Role of Endothelium in Abnormal Cannabidiol-Induced Vasoactivity in Retinal Arterioles

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Purpose. Cannabinoids have been reported to mediate changes in vascular resistance through endothelial receptor targets. We examined involvement of the endothelium in cannabinoid-mediated vasoactive responses in resistance arterioles of the retina.

Methods. Vascular responses to both intraluminal (IL) and extraluminal (EL) administration of the atypical cannabinoid, abnormal cannabidiol (abn-CBD), a prototypical agonist at the non-CB_1/CB_2 endothelial cannabinoid receptor (CB_eR), were studied in endothelial intact and endothelial denuded, isolated perfused porcine retinal arterioles with and without endothelin-1 (ET-1) precontraction. The effects of AM251, a CB_1 receptor antagonist, and O-1918, an analog of CBD reported to antagonize CBeR, were also studied.

Results. Dose-dependent vasocontractile responses were induced by both IL and EL administration of abn-CBD in the absence of precontraction. Significantly greater vasoconstriction was induced by IL administration of abn-CBD than with EL administration. In contrast, only vasodilation to abn-CBD was observed in ET-1 precontracted retinal arterioles. Endothelium removal significantly reduced abn-CBD–induced vasoactivity when abn-CBD was used IL but not when applied EL. IL abn-CBD–induced vasoactivity was antagonized by O-1918 and AM251.

Conclusions. Cannabinoids show complex vasoactive actions in isolated perfused retinal arterioles. The fact that abn-CBD-mediated vasorelaxation was seen only in precontracted retinal vessels indicates that the abn-CBD–induced vasoactive response is highly dependent on vascular tone. Furthermore, IL and EL administration produced differential responses, and removal of endothelium blunted abn-CBD vasoactivity, highlighting the critical role of endothelium in abn-CBD vasoactivity. AM251 and O-1918 inhibition of abn-CBD–induced vasoactivity suggests the possibility of modulating abn-CBD–induced vasoactivity.

Keywords: abn-CBD, AM251, endothelium, O-1918, porcine, retinal arteries, vasoactivity

Endocannabinoids and their receptors are present in ocular tissues, including the ciliary body, iris, choroid, and trabecular meshwork, and cannabinoid effects have been studied in numerous species including monkey and human tissues.[1–4] It has been reported that the retinal endocannabinoid system has much in common with other regions of the central nervous system (CNS).[4] However, there is still less research work in this field in the eye and retina than in the CNS and other organ systems.[4] Given increasing reports of therapeutic benefits of targeting the endocannabinoid system,[5–7] it is important to characterize the effects of cannabinoids on specific cells and tissues within the retina in order to provide some insight into pathogenic mechanisms and to identify potential therapeutic targets for treating retinal and ocular diseases.

The cardiovascular effects of cannabinoids are increasingly interesting.[8,9] Both the endocannabinoids, including the prototypical endocannabinoid, anandamide (*N*-arachidonoylethanolamide), and the exogenous cannabinoid ligands have been demonstrated to produce complex vascular effects which vary under different experimental conditions. In vitro studies appear to overwhelmingly suggest that cannabinoids act as vasodilators in systemic vascular beds.[8] Although cannabinoid-induced hypotension has been observed in anesthetized animals,[8] supporting the notion that cannabinoids are vasodilator agents,[6] this notion has been challenged by results obtained from conscious animals after administration of cannabinoids[10] and in anesthetized animals after injection of anandamide, in which multiphasic blood pressure responses were observed.[11] These and other findings indicate that cannabinoid-induced cardiovascular responses are influenced by a number of factors including anesthesia and vascular tone. For example, vasodilation induced by cannabinoids was often found in precontracted conditions in isolated vessel preparations,[12–14] and anandamide-induced hypotension in conscious rats was found in drug-induced acute hypertensive rats but not in normotensive rats.[10] These previous studies have highlighted the fact that clear differences exist in cannabinoid-induced cardiovascular effects depending on a variety of conditions, including the vascular bed and species studied, the experimental conditions (i.e., whether in vivo [anesthetized and conscious] or in vitro [isolated arterial segments] and intact perfused vascular beds), the presence or absence of inhibitors and spasmogens, and the route of drug administration, and

postjunctional influences or control systems such as the autonomic nervous system.[8] Furthermore, the role of the endothelium in relaxant responses to cannabinoids is still controversial, and the molecular targets for cannabinoid actions, including both cannabinoid (CB_1 and CB_2) and noncannabinoid (endothelial CB_eR, transient potential 1 receptor, and others) receptors under the various conditions and in different vascular beds remain to be clearly defined.[8]

Complex cardiovascular effects of cannabinoids, involving diverse molecular targets with uncertain relative contributions from local influences as well as from the central and peripheral nervous system, require more precise investigation.

The retinal arteriole could be a suitable model with which to discern the vascular effects of cannabinoids. Unlike that of many vascular beds, retinal arteriolar tone is not influenced by autonomic innervation as retinal blood vessels are not innervated adrenergically, cholinergically, or peptidergically.[15–19] Retinal arteriole tone is regulated mainly by local factors.[20] Mediators derived from endothelial cells and retinal tissue can be considered local regulators, in addition to physical and metabolic influences.[21,22] Within these local factors, one can distinguish physical (e.g., variations in perfusion pressure) and metabolic (variations in pO_2, pCO_2, and pH) influences. In addition, NO, prostaglandins, adenosine, endothelin, and other mediators derived from endothelial cells and retinal tissue can be considered local regulators of retinal blood flow.

In this study, we examined vasoactive effects of the nonpsychoactive atypical cannabinoid abnormal-cannabidiol (*trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol [abn-CBD]), a synthetic analog of the cannabinoid cannabidiol that is purported to act as an agonist at the anandamide-activated endothelial CBeR receptor but is devoid of activity at the CB_1 or CB_2 receptor.[23,24] We studied the effects of abn-CBD by using an isolated perfused porcine retinal arteriole preparation in which concentration-dependent vasoactivity can be observed in vessels with basal tone or in endothlin-1 (ET-1) precontracted arterioles, following both intraluminal (IL) and extraluminal (EL) drug delivery. The effects of endothelial removal on abn-CBD–induced vasoactivity was also studied, as well as the effects of the CB_1 receptor antagonist AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] and the analog of cannabidiol (-)-1,3-dimethoxy-2-(3-3,4-*trans*-*p*-menthadien-(1,8)-yl)-orcinol (O-1918), which is a neutral antagonist at CBeR.[11,25]

Methods

Isolated Perfused Retinal Arteriole

Dissection, cannulation, perfusion, monitoring and vessel diameter measuring system are fully described in our previous publications, using isolated perfused retinal arterioles[26–29] and will be only briefly described here. Pig eyes were obtained from a local abattoir. Following enucleation, the eyes were placed in a sealed bottle of oxygenated Krebs solution and kept on ice during transfer to the laboratory (~60 minutes). All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee of The University of Western Australia.

Dissection and Cannulation of Vessels

The eyes were sectioned at pars plana ciliaris, separating the anterior segment and adherent vitreous body from the posterior pole with the aid of a dissecting microscope. The retina, choroid, and sclera were divided into quadrants. The retina was then separated from the underlying choroid and sclera. A quadrant of retina was placed on a hollowed glass slide containing Krebs solution and individual first-order retinal arteries were dissected free of retinal tissue with a micropipette. A segment of retinal arteriole (~100-μm outer diameter) approximately 800- to 1500-μm long and containing only one relatively large side branch was selected. This arteriole segment was then relocated to an incubation chamber (PDMI-2; Medical System Corp, New York, NY, USA) mounted on the stage of an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan). The chamber contained 5 mL of Krebs solution. Temperature was maintained at 37°C, and the incubating solution was equilibrated with 95% O_2 and 5% CO_2 to maintain pO_2, partial pressure of CO_2 (pCO_2), and pH of the incubating solution.

The arteriolar segment was cannulated at both ends using a customized pipette and manipulating system (Fig. 1A, schematic). The vessel was then perfused through the proximal end in the orthograde direction at a constant flow of 5 μL min^{-1}. The distal end was perfused at 0.3 μL min^{-1} in the retrograde direction to avoid drug entrapment. Both flow streams exited through the side branch. With constant flow within the physiological range, the retinal arteriole was under a basal or baseline tone. We have previously shown that vascular constriction or dilation can be induced from the basal tone condition.[27,29] IL saponin (0.125 mg/mL for 10 minutes) was used for functional endothelial removal. Endothelial cell layer can be seen under the microscope, but loss of normal function can be confirmed by the lack of response to the endothelium-dependent vasodilator acetylcholine.[30]

The vessel was visualized on a video monitor, and a preprogrammed computer algorithm was used to measure the external vessel diameter at user-selected locations from frame images grabbed at 2-second intervals. The vessel was left to stabilize for 30 minutes prior to any drug study. Figure 1B shows an image of a cannulated retinal arteriole held by the outer and inner pipettes in an incubation bath. Drugs can be reliably delivered by IL or EL or both routes as required in order to determine abn-CBD–induced vasoactive changes.

IL and EL Drug Delivery

IL drug delivery was administered as a 5-μL bolus into the perfusate stream, using an HPLC-type sample injector valve. This system allowed the bolus to enter the perfusate stream without pressure artifacts. A switch on the HPLC valve signaled the time of injection of the bolus to the computer and chart recorder. The size and, hence, duration of the bolus was sufficient for vasoactive responses of the vessel to stabilize. EL drug delivery was accomplished by direct pipetting into the incubating solution to achieve the required concentration without washing out the bath. The concentration range of abn-CBD used was 10^{-10} to 10^{-4} M. This dose range covers the doses used for abn-CBD vasoactivity studies in other organs,[13,31,32] including rat retinal arterioles.[33] All data are normalized vessel diameter percentages, where data were normalized to the diameter of the vessel prior to any drug administration.

Solutions and Agents

Vessels were usually bathed and perfused with normal Krebs solution composed of 119 mM NaCl; 4.6 mM KCl; 1.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 15 mM $NaHCO_3$; 1.2 mM NaH_2PO_4; and glucose 6. All chemicals and vasoactive agents used were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) except for abn-CBD, AM251, and O-1918, which were obtained from Tocris Bioscience (Bristol, UK). abn-CBD, AM251, and O-1918

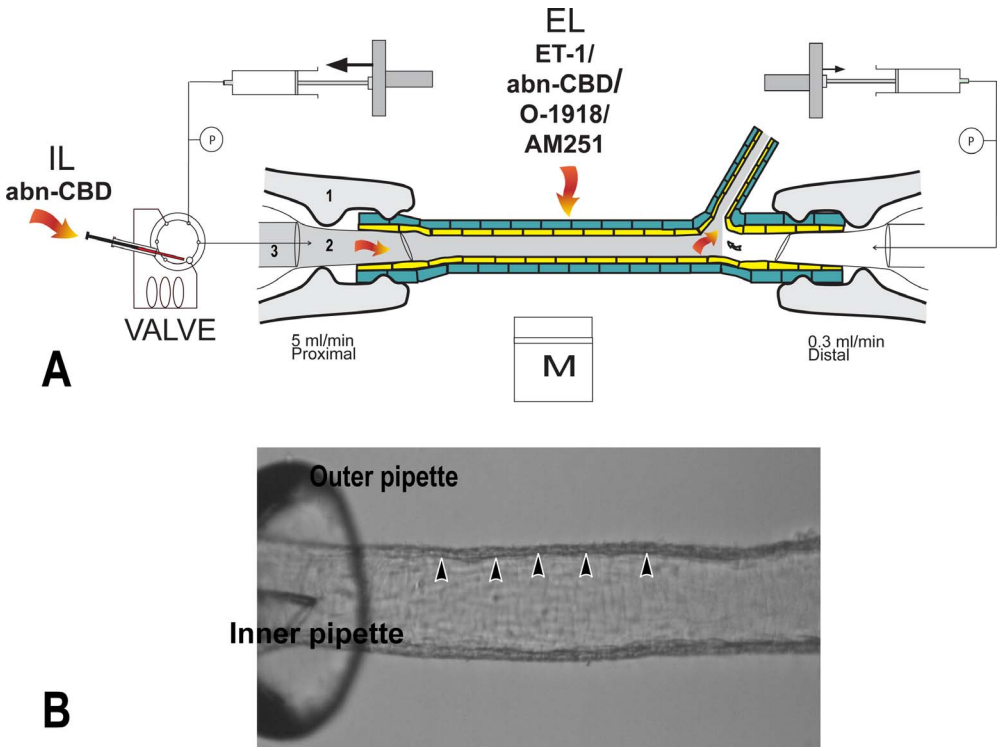

Figure 1. Schematic of retinal arteriole experiment. (**A**) Representation of the isolated perfused vessel system. The isolated pig retinal arteriole is cannulated at both ends and perfused intraluminally in a temperature-controlled bath on the stage of an inverted microscope. Drugs, including abn-CBD, can be administered intraluminally via HPLC valve or delivered extraluminally by addition to the bathing solution (e.g., ET-1, O-1918, and AM251). The retinal arteriole is held firmly between the outer (*1*) and inner (*2*) pipettes. There is a third pipette (*3*) used for intraluminal perfusion. The tip of the third pipette is pushed down close to the tip of the inner pipette in order to maximally avoid dilution of the abn-CBD bolus. Vessel diameter is monitored every 2 seconds by an automated frame-grabbing and vessel diameter measurement routine. (**B**) Photomicrograph of a cannulated retinal arteriole held by the outer and inner pipettes. A thin layer of the endothelium is clearly located along the inside of the vessel wall and indicated by *arrowheads*. On the outside of the vessel wall, a single layer of smooth muscle cells can be seen, with only the occasional overlap with processes of another smooth muscle cell.

were prepared using standard procedures provided by Tocris Bioscience. Stock solutions were stored at −70°C, and fresh dilutions were made daily. Ca^{2+}-free solution was prepared by omitting the $CaCl_2$ and adding ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA).[34]

Experimental Protocol

After equilibration, an IL injection of 124 mM K^+ Krebs solution was given to confirm retinal vessel viability. Vessels were rejected if the contraction response did not result in a diameter of less than 85% of the uncontracted baseline diameter.

To quantify uncontracted retinal arterioles, that is, the basal tone, normal perfusion solution was replaced with Ca^{2+}-free solution, resulting in an averaged diameter that was significantly increased from 107.1 ± 2.7 μm to 123.3 ± 0.8 μm ($n = 18$, $P < 0.001$). It suggests that the basal tone in our preparation was 13.1 ± 2.3% from maximal passive diameter.

The effect of a wide range of IL abn-CBD concentration (10^{-10} to 10^{-4} M) was determined in uncontracted retinal arterioles and in arterioles contracted with EL ET-1. When EL application of ET-1 (3×10^{-9} M) was used to pre-contract the vessels, the ET-1 remained in the bath during all subsequent drug administrations. Pre-contracted vessels could be sustained over the experimental period.[29,29,35,36] AM251 and O-1918 were applied EL and remained in the bath. The specificity of O-1918 for the endothelial abn-CBD-sensitive cannabinoid receptor CBeR is now well established by several reputable studies in several different vascular beds, including the retina,[33] the human pulmonary artery,[13] and in cell and expression system studies.[37–39]

Statistics

All statistical testing was performed using SigmaStat software (Jandel Scientific Software, San Raphael, CA, USA). The significance of any drug-induced concentration-dependent change was tested using one-way ANOVA, with a significance acceptance P level of <0.05 for the F value. When we compared dose response curves, the 2-way ANOVA using drug concentration as the second factor was used with an acceptance P level of <0.05. When appropriate, Student's t-test was used. All mean data are means ± standard error, and all error bars on graphs are also standard errors.

Results

Dose Response for IL and EL Administrations of abn-CBD in Retinal Arterioles With Basal Tone or Precontracted With ET-1

To determine vasoactive responses induced by abn-CBD, we used the perfused retinal arteriole preparation with basal tone (no exogenous vasoconstrictor). A perfused arteriole corresponds more closely to the in vivo situation, allows differentiation of IL or EL responses, may cause less endothelial cell damage, especially for small vessel of outside diameter ~100

μm, and is more readily applied to smaller vessels such as human or porcine retinal arteries and arterioles. Moreover, the use of a controlled perfusion technique means that the flow rate and IL pressure are well controlled and that the vessel diameter can be measured. abn-CBD was administered IL and EL in perfused vessels. With IL delivery, abn-CBD initially contacts the surface of vascular endothelium, whereas with EL delivery, abn-CBD initially contracts the smooth muscle cells.[27,29,40–43] Figure 2A shows the average vasoactive responses to IL or EL delivery ($n = 53$) of increasing doses of abn-CBD (10^{-10} to 10^{-4} M). The outer vessel diameters were normalized in order to compare dose-response for IL and EL administrations of abn-CBD. Both the IL and the EL delivery of abn-CBD induced dose-dependent vasoconstriction. However, IL induced a significantly greater contraction than that with EL delivery when the 2 groups were compared ($P < 0.05$). In both cases, the contraction was significant at doses of 10^{-9} M and above ($P < 0.05$), reaching a maximum contraction of 78.5 ± 1.3% and 86 ± 1.0% at doses of 10^{-6} M (IL) and 10^{-5} M (EL), respectively. Results clearly demonstrate a differential response to IL and EL delivery of abn-CBD, with a significantly greater contraction induced by IL delivery of abn-CBD. Results also suggest the presence of an intact barrier and functional endothelial cells in our preparation.

ET-1 is a powerful vasoconstrictor in retinal and other arteriole preparations,[36,44] acting primarily at ETA receptors to produce increases in intracellular Ca^{2+} of smooth muscle cells. In the retinal arteriole preparation, in contrast to results seen with abn-CBD, EL delivery of ET-1–induced vasoconstriction is significantly greater than IL delivery of ET-1.[29] EL application of ET-1 produced a stable vasoconstriction in the pig and human retinal arterioles.[35,36] Figure 2B shows the response of retinal arterioles precontracted with ET-1, to increasing doses of IL and EL abn-CBD. In contrast to basal tone vessels in the absence of exogenous ET-1, abn-CBD delivered IL at doses of 10^{-9} M and above (10^{-9} to 10^{-4} M) in ET-1–constricted vessels produced a dose-dependent dilation from the precontracted baseline (63.2 ± 1.6%) with maximum vasodilation (85.3 ± 2.3%) seen at doses of 10^{-6} M and above ($n = 30$). EL delivery of abn-CBD resulted in slightly less dilation from the precontracted baseline (61.6 ± 1.3%), with the dilation becoming significant at 10^{-8} M and above and reaching 76.0 ± 1.82% at 10^{-4} M ($n = 38$). There were no statistically significant differences in ET-1 precontracted baseline between abn-CBD delivered IL and abn-CBD delivered EL ($P = 0.436$). However, abn-CBD delivered IL produced more dilation than abn-CBD delivered EL ($P < 0.001$). Therefore, the effect of abn-CBD on ET-1 precontracted retinal arterioles differs from that observed in the retinal arterioles with basal tone; abn-CBD induces dose-dependent vasodilation in the precontracted vessels, whereas dose-dependent vasoconstriction is observed in basal tone vessels.

Effects of Denuded Endothelium on abn-CBD-Induced Vasoactive Responses

To define whether the abn-CBD–induced vasoactive response is endothelium dependent, IL saponin was used to chemically denude the vascular endothelium.[30,45–47] The vessel diameter was not significantly changed before and after endothelial denuding (100.6 ± 4.8 μm and 98.7 ± 4.7 μm, respectively, $n = 24$, $P = 0.203$). Both IL and EL delivery of abn-CBD was then tested in the endothelium-denuded retinal arteriole preparation and was compared with intact retinal arteriole. Figure 3A shows that, in vessels denuded of endothelial cell function (saponin treated; DE-IL), the vasoconstriction seen with IL abn-CBD was significantly suppressed ($n = 12$) in comparison to endothelium-intact vessels (IE-IL) with normal

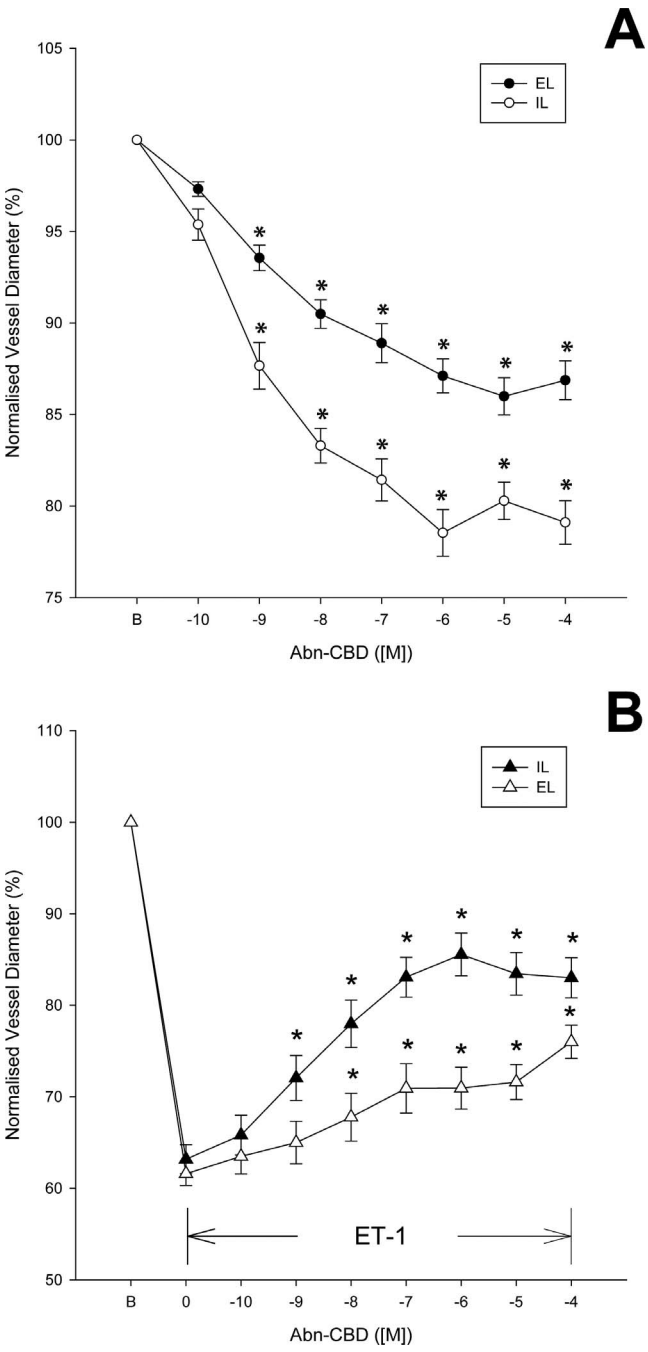

Figure 2. Dose-response for intra- and extraluminal administrations of abn-CBD (10^{-10} to 10^{-4} M) in retinal arterioles with basal tone (**A**) or precontracted with ET-1 (**B**). With normal tone, significant vasoconstriction was seen at 10^{-9} M abn-CBD, with contraction increasing at higher intraluminal and extraluminal abn-CBD concentrations. Intraluminal administration of abn-CBD produced a greater contraction than extraluminal delivery of abn-CBD ($P < 0.001$). *Significant contraction compared to initial baseline ($P < 0.05$). In vessels contracted by ET-1, abn-CBD produced significant dilation at 10^{-9} M and above with intraluminal delivery and at 10^{-8} M and above with extraluminal delivery.

tone ($P < 0.05$). In contrast to IL application of abn-CBD, the vasoconstriction of vessels to EL delivered abn-CBD was unaffected by the saponin treatment ($n = 12$; $P > 0.05$) (Fig. 3B). It is clear that after denudation of the endothelium, the abn-CBD–induced vasoconstriction response is substantially

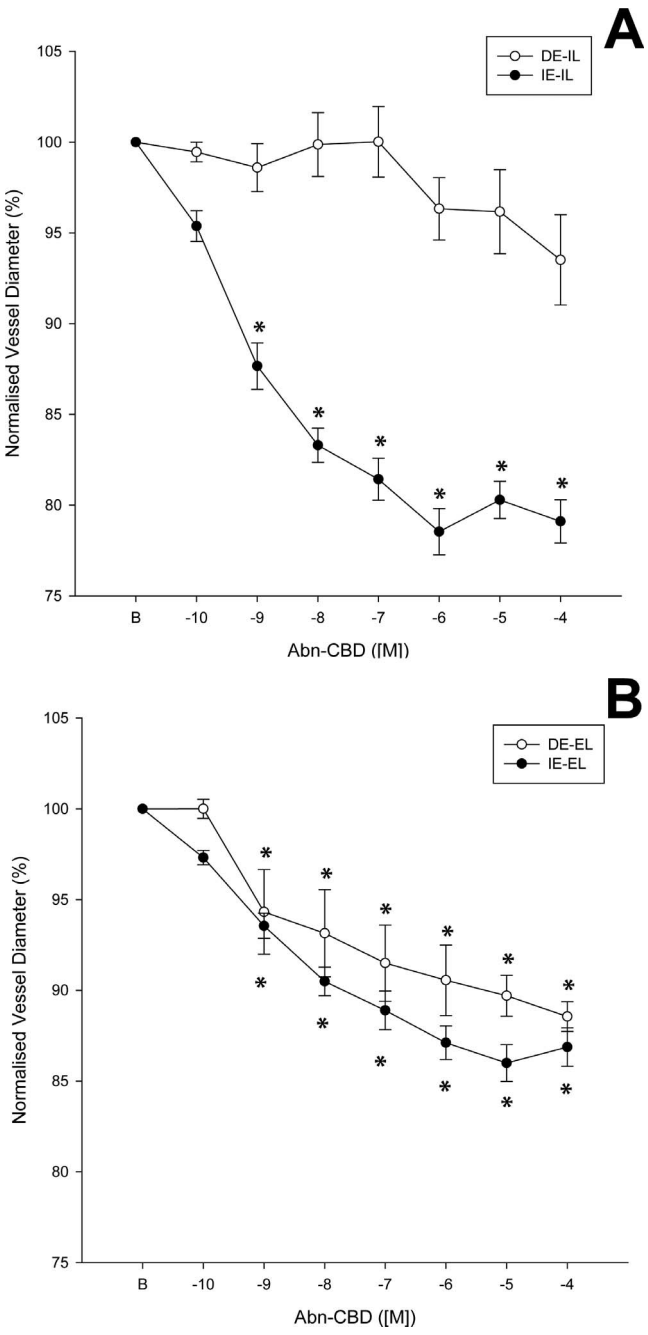

Figure 3. Intraluminal (**A**) or extraluminal (**B**) administration of abn-CBD (10^{-10} to 10^{-4} M) in retinal arterioles with normal tone (**A**) with or without saponin denudation of the endothelium. *Significant differences between the response of intact and denuded arterioles ($P < 0.05$).

reduced following abn-CBD IL delivery. This suggests that the endothelium plays a significant role in abn-CBD–induced vasoconstriction in retinal arterioles.

Effects of O-1918 on abn-CBD-Induced Vasoactive Responses With Basal Tone or Precontracted With ET-1

As CBeR (O-1918-sensitive) receptor has been implicated in the vasoactive actions of cannabinoids, including abn-CBD,[48] the effects of the CBeR antagonist O-1918 were examined on abn-CBD–mediated vasoactivity in retinal vessels with basal tone (Fig. 4A) or precontracted with ET-1 (Fig. 4B). In vessels with basal tone, the effects of IL abn-CBD in the presence of the antagonist, O-1918, showed dose-dependent effects (Fig. 4A). In the presence of the O-1918 at a dose of 10^{-6} M, the IL vasoconstrictor effect of abn-CBD was maintained to some degree at lower doses, with the maximum contraction (75.7 ± 2.2%, $n = 27$) seen at 10^{-8} M abn-CBD. However, increasing antagonism of abn-CBD vasoconstriction was observed at higher doses of abn-CBD. However, in the presence of higher doses of O-1918 (10^{-5} M), complete block of the abn-CBD response was seen, with the average response failed to reach significance at any of the doses tested ($P > 0.05$, $n = 21$).

Figure 4B shows the response of retinal arterioles precontracted with ET-1, to increasing doses of IL abn-CBD in the presence of 10^{-5} M O-1918. abn-CBD delivered IL at doses of 10^{-5} M and 10^{-4} M in ET-1 constricted vessels, produced dilation from the precontracted baseline (62.6 ± 1.4%), with maximum vasodilation (70.5 ± 1.7%) seen at doses of 10^{-5} M ($n = 24$). The abn-CBD induced dose-dependent vasodilation in the precontracted vessels in the presence of O-1918 was significantly reduced compared to the abn-CBD–induced vasorelaxation seen in ET-1 constricted vessels in the absence of O-1918 (Fig. 2B, $P < 0.05$).

Effects of AM251 on abn-CBD-Induced Vasoactive Responses With Basal Tone or Precontracted With ET-1

As CB_1 receptor has been implicated in the vasoactive actions of cannabinoids,[11] the effects of the CB_1 antagonist, AM251, was examined on abn-CBD-mediated vasoactivity in retinal vessels (Fig. 5). In vessels with basal tone and in the presence of the CB_1 antagonist, AM251 (10^{-5} M), the vasoactive response of IL abn-CBD in perfused vessels was suppressed (Fig. 5A, $P < 0.001$). The average response in the presence of AM251 failed to reach significance at any of the doses of abn-CBD tested ($P > 0.05$; $n = 42$). Figure 5B shows the response of retinal arterioles precontracted with ET-1, to increasing doses of IL abn-CBD in the presence of AM251 at dose of 10^{-5} M. abn-CBD delivered IL at doses of 10^{-10} M and 10^{-4} M in ET-1 constricted vessels, in the presence of AM251, produced only slight changes from the precontracted baseline (60.1 ± 1.0%) with maximum diameter (62.7 ± 2.0%) seen at doses of 10^{-4} M ($n = 24$). However, these changes did not reach statistical differences ($P = 0.131$). Therefore, AM251 blocked the vasoactive response of IL abn-CBD in basal tone or ET-1 precontracted perfused retinal arterioles (Figs. 5A, 5B).

Discussion

Using the perfused retinal arteriole system, the major findings from this study are: (1) abn-CBD induced dose-dependent vasocontractile responses in basal tone retinal arterioles following both IL and El administration; (2) vasocontractile responses were significantly greater with IL administration of abn-CBD than with EL administration; (3) endothelium removal reduced abn-CBD–induced vasoactivity to a greater extent with IL than with EL abn-CBD administration; (4) dose-dependent vasodilation responses to abn-CBD occurred in ET-1 precontracted retinal arterioles; and (5) both the CBeR antagonist O-1918 and the CB_1 receptor antagonist AM251 blocked abn-CBD–induced vasoactivity in normal tone and ET-1–constricted vessels.

In our previous study, Macintyre et al.,[33] we examined the vasoactivity of abn-CBD and *N*-arachidonoyl glycine (both

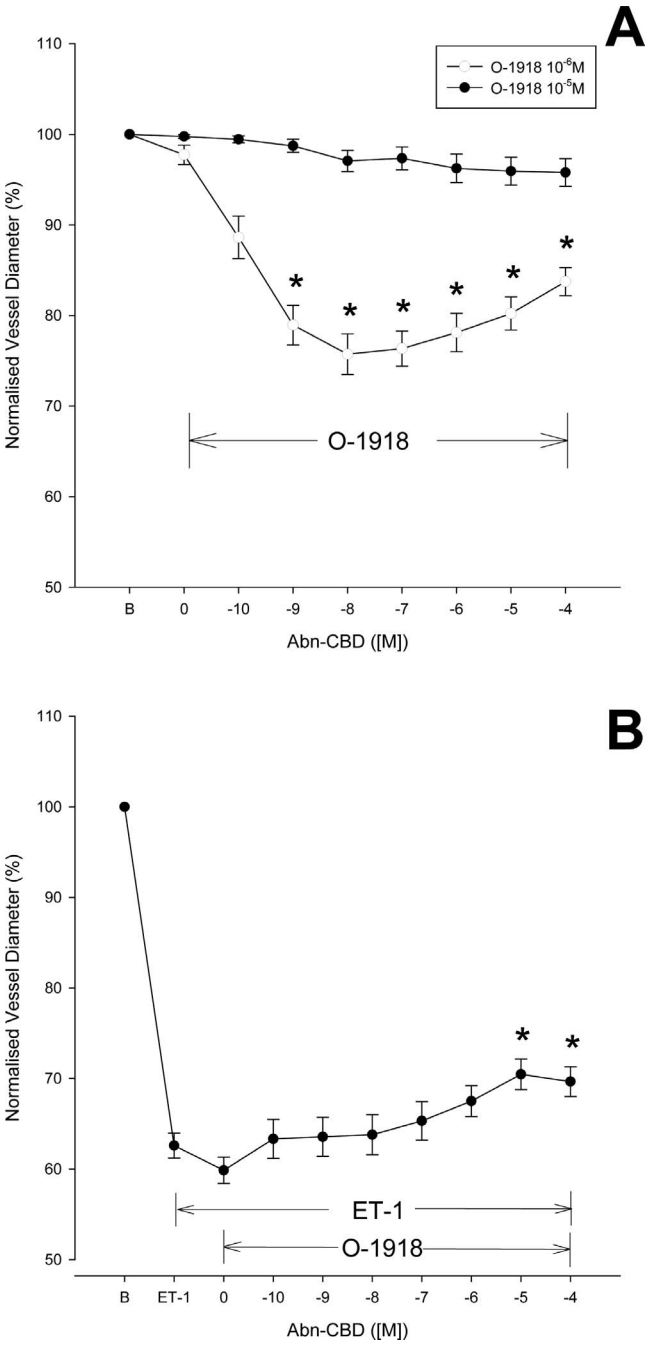

FIGURE 4. Average vessel diameter in normal tone (**A**) or ET-1-contracted (**B**) vessels in response to increasing doses of abn-CBD (10^{-10} to 10^{-4} M) in the presence of 10^{-6} M or 10^{-5} M O-1918. *Significant vasoconstriction compared to baseline vessel diameter ($P < 0.05$).

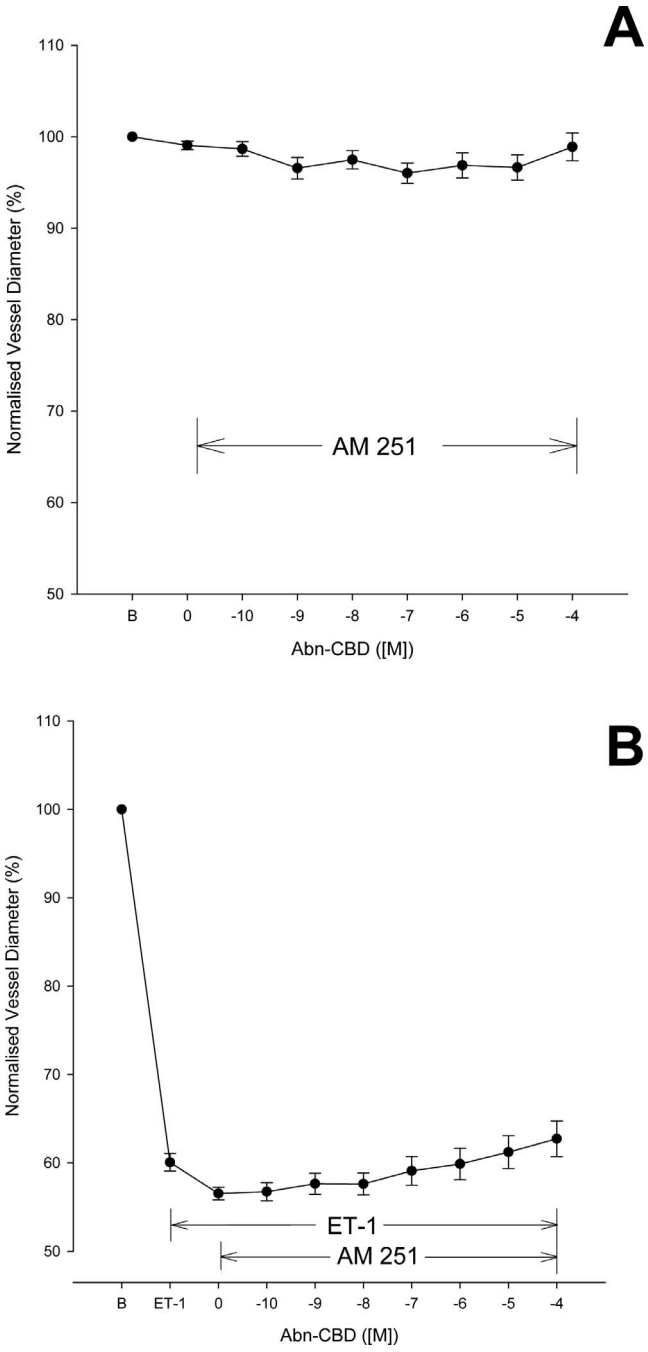

FIGURE 5. Average vessel diameter in normal tone (**A**) or ET-1-contracted (**B**) vessels in response to increasing doses of abn-CBD (10^{-10} M to 10^{-4} M) in the presence of the CB_1 antagonist AM251 (10^{-5} M).

agonists at GPR18 and CBeR). This study was conducted using rat retinal arterioles (<20 μm), hence the vessel segments were too small to be perfused, and therefore, this study examined only vessels in the presence of ET-1 to generate tone. Under those conditions, both abn-CBD and *N*-arachidonoyl glycine produced 0-1918–sensitive vasodilation of ET-1–constricted vessels. What is novel about the present study is the opportunity to examine the actions of cannabinoids that act at the CBeR in perfused vessels in the absence and presence of vasoconstriction and also with IL and EL drug application in both endothelial intact and endothelial denuded retinal arteriole preparations. In the absence of vasoconstriction (basal tone), the atypical cannabinoid, abn-CBD, produces vasoconstriction in perfused arterioles that is sensitive to both O-1918 and the CB_1 antagonist AM251. In contrast, in ET-1–precontracted perfused vessels, we observed only vasodilation, which was also sensitive to O-1918 and AM251. This suggests that ET-1 vasoconstriction may amplify the endothelial-dependent abn-CBD vasodilatory response, that is, ET-1 vasoconstriction may activate additional endothelial signaling pathways that further promote vasodilation (i.e., prostanoid, NO, or other

signaling pathways). As perfused porcine retinal vessel preparations represent CNS vasculature (devoid of neurogenic input) and lack the confounders of blood pressure and anesthesia reported in previously published in vivo studies, this finding suggests that the level of vessel tone is a primary dictator of response to atypical cannabinoids previously reported to act at the non-CB1/CB2 endothelial cannabinoid receptors.

Given the complexities of the cardiovascular effects of both synthetic and endogenous cannabinoids,[8,9] we used our established isolated perfused pig arteriole preparation to address the direct vasoactive response of the atypical cannabinoid abn-CBD, an agonist at the endothelial non-CB1/CB2 cannabinoid receptor CBeR, avoiding the confounding influence of systemic factors.[8] The retina is known as an extension of the CNS anatomically and developmentally. It consists of retinal ganglion cells, the axons of which form the optic nerve, whose fibers are central nervous axons. The retina has been described as a part of the brain[49]; therefore, the retinal arteriole preparation can provide valuable information on endocannabinoid regulation in an accessible microvascular bed in the CNS. In addition, lack of autonomic innervation of the retinal microvasculature[15–19] enhances the advantage of using the retinal arteriole as a model to study the vasoactivity of cannabinoids. Furthermore, the experimental setup is designed to provide a well-controlled environment, particularly with regard to endothelial cells; the constant perfusate flow through the vessel maintains a physiological shear stress at the innermost endothelial cell wall. This may be important in maintaining normal responsiveness of the endothelium. The cannulation and perfusion system is capable of delivering an IL administration of vasoactive solutions with minimal accumulation of vasoactive agent. We are, therefore, confident that the observed vasoactive responses to abn-CBD are a feature of the action of abn-CBD on the endothelium.

Differential responses to IL and EL abn-CBD application are demonstrated in our results. Such differential responses to IL and EL drug application have been found not only in retinal arteriole preparations but also in other organs,[27,29,40–43] although for some drugs, responses to IL are greater than EL responses, and in other drugs, EL response is greater than IL response. This dichotomy has been demonstrated in brain vessels, where the basilar artery had stronger IL responses to K^+ and 5-HT,[50] whereas smaller intracerebral arteries had stronger EL responses to adenosine and its analogs.[51] In the latter study, this asymmetry between EL and IL was found to be independent of the vascular endothelial cells. The mechanisms involved in the differential responses to IL and EL drug application are still not exactly known. It depends on the drug and may be related to the distribution of relevant receptors in the endothelium and smooth muscle cells. In terms of whether the actions of abn-CBD are endothelium dependent, the denuded endothelium preparation provides an optimum approach to verify endothelial targets. The effects of denuded endothelium on vasocontractile responses induced in basal tone vessels by both the IL and EL delivery of increasing doses of abn-CBD suggest that the abn-CBD–induced vasoactive response is endothelium-dependent. In vessels denuded of endothelial cell function (Fig. 3, saponin-treated, D, E-IL) the vasoconstriction seen with IL abn-CBD was significantly suppressed in comparison to that of endothelium intact vessels (IE-IL) with basal tone. In contrast to IL application of abn-CBD, the vasoconstriction of vessels to EL-delivered abn-CBD was unaffected by saponin treatment (Fig. 3). It is clear that after denudation of the endothelium, the abn-CBD–induced vasoconstriction response is substantially reduced following abn-CBD IL delivery. This suggests that the endothelium plays a significant role in abn-CBD induced vasoconstriction in retinal arterioles. In fact, the endothelial cell layer of the retinal arteriole was still present after functional removal of the endothelium by saponin. Therefore, it is possible that while the saponin-treated endothelium may be functionally compromised and failing to release some vasoactive factors after IL application of abn-CBD, it may still provide a diffusion barrier to abn-CBD. Another possibility could be that the distribution of the receptors of the vascular smooth muscle cells is different between the abluminal and EL sides.

Taken together, the results from this study revealed a significant level of complexity with regard to the actions of abn-CBD in retinal arterioles. On the one hand, consistent with previous studies,[12–14] we found that abn-CBD is an endothelium-dependent drug and that abn-CBD–induced vasodilation in ET-1 precontracted vessels was eliminated by the putative non-CB_1 CBeR receptor antagonist O-1918. This is consistent with previous studies in isolated ET-1 precontracted retinal arterioles that reported that abn-CBD vasorelaxation, like NAGly-mediated vasodilation, is endothelium dependent and involves activation of small Ca^{2+}-sensitive K channels and nitric oxide.[33] However, the abn-CBD–induced contraction observed in perfused retinal arterioles with basal tone in the absence of precontraction has not been reported before and further suggests that it is the vascular contractile state and local mediators and not neural regulation that is a major determinant of the response of this CNS microvasculature to cannabinoids. Although the mechanisms underlying abn-CBD–induced contraction in retinal arterioles remains to be fully clarified, this contraction was reduced, albeit with a delayed block, by O-1918, and was also sensitive to CB_1 receptor antagonism, suggesting the potential involvement of both CB_1 and non-CB_1 (CBeR) receptors. Although only vasorelaxant responses have been reported previously for abn-CBD, both vasocontractile and vasorelaxant responses are seen in other vascular beds for cannabinoids, including THC, as well as endocannabinoids (or their metabolites). These responses were mediated by both CB_1 and non-CB_1 receptors, including prostanoid receptors.[52] It is, therefore, possible that the vasocontraction seen following abn-CBD application in perfused retinal vessels with basal tone may arise from the direct or indirect actions of abn-CBD; activation of CBeR by abn-CBD may give rise to release of endothelial mediators, including endocannabinoids and/or metabolites, that activate vascular smooth muscle CB_1 receptors providing increases in tone. Furthermore, signaling crosstalk between cannabinoid systems and other Gq-coupled systems that are coexpressed in smooth muscle has been recently reported to produce synergistic contractile effects in ocular smooth muscle, with sequential activation of Rho-kinase and PLC signaling pathways.[53] A similar cross talk in retinal arterioles with basal tone may amplify vasocontractile responses giving rise to abn-CBD induced increased tone.

It has also been reported that some cannabinoids can have off-target effects. For example, AM251 has been noted to act as a mixed CB_1/GPR18 antagonist in cell culture experiments.[38] It is interesting that the AM251 partially blocked the abn-CBD–induced vasoconstrictor responses (Fig. 5). A possible explanation could be that AM251 is acting as a mixed CB1/GPR18 antagonist, as detailed above. Alternatively, abn-CBD may be acting at other noncannabinoid receptors, or resulting in the release of mediators that act at distinct receptors, including CB_1 to cause vasoconstriction. In all cases, one would expect the CB_1 antagonist AM251 to reduce the response.

In keeping with the findings reported in this study, differential vasoactive properties have also been reported for the vasoactive actions of prostanoids in the retinal arteriole preparations.[29,35] In the case of prostaglandin (PG)$F_{2\alpha}$, vasocontraction was observed with this PG in retinal arterioles with normal tone, with IL administration producing the greatest

response, while $PGF2_{\alpha}$-induced vasodilation was found in ET-1 precontracted retinal arterioles. The vasoactive responses induced by $PGF_{2\alpha}$ were weaker than that induced by abn-CBD in the same retinal arteriole preparation. A potential interrelationship between the endocannabinoid and eicosanoid signaling systems has been described.[4,54]

In conclusion, our results suggest that abn-CBD–induced vasoactivity in retinal arterioles is predominantly endothelium-dependent and also highly dependent on vessel tone (normal or precontracted), as well as site of drug administration (IL or EL). The underlying molecular mechanisms of such complicated abn-CBD–induced vasoactivity, including drug targets and signaling pathways, still needs to be further defined. However, given that AM251 and O-1918 blocked abn-CBD–induced vasoactivity suggest that drugs that target the endocannabinoid system, including CB_1 and the endothelial cannabinoid receptor CBeR could be used to modulate retinal vasoactivity and blood flow.

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References

1. Chen J, Matias I, Dinh T, et al. Finding of endocannabinoids in human eye tissues: implications for glaucoma. *Biochem Biophys Res Commun*. 2005;330:1062–1067.
2. Stumpff F, Boxberger M, Krauss A, et al. Stimulation of cannabinoid (CB1) and prostanoid (EP2) receptors opens BKCa channels and relaxes ocular trabecular meshwork. *Exp Eye Res*. 2005;80:697–708.
3. Njie YF, Qiao Z, Xiao Z, Wang W, Song ZH. N-arachidonylethanolamide-induced increase in aqueous humor outflow facility. *Invest Ophthalmol Vis Sci*. 2008;49:4528–4534.
4. Yazulla S. Endocannabinoids in the retina: from marijuana to neuroprotection. *Prog Retin Eye Res*. 2008;27:501–526.
5. Jarvinen T, Pate DW, Laine K. Cannabinoids in the treatment of glaucoma. *Pharmacol Ther*. 2002;95:203–220.
6. Hogestatt ED, Zygmunt PM. Cardiovascular pharmacology of anandamide. *Prostaglandins Leukot Essent Fatty Acids*. 2002; 66:343–351.
7. Cunha P, Romao AM, Mascarenhas-Melo F, Teixeira HM, Reis F. Endocannabinoid system in cardiovascular disorders—new pharmacotherapeutic opportunities. *J Pharm Bioallied Sci*. 2011;3:350–360.
8. Randall MD, Kendall DA, O'Sullivan S. The complexities of the cardiovascular actions of cannabinoids. *Br J Pharmacol*. 2004; 142:20–26.
9. Pacher P, Kunos G. Modulating the endocannabinoid system in human health and disease—successes and failures. *FEBS J*. 2013;280:1918–1943.
10. Ho WS, Gardiner SM. Acute hypertension reveals depressor and vasodilator effects of cannabinoids in conscious rats. *Br J Pharmacol*. 2009;156:94–104.
11. Zakrzeska A, Schlicker E, Baranowska M, Kozlowska H, Kwolek G, Malinowska B. A cannabinoid receptor, sensitive to O-1918, is involved in the delayed hypotension induced by anandamide in anaesthetized rats. *Br J Pharmacol*. 2010;160: 574–584.
12. Ho WS, Hiley CR. Vasodilator actions of abnormal-cannabidiol in rat isolated small mesenteric artery. *Br J Pharmacol*. 2003; 138:1320–1332.
13. Kozlowska H, Baranowska M, Schlicker E, Kozlowski M, Laudanski J, Malinowska B. Identification of the vasodilatory endothelial cannabinoid receptor in the human pulmonary artery. *J Hypertens*. 2007;25:2240–2248.
14. Su JY, Vo AC. 2-Arachidonylglyceryl ether and abnormal cannabidiol-induced vascular smooth muscle relaxation in rabbit pulmonary arteries via receptor-pertussis toxin sensitive G proteins-ERK1/2 signaling. *Eur J Pharmacol*. 2007;559: 189–195.
15. Ehinger B. Adrenergic nerves to the eye and its adnexia in rabbit and guinea-pig. *Acta Univ Lundensis*. 1964;2:5–23.
16. Hoste AM, Boels PJ, Andries LJ, Brutsaert DL, de Laey JJ. Effects of beta-antagonists on contraction of bovine retinal microarteries in vitro. *Invest Ophthalmol Vis Sci*. 1990;31:1231–1237.
17. Laties AM. Central retinal artery innervation. *Arch Ophthalmol*. 1967;77:405–409.
18. Ye X, Laties AM, Stone RA. Peptidergic innervation of the retinal vasculature and optic nerve head. *Invest Ophthalmol Vis Sci*. 1990;31:1731–1737.
19. Ferrari-Dileo G, Davis EB, Anderson DR. Biochemical evidence for cholinergic activity in retinal blood vessels. *Invest Ophthalmol Vis Sci*. 1989;30:473–477.
20. Yu DY, Cringle SJ, Balaratnasingam C, Morgan WH, Yu PK, Su EN. Retinal ganglion cells: energetics, compartmentation, axonal transport, cytoskeletons and vulnerability. *Prog Retin Eye Res*. 2013;36:217–246.
21. Delaey C, Van de Voorde J. Retinal arterial tone is controlled by a retinal-derived relaxing factor. *Circ Res*. 1998;83:714–720.
22. Delaey C, Van de Voorde J. Regulatory mechanisms in the retinal and choroidal circulation. *Ophthalmic Res*. 2000;32: 249–256.
23. Offertaler L, Mo FM, Batkai S, et al. Selective ligands and cellular effectors of a G protein-coupled endothelial cannabinoid receptor. *Mol Pharmacol*. 2003;63:699–705.
24. Begg M, Pacher P, Batkai S, et al. Evidence for novel cannabinoid receptors. *Pharmacol Ther*. 2005;106:133–145.
25. DeGrado TR, Holden JE, Ng CK, Raffel DM, Gatley SJ. Measurement of O_2 consumption in isolated organs without venous cannulation. *J Appl Physiol*. 1989;66:1316–1320.
26. Su EN, Yu D-Y, Cringle SJ. Histamine induces opposing vasoactive effects at different levels of the ocular vasculature. *Curr Eye Res*. 2005;30:205–212.
27. Yu DY, Alder VA, Cringle SJ, Su EN, Yu PK. Vasoactivity of intraluminal and extraluminal agonists in perfused retinal arteries. *Invest Ophthalmol Vis Sci*. 1994;35:4087–4099.
28. Yu D-Y, Su EN, Cringle SJ, Alder VA, Yu PK, DeSantis L. Effect of beta blockers and Ca2+ entry blockers on ocular vessels. In: Drance S, ed. *Glaucoma Ocular Blood Flow and Drug Treatment*. Amsterdam: Kugler Publications; 1997;123–134.
29. Yu DY, Su EN, Cringle SJ, Yu PK. Isolated preparations of ocular vasculature and their applications in ophthalmic research. *Prog Retin Eye Res*. 2003;22:135–169.
30. Yu CY, Boyd NM, Cringle SJ, Su EN, Alder VA, Yu DY. Acetylcholine-induced vasodilation of isolated pulpal arterioles. *J Dent Res*. 2001;80:1995–1999.
31. Jarai Z, Wagner JA, Varga K, et al. Cannabinoid-induced mesenteric vasodilation through and endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci U S A*. 1999; 24:14136–14141.
32. Su JY, Vo AC. 2-Arachidonylceryl ether and abnormal cannabinoid-induced vascular smooth muscle relaxation in rabbit pulmonary arteries via receptor-pertussis toxin sensi-

tive G proteins-ERK1/2 signaling. *Eur J Pharmacol*. 2007;559:189–195.

33. Macintyre J, Dong A, Straiker A, et al. Cannabinoid and lipid-mediated vasorelaxation in retinal microvasculature. *Eur J Pharmacol*. 2014;735:105–114.
34. Barrus MT, Reviriego J, Marin J. Effect of Ca^{2+} agonist Bay K 8644 in human placental arteries. *Gen Pharmacol*. 1995;26:989–996.
35. Yu D-Y, Su EN, Cringle SJ, Schoch C, Percicot CP, Lambrou GN. Comparison of the vasoactive effects of the docosaoid unoprostone and selected prostanoids on isolated perfused retinal arterioles. *Invest Ophthalmol Vis Sci*. 2001;42:1499–1504.
36. Yu DY, Su EN, Cringle SJ, Alder VA, Yu PK, DeSantis L. Effect of betaxolol, timolol and nimodipine on human and pig retinal arterioles. *Exp Eye Res*. 1998;67:73–81.
37. Kreutz S, Koch M, Bottger C, Ghadban C, Korf HW, Dehghani F. 2-Arachidonoylglycerol elicits neuroprotective effects on excitotoxically lesioned dentate gyrus granule cells via abnormal-cannabidiol-sensitive receptors on microglial cells. *Glia*. 2009;57:286–294.
38. McHugh D, Hu SS, Rimmerman N, et al. N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC Neurosci*. 2010;11:44.
39. McHugh D, Page J, Dunn E, Bradshaw HB. Delta(9)-tetrahydrocannabinol and N-arachidonyl glycine are full agonists at GPR18 receptors and induce migration in human endometrial HEC-1B cells. *Br J Pharmacol*. 2012;165:2414–2424.
40. Alder VA, Su EN, Yu D-Y, Cringle SJ, Yu PK. Asymmetrical response of the intraluminal and extraluminal surfaces of the porcine retinal artery to exogenous adenosine. *Exp Eye Res*. 1996;63:557–564.
41. Duling BR, Matsuki T, Segal SS. Conduction in the resistance vessel wall: contributions to vasomotor tone and vascular communication. In: Bevan JA, Halpern W, Mulvany MJ eds. *The Resistance Vasculature*. Humana Press; 1991:193–215.
42. Matsuki T, Hynes MR, Duling BR. Comparison of conduit vessel and resistance vessel reactivity: influence of intimal permeability. *Am J Physiol*. 1993;33:H1251–H1255.
43. Kaul S, Waack BJ, Heistad DD. Asymmetry of vascular responses of perfused rabbit carotid artery to intraluminal and abluminal vasoactive stimuli. *J Physiol*. 1992;458:223–234.
44. Su EN, Cringle SJ, McAllister IL, Yu DY. An experimental study of VEGF induced changes in vasoactivity in pig retinal arterioles and the influence of an anti-VEGF agent. *BMC Ophthalmol*. 2012;12:10.
45. Itoh T, Sasaguri T, Makita Y, Kanmura Y, Kuriyama H. Mechanisms of vasodilation induced by vasoactive intestinal polypeptide in rabbit mesenteric artery. *Am J Physiol*. 1985;18:H231–H240.
46. Samata K, Kimura T, Satoh S, Watanabe H. Chemical removal of the endothelium by saponin in the isolated dog femoral artery. *Eur J Pharmacol*. 1986;128:85–91.
47. Tsuji T, Cook DA. Vasoconstrictor mechanism of neuropeptides augmented after endothelial removal in isolated, perfused canine basilar arteries. *Neurol Res*. 1995;17:193–200.
48. Hoi PM, Visintin C, Okuyama M, et al. Vascular pharmacology of a novel cannabinoid-like compound, 3-(5-dimethylcarbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide (VSN16) in the rat. *Br J Pharmacol*. 2007;152:751–764.
49. London A, Benhar I, Schwartz M. The retina as a window to the brain—from eye research to CNS disorders. *Nat Rev Neurol*. 2013;9:44–53.
50. Ogawa R, Ohta T, Tsuji M, Mori M. Role of the endothelium on extraluminal and intraluminal vasoactive mechanisms in the perfused rabbit basilar artery. *Neurol Res*. 1993;15:154–159.
51. Ngai AC, Winn HR. Effects of adenosine and its analogues on isolated intracerebral arterioles—extraluminal and intraluminal application. *Circ Res*. 1993;73:448–457.
52. Stanley C, O'Sullivan SE. Vascular targets for cannabinoids: animal and human studies. *Br J Pharmacol*. 2014;171:1361–1378.
53. Romano MR, Lograno MD. Signaling cross-talk between cannabinoid and muscarinic systems actives Rho-kinase and increases the contractile responses of the bovine ciliary muscle. *Eur J Pharmacol*. 2013;702:174–179.
54. Rouzer CA, Marnett LJ. Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways. *Chem Rev*. 2011;111:5899–5921.

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