were examined. At the end of each study, a complete dose-dependent vasodilative response to sodium nitroprusside was examined to ensure that the vasodilative function (or vessel preparation) had not deteriorated. Because >90% of glibenclamide binds albumin,25 the series of experiments using this drug were performed in PSS without albumin. All drugs were administered extraluminally, and each antagonist was incubated for at least 30 minutes.

Chemicals

Drugs were obtained from Sigma-Aldrich (St. Louis, MO) except when specifically stated otherwise. Adenosine, bradykinin, sodium nitroprusside, and t-NAME were dissolved in PSS. CPA, CPX, indomethacin, pinacidil, and sulfaphenazole were dissolved in ethanol, and CGS21680, glibenclamide, and ZM241385 were dissolved in dimethylsulfoxide (DMSO) as stock solutions (10 mM). Subsequent concentrations of these drugs were diluted in PSS. The final concentration of ethanol or DMSO in the vessel bath was 0.1%. Vehicle control studies indicated that this final concentration of solvent had no effect on the arteriolar function.

Data Analysis

At the end of each experiment, the vessel was relaxed with 100 µM sodium nitroprusside in EDTA (1 mM) and calcium-free PSS, to obtain its maximal diameter at 55 cmH2O intraluminal pressure. All diameter changes in response to agonists were normalized to this maximal vasodilation.26 Data are reported as the mean ± SEM and n values represent the number of vessels studied. In each set of interventions, the vessels have their own control, with each vessel being from a different eye. The control vessels were pooled for comparison of a series of experiments examining the effect of antagonists on vasodilation. Statistical comparisons of vasoresponse responses to the same agonist under various treatments were performed using two-way analysis of variance, with or without repeated measures when appropriate, followed by a Bonferroni multiple-range test. Comparisons of basal tone and vasodilations to pinacidil, and L-NAME were dissolved in PSS. CPA, CPX, indomethacin, pinacidil, and sulfaphenazole were dissolved in ethanol, and CGS21680, glibenclamide, and ZM241385 were dissolved in dimethylsulfoxide (DMSO) as stock solutions (10 mM). Subsequent concentrations of these drugs were diluted in PSS. The final concentration of ethanol or DMSO in the vessel bath was 0.1%. Vehicle control studies indicated that this final concentration of solvent had no effect on the arteriolar function.

Results

Vasodilation of Retinal Arterioles to Adenosine and Adenosine Receptor Agonists

In this study, all vessels (n = 47) showed a similar level of basal tone (constricted to 58% ± 2% of their maximal diameter) at
36°C to 37°C bath temperature with 55 cmH₂O intraluminal pressure. The average resting and maximal diameters of the vessels were 60 ± 3 and 101 ± 3 μm, respectively. Adenosine, the A₁ agonist CPA, and the A₂A agonist CGS21680 produced dose-dependent dilation of isolated retinal arterioles (Fig. 2). The relative order of potency of these agonists was CGS21680 > adenosine > CPA. The higher concentrations of CPA and CGS21680 (i.e., >10 μM) could not be evaluated because of the toxic effect of solvent (>0.1% ethanol and DMSO, respectively), which caused nonspecific dilation of arterioles and loss of basal tone.

**Role of Adenosine A₁ and A₂A Receptors in Retinal Arteriolar Dilation Induced by Adenosine and Adenosine Receptor Agonists**

To evaluate the contribution of A₁ and A₂A adenosine receptors to retinal arteriolar dilation in response to adenosine, dose-dependent responses to adenosine analogues were examined in the absence and presence of competitive adenosine receptor antagonists. Blockade of A₁ receptors by CPX had no effect on vasodilation in response to adenosine (Fig. 2A). In contrast, the A₂A receptor antagonist ZM241385 abolished vasodilation induced by the lower concentrations of adenosine (≤10 μM) and reduced the response to the highest concentration of adenosine (0.1 mM) from 81% (control) to 25% (Fig. 2A). The dilation of arterioles after addition of CPA was not altered by CPX, but was abolished by ZM241385 (Fig. 2B). Retinal arteriolar dilation in response to CGS21680 also was not altered by CPX, but was almost completely inhibited by ZM241385 (Fig. 2C). It should be noted that ZM241385 did not significantly alter resting basal tone (control: 60% ± 3% versus ZM241385: 58% ± 4%).

**Role of KₐTP Channels in Adenosine-Induced Retinal Arteriolar Dilation**

To determine the relative contribution of KₐTP channels to adenosine-mediated vasodilation, dose-dependent responses to adenosine were examined in the absence and presence of the KₐTP channel inhibitor glibenclamide. As shown in Figure 3A, glibenclamide abolished vasodilation induced by the lower concentrations of adenosine (≤1 μM) and reduced the response to the highest concentration (0.1 mM) from 87% (control) to 42%. Glibenclamide did not significantly alter resting basal tone (control: 60% ± 3% versus glibenclamide: 58% ± 4%). The concentration of glibenclamide used in this study appears to be effective, because this antagonist abolished vasodilation in response to the KₐTP channel opener pinacidil (Fig. 3B).

**Role of Endothelium-Derived Factors in Retinal Arteriolar Dilation in Response to Adenosine and CGS21680**

The relative contribution of prostaglandins, cytochrome P-450 metabolites, and NO to adenosine- and CGS21680-induced vasodilations was examined and compared in the absence and presence of their respective inhibitors. Neither indomethacin nor sulfaphenazole inhibited the vasodilation elicited by adenosine (Fig. 4A). However, l-NAME significantly shifted the vasodilative response curves of adenosine (Fig. 4A) and CGS21680 (Fig. 4B) to the right. The l-NAME concentration was effective for blocking NO synthase because it significantly reduced the retinal arteriolar dilation by the NO-mediated agonist bradykinin (10 nM; control: 79% ± 4% of maximal dilation versus l-NAME: 30% ± 5% of maximal dilation; n = 5). The resting basal tone was slightly increased by l-NAME, but not significantly (control: 61% ± 4% versus l-NAME: 58% ± 4%; P = 0.07). The residual vasodilative responses to adenosine (Fig. 4A) and CGS21680 (Fig. 4B) in the presence of l-NAME
were further reduced by subsequent treatment with glibenclamide. It does not appear that L-NAME/glibenclamide or ZM241385 influenced retinal arteriolar function through a non-specific effect, because vasodilation by sodium nitroprusside was not altered by these agents (Fig. 5).

**DISCUSSION**

Adenosine, a breakdown product of cellular adenosine triphosphate, is a potent vasodilator in most vascular beds, including the retinal circulation. This nucleoside has been proposed to play a significant role in the metabolic regulation of retinal blood flow. The putative regulatory role of adenosine is based on the observations that intravitreal administration of adenosine and adenosine reuptake inhibitors evokes dilation of retinal microvessels in vivo. In addition, direct evidence of adenosine’s vasodilative action has been provided.

**FIGURE 3.** Role of K<sub>ATP</sub> channels in adenosine-induced dilation of isolated retinal arterioles. (A) The dose-dependent vasodilative response to adenosine was examined before and after incubation with the K<sub>ATP</sub> channel inhibitor glibenclamide (5 μM). (B) Vasodilation induced by pinacidil was examined before (control) and after incubation with glibenclamide (5 μM). n, number of vessels. *P < 0.05 versus control.

**FIGURE 4.** Roles of endothelium-derived factors and K<sub>ATP</sub> channels in isolated retinal arteriolar dilation in response to adenosine and CGS21680. (A) Dose-dependent vasodilation after exposure to adenosine was examined before (control) and after incubation with the cyclooxygenase inhibitor indomethacin (10 μM), the cytochrome P-450 inhibitor sulfaphenazole (1 μM), or the NO synthase inhibitor L-NAME (10 μM). Residual vasodilation in the presence of L-NAME was examined after coincubation with glibenclamide (5 μM). (B) Dose-dependent vasodilation induced by CGS21680 was examined before (control) and after incubation with L-NAME (10 μM). Residual vasodilation in the presence of L-NAME was examined after coincubation with glibenclamide (5 μM). n, number of vessels. *P < 0.05 L-NAME/glibenclamide versus L-NAME; †P < 0.05 versus control.

Our present results demonstrate that the dilation of isolated porcine small retinal arterioles induced by adenosine is mediated by the activation of A<sub>2A</sub> receptors and subsequent production of NO and opening of K<sub>ATP</sub> channels.

Four adenosine receptor subtypes (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>) have been cloned from various cell types, tissues, and species. In microvascular beds that are highly regulated by local metabolism, the A<sub>1</sub> and A<sub>2</sub> receptors have been shown to...
mediate adenosine-induced dilation. Specifically, coronary arterioles dilate in response to \( A_{2\alpha} \) and \( A_{2\beta} \) receptor activation, and skeletal muscle arterioles dilate after \( A_1 \) receptor activation. The receptor subtypes involved in adenosine-induced dilation of retinal arterioles have not been clearly identified. For example, in vivo studies in pigs have shown that intravitrreal administration of a nonselective adenosine \( A_2 \) antagonist inhibited the dilation of retinal arterioles to adenosine. CPA has been shown to produce three major vasodilators: NO, prostaglandins, and cytochrome P-450 metabolites. These factors are confounded vasomotor function, as demonstrated in various vascular beds is the opening of smooth muscle \( \mathrm{K}^{+} \) channels. The smooth muscle \( \mathrm{K}_{\mathrm{ATP}} \) channels are important in the dilation of coronary and skeletal muscle arterioles. These earlier studies, as well as numerous others, have investigated the possible functional role of \( \mathrm{K}_{\mathrm{ATP}} \) channels using the pharmacological blocker glibenclamide. An in vivo study in pigs has shown that intravitrreal administration of glibenclamide attenuates retinal blood flow in response to exogenous adenosine, indicating that activation of these \( \mathrm{K}^{+} \) channels may be involved in retinal vasodilation caused by adenosine. However, these results are difficult to interpret because the in vivo administration of glibenclamide may affect the activity of retinal pigment epithelium and retinal neurons. The evidence for a possible role of \( \mathrm{K}_{\mathrm{ATP}} \) channels was recently suggested by an in vitro study showing that glibenclamide abolished the dilation of isolated porcine retinal arterioles induced by adenosine. However, a pharmacological preconstrictor was used in that study for vascular tone development, which could have altered the signaling pathways and confounded vasomotor function, as demonstrated in various isolated vessel preparations. Our present results showing that glibenclamide attenuated, but did not abolish, the adenosine-induced dilation of porcine retinal arterioles that developed spontaneous myogenic tone supports this idea. Thus, it appears that activation of \( \mathrm{K}_{\mathrm{ATP}} \) channels may not be the sole signaling pathway in the vasodilative response of retinal arterioles to adenosine.

Another signaling pathway that may be involved in retinal arteriolar dilation in response to adenosine is through an endothelium-dependent mechanism. The endothelium has been shown to produce three major vasodilators: NO, prostaglandins, and cytochrome P-450 metabolites. These factors are worth investigating, because the endothelial release of the potent vasodilator NO has been shown to play a role in the dilation of coronary and skeletal muscle arterioles in response to adenosine. Because it is difficult to distinguish between possible neuronal- or vascular-mediated mechanisms of action of NO in the retina in vivo, the isolated vessel preparation provides the most appropriate approach for unambiguous identification of this signaling pathway. In the present study, it does not appear that prostaglandins or cyto-

![Figure 5](https://iovs.arvojournals.org/)
chrome P-450 metabolites are involved in retinal arteriolar dilation in response to adenosine because blockade of cyclooxygenase or cytochrome P-450 epoxygenase did not alter the response. In contrast, the blockade of NO synthase with l-NAME reduced the adenosine-induced vasodilation, suggesting that NO contributes in part to the response. Likewise, l-NAME attenuated vasodilation after application of CGS21680, indicating that the adenosine-induced NO production is mediated by A2A receptors. In contrast to in vivo findings in the retinal circulation, \cite{49, 45} it is important to note that l-NAME (10 \( \mu \)M) did not significantly increase basal tone of isolated retinal arterioles. This may be due to the absence of luminal flow in our in vitro study, since it has been shown that endothelial cells respond to increased flow (or shear stress) by releasing NO. \cite{23, 46} In this regard, it is expected that the NO component would be more pronounced in vivo (i.e., with luminal flow) compared with that in vitro (i.e., without luminal flow) in resting conditions. Therefore, the effect of l-NAME on basal vascular tone would be less apparent in our in vitro study. The present results are consistent with our previous findings in isolated porcine coronary arterioles. \cite{10, 13} In the presence of l-NAME, we found that the residual dilation of retinal arterioles to both adenosine and CGS21680 was further reduced by glibenclamide. These data support the idea that both endothelium-derived NO and smooth muscle KATP channels contribute to the adenosine receptor-activated dilation of retinal arterioles.

Our current findings may provide further insight into the possible adenosine receptor signaling mechanisms associated with retinal ischemia–reperfusion injury. One of the manifestations of ischemia–reperfusion injury in the retinal vasculature is the reduction of retinal blood flow. \cite{47, 48} Increased blood flow during early reperfusion is a compensatory reactive hyperemic response to ischemia; however, it may also promote edema, which can directly compress the blood vessels and contribute to hyperperfusion. \cite{49} In addition, postischemic hyperperfusion may result from altered vascular reactivity during prolonged periods of reperfusion. \cite{47} Although retinal microvessels are exposed to an elevated level of adenosine during experimental ischemia \cite{50} and reperfusion, \cite{51} the role of specific vascular adenosine receptors in ischemia–reperfusion injury remain unclear. Previous studies in cats have shown that the nonselective adenosine receptor antagonist 8-sulphophenyltheophylline attenuates postischemic retinal hyperemia, suggesting the involvement of adenosine. \cite{52, 53} Based on our results, it is reasonable to speculate that the activation of arteriolar adenosine A2A receptors could contribute to the hyperemic response. An in vivo study in rats has shown that A2A receptor blockade protects retinal function (i.e., recovery of electroretinogram a- and b-waves) and structure after ischemia–reperfusion. \cite{54} Although retinal blood flow was not measured, a possible explanation for the postsischemic damage could be hyperemia-induced retinal edema. It is also plausible that the activation of A2A receptors on retinal neuronal cells caused the deleterious effect. However, the direct role of neuronal or vascular A2A receptors in the ischemia–reperfusion injury was not determined in this earlier study. Future studies examining the function of small retinal arterioles after ischemia and reperfusion are necessary to identify the precise role of vascular adenosine A2A receptor activation, as well as NO synthase/KATP channel signaling, in retinal ischemia–reperfusion injury.

In summary, the results of this study provide the first evidence that retinal arteriolar dilation in response to adenosine is mediated predominantly by A2A receptors. The stimulation of NO synthase and the opening of KATP channels appear to be two independent mechanisms responsible for the dilation of retinal arterioles in response to the activation of adenosine A2A receptors. Because modulation of retinal vascular tone is fundamentally dependent on local control mechanisms such as metabolic regulation, these findings provide the framework for investigating potential vascular signaling pathways involved in impaired retinal blood flow after retinal ischemia.

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References


