

The Retinal Relaxing Factor: Update on an Enigmatic Regulator of the Retinal Circulation

Laura Vanden Daele, Charlotte Boydens, Bart Pauwels, and Johan Van de Voorde

Department of Pharmacology, Ghent University, Ghent, Belgium

Correspondence: Johan Van de Voorde, Department of Pharmacology-Vascular Research Unit, De Pintelaan 185, 9000 Ghent, Belgium; johan.vandevoorde@UGent.be.

Submitted: October 11, 2016

Accepted: February 14, 2017

Citation: Vanden Daele L, Boydens C, Pauwels B, Van de Voorde J. The retinal relaxing factor: update on an enigmatic regulator of the retinal circulation. *Invest Ophthalmol Vis Sci*. 2017;58:1702-1708. DOI: 10.1167/iovs.16-20904

The retinal circulation is regulated by different local factors and might include the retinal relaxing factor (RRF). This factor is found to be continuously released by the retina and relaxes smooth muscle cells. This review describes the current knowledge about the RRF. Despite many research efforts, the cellular source, identity, mechanism, and physiological role of the RRF remain largely unknown. Thus far, it seems that the RRF is a hydrophilic, thermostable, diffusible chemical messenger, which characteristics do not correspond with most well-known endogenous vasorelaxants. The RRF-induced relaxation seems to rely on activation of the inward rectifier K⁺ channels and the Rho kinase Ca²⁺ sensitization mechanism. Voltage-dependent K⁺ channels and plasma membrane Ca²⁺-ATPase might also be involved, whereas the involvement of cyclooxygenase is still a point of discussion. Furthermore, it appears that the RRF is involved in other relaxation pathways, namely those of hypoxia, adenosine, and adenosine triphosphate, hydrogen sulfide, γ -aminobutyric acid, and dorzolamide.

Keywords: retina, retinal blood flow, vasorelaxation, retinal relaxing factor, review

The retina is a layer of the eye with an important role in vision as it converts the incoming light into a neuronal signal. The high demands of oxygen and metabolic substrates require that the blood flow can be regulated to maintain the retinal structure and function. The retina is vascularized by two blood circulations, the choroidal and retinal circulation, with different functional and anatomical characteristics. The choroidal circulation has a superfluous blood flow and is regulated by the autonomic nervous system. The retinal circulation lacks adrenergic, cholinergic, or peptidergic innervation and is mainly regulated by autoregulatory mechanisms and local paracrine factors, such as nitric oxide (NO) and prostaglandins.¹⁻³

In 1998, a new paracrine factor was discovered during experiments on isolated retinal arteries. A huge variation in contractile response to prostaglandin F_{2 α} (PGF_{2 α}) was observed in a study on isolated bovine retinal arteries. Because damage of smooth muscle cells of the arteries during the isolation was thought to be the reason for this variation, efforts were made to reduce damage by cleaning them not too rigorously from adherent tissue. Surprisingly, the arteries then no longer contracted to PGF_{2 α} . In further experiments, it was found that the contraction level of bovine retinal arteries with adhering retinal tissue was significantly lower than of those completely cleaned of adhering retinal tissue. Similar results were obtained when contracting the bovine retinal arteries with other contractile substances than PGF_{2 α} , such as serotonin, endothelin-1, or the thromboxane A₂ mimetic U-46619. One explanation for this observation could be that the retina, like the endothelium releases NO, continuously releases a relaxing factor. To test this hypothesis, a bioassay was set up in which a bovine retinal artery was mounted for isometric tension measurements and contracted with PGF_{2 α} . Once the contraction was stabilized, a piece of bovine retina was brought in proximity of the bovine retinal artery. This elicited a rapid,

complete, and stable relaxation. When the retina was removed, the relaxing influence completely disappeared. The relaxing influence of retinal tissue could be reproduced using retinal tissue of pigs, dogs, and sheep.⁴ A relaxing effect was not seen when bringing bovine choroid tissue in proximity of the bovine retinal artery, illustrating the tissue specificity. On the other hand, retinal tissue also relaxes nonretinal arteries, such as rat renal and mesenteric arteries, and even nonvascular smooth muscle preparations, such as rat main bronchi. This more general relaxing factor was coined the name retinal relaxing factor (RRF).⁴

In the years following these initial observations, a lot of effort was made to reveal the secrets of the RRF. Several studies by different research groups have tried to find out the identity of RRF and the mechanism of the RRF-induced relaxation. However, until now, these questions remain largely unanswered. This review aims to overview the actual knowledge about the RRF.

IDENTITY OF THE RRF

One of the first steps in the effort to discover the identity of the RRF was to incubate a bovine retina in a physiologic solution and to test then whether this solution could elicit a vasorelaxation. This solution could indeed induce vasorelaxation.⁴ Similar results were obtained by Lee et al. using rat retinas.⁵ These experiments prove that the RRF is a diffusible and stable chemical messenger. Hexane extraction, heating the solution up to 70°C or treatment with trypsin did not change the vasorelaxing effect of the RRF, suggesting that the RRF is hydrophilic, thermostable, and not a polypeptide or protein.⁴

Further research concentrated on the possibility that the RRF might be identified as NO, cyclooxygenase (COX) metabolites, or other known vasorelaxants formed in the retina. Based on the characteristics, some molecules could



TABLE. Overview of the Knowledge About the Identity of the RRF

Excluded Molecules	Characteristics
Nitric oxide (confirmed on bovine, rat and mice retina)	Diffusible, stable chemical messenger (confirmed on bovine and rat retina)
Cyclooxygenase products (confirmed on bovine, rat and mice retina, but <i>not</i> on porcine retina)	Hydrophilic (confirmed on bovine retina, but not on rat retina)
Epoxyeicosatrienoic acids (confirmed on rat retina)	Thermostable (confirmed on bovine and rat retina)
Glutamate, glycine, GABA, melatonin, dopamine, l-lactate, adenosine, adrenomedullin, calcitonin gene-related peptide, natriuretic peptides ANP, BNP, and CNP (confirmed on bovine retina)	Resistant to trypsin (confirmed on bovine retina)
Acetylcholine, histamine or other endothelium-dependent vasodilators (confirmed on bovine retina)	
Aspartic acid and taurine (confirmed on rat retina)	

already be excluded from being the RRF. NO can be excluded, because it is not a thermostable molecule. This finding is confirmed by the observation that N ω -Nitro-L-arginine (L-NA), a blocker of NO synthase, does not reduce the relaxation.^{4,6,7} The RRF response was also observed in neuronal NO synthase (nNOS) knockout mice.⁷ Also, the observation that NO as such has a much smaller relaxing influence than the RRF excludes NO as being the RRF.⁸

Whether the RRF is a COX product or not is unclear. In bovine retina indomethacin, a COX inhibitor did not reduce the RRF effect, proving that COX products are not involved. This was confirmed on bovine, rat, and mice retina by using the COX inhibitors indomethacin and sodium diclofenac.^{4,6,7} However, on porcine tissues, the RRF-induced relaxation was found to be completely blocked by ibuprofen, another COX inhibitor.⁹ Epoxyeicosatrienoic acid (EET) could be excluded from being the RRF, because blocking EET synthase with the inhibitors SKF-525A and miconazole had no effect.⁵

The vasorelaxing capacity of different vasorelaxants, known to be released in the retina, were tested on bovine retinal arteries to see whether the RRF corresponded with one of them. Glutamate, glycine, γ -aminobutyric acid (GABA), melatonin, and dopamine all failed to relax the retinal arteries.⁴ Also aspartic acid and taurine failed to relax rat carotid arteries in contrast to the RRF.⁶ l-Lactate was able to relax retinal arteries, but the concentration needed for this relaxation was much higher than usually present in the retina.¹⁰ In addition, the relaxation induced by l-lactate is NO dependent, whereas the RRF-induced relaxation is not.^{11,12} Acetylcholine, histamine, and other endothelium-dependent vasodilators could also be excluded, because the RRF relaxation is endothelium independent. Adenosine did relax bovine retinal arteries,^{2,4} but the adenosine receptor blocker 8-phenyltheophylline could not reduce the RRF relaxation. In addition, adenosine could not relax rat main bronchi, whereas the RRF did.⁴

Also, adrenomedullin was excluded. Adrenomedullin relaxes bovine retinal arteries via an endothelium-dependent mechanism and is inhibited by calcitonin gene-related peptide (CGRP) 8-37, an antagonist of the CGRP₁ receptor. However, the vasorelaxing mechanism of the RRF is not endothelium dependent and is not inhibited by CGRP 8-37.¹³ Also CGRP was studied because it relaxes bovine retinal arteries. However, its mechanism is endothelial NO dependent, in contrast to the RRF. Natriuretic peptides ANP (atrial), BNP (brain), and CNP (C-type) have also been tested, but they were not able to elicit a substantial relaxation.¹⁴ It is also unlikely that proteins, such as vasoactive intestinal peptide, substance P, or somatostatin, are the RRF, because the RRF is not destroyed by trypsin.⁴

In 2010, Lee et al.⁵ reported evidence that the RRF might be identified as methyl palmitate. This was based on analysis by gas chromatography/mass spectrometry of a RRF-containing

solution, obtained by incubation of rat retina. Further evidence was derived from the fact that the release of the RRF and methyl palmitate are both calcium-dependent, that their relaxations rely on activation of voltage-dependent potassium (K_v) channels, and that their relaxations remain after being heated for 1 hour at 70°C.⁵ Furthermore, extraction with hexane reduces the relaxing effect of the RRF and methyl palmitate to the same degree. This result is in contrast to the findings of Delaey et al., who found that the RRF relaxation was similar after hexane extraction.⁴ As yet, the identification of the RRF as methyl palmitate has not been confirmed by other research groups. In contrast, Takir et al. could not detect a relaxing effect of methyl palmitate on bovine retinal arteries.¹⁵ Also, we were unable to confirm that methyl palmitate relaxes arteries and can be identified as the RRF (unpublished observations). Thus, the identity of the RRF remains unknown, which probably means that the right suspect has not been investigated yet. However, the possibility that the right one has been excluded wrongly should also be kept in mind. An overview of what is known about the identity of the RRF thus far is summarized in the Table.

MECHANISMS MEDIATING THE RRF-INDUCED RELAXATION

Adhering retinal tissue causes reduced contraction of arteries, and when a piece of retina is placed on top of an artery, a relaxation is seen. The percentage relaxation depends on the species and arteries being used. When a bovine retina is placed on a bovine retinal artery a complete relaxation is caused. Placing a rat retina on a rat carotid artery causes 33.24% relaxation and mouse retina on a mouse aorta 21.57% relaxation. All relaxations in these studies seem to be monophasic.^{4,6,7} In the study of Takir et al., the relaxation seen by placing a bovine retina on a bovine retinal artery is biphasic, suggesting that there are two mechanisms or factors involved.¹⁵

One of the first mechanisms to be tested was the involvement of the endothelium. Removal of the endothelium did not change the effect of the RRF on bovine retinal arteries.^{4,15} This finding was confirmed for the rat RRF.^{6,15} There is evidence that NO synthase (NOS) and COX are not involved in the RRF-induced relaxation. However, Holmgaard et al. suggested the involvement of a COX product, because inhibition of COX blocked the RRF-induced relaxation completely.⁹ Cyclic guanosine monophosphate (cGMP) seems not to be involved in the RRF-induced relaxation, because no differences in the RRF-induced relaxation were seen by using methylene blue, a blocker of guanylyl cyclase.⁴ 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase (sGC) blocker, also had no effect. This is in accordance

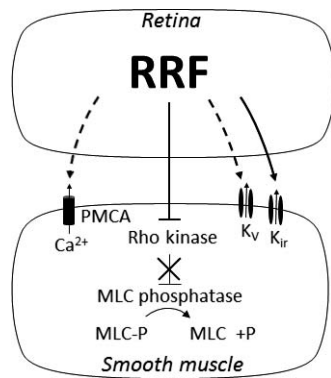


FIGURE 1. Overview of the mechanisms involved in the RRF-induced relaxation. Uncertain connections are represented by *dotted lines* and more confirmed connections by *solid lines*. The RRF may induce relaxation via activation of PMCA, K_v , K_{ir} , and inhibition of Rho kinase, which then no longer inhibits MLC phosphatase, so MLC-P can be dephosphorylated resulting in relaxation.

with the observation that NO donors, which activate sGC, elicit only a small relaxing effect on bovine retinal arteries.^{6–8} Furthermore, the RRF response is still present in sGC α_1 -knockout mice, whereas sGC α_1 is the most important isoform of sGC present in vascular smooth muscle.² Also cAMP is probably not involved, because its levels were not elevated in rat arteries that were relaxed by the RRF.² Holmgaard et al. demonstrated that the RRF-induced relaxation is reduced by DL-amino-5-phosphonovaleric acid, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, suggesting the involvement of NMDA receptors.⁹

The Ca^{2+} -lowering mechanisms leading to smooth muscle relaxation were also studied. Blockade of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) with thapsigargin and cyclopiazonic acid did not influence the RRF-induced relaxation.^{6,16} Also, blocking the Na^+ - Ca^{2+} exchanger with dimethyl-2-thiourea (DMTU) or amiloride had no influence.⁶ L-type Ca^{2+} channels are also not involved, because blocking them with nifedipine did not change the RRF relaxation.¹⁶ Surprisingly, blocking the plasma membrane Ca^{2+} -adenosine triphosphate (ATPase) (PMCA) with vanadate inhibited the RRF-induced relaxation (Fig. 1). However, it should be noted that vanadate is not a specific blocker of PMCA, so the conclusion that PMCA is involved in the RRF-induced relaxation requires further investigation.⁶

Besides Ca^{2+} -lowering mechanisms, a Ca^{2+} sensitization mechanism could be involved in the relaxation caused by the RRF. Inhibition of myosin light chain phosphatase by calyculin A abolished the RRF-induced relaxation completely. There are two important Ca^{2+} sensitization pathways: one via Rho kinase and one via protein kinase C (PKC). Calphostin C, a PKC inhibitor, induced only a weak relaxation in PGF_{2 α} or 120 mM K^+ Krebs-Ringer bicarbonate (KRB) contracted arteries, indicating that the RRF does not act via PKC. On the other hand, Y-27632, a Rho kinase inhibitor, induced a complete relaxation in PGF_{2 α} -contracted arteries and a moderate relaxation in 120 mM K^+ KRB-contracted arteries. This difference in the vasorelaxing effect of Y-27632 is comparable to the vasorelaxing effect of the RRF, indicating that the RRF might interfere with Rho kinase (Fig. 1). Interestingly, the retinal adhering tissue together with Y-27632 can cause an almost complete relaxation in 120 mM K^+ KRB-contracted arteries.¹⁶

Potassium (K^+) channels could be involved in the RRF relaxation, because bovine retinal arteries relax less when contracted with a 120 mM K^+ KRB solution.⁴ It even changes the biphasic RRF-induced relaxation into a monophasic one.¹⁵

Lee et al. tested different K^+ channels inhibitors and observed that blocking voltage-dependent K^+ (K_v) channels with 4-aminopyridine (4-AP) inhibited the RRF-induced relaxation on rat aortas (Fig. 1). A more general K^+ channel blocker, tetraethylammonium (TEA), did the same.⁵ However, Takir et al. could not confirm these observations for bovine RRF, because 4-AP and TEA were unable to block the RRF-induced relaxation.¹⁵ The contradictory results about the involvement of K_v channels could be attributed to the use of different contractile substances (phenylephrine versus PGF_{2 α}) and different species or arteries (rat aorta versus bovine retinal artery).¹⁵ Both studies also tested ATP-sensitive K^+ (K_{ATP}) channels with the blocker glibenclamide and concluded that it had no effect. Calcium-activated K^+ (K_{Ca}) channels were tested by using iberiotoxin on rat retina and charybdotoxin (big and intermediate K_{Ca} blocker) and apamin (small K_{Ca} blocker) on bovine retina. All these blockers did not reduce the RRF-induced relaxation. However, inward rectifier K^+ (K_{ir}) channels seem to be involved (Fig. 1). Their inhibition with barium chloride (BaCl₂) reduced the RRF-induced relaxation and changed the biphasic relaxation in a monophasic one.¹⁶

RETINAL RELAXING FACTOR IN HYPOXIA-INDUCED RELAXATION

Development of several eye diseases, such as glaucoma or diabetic retinopathy, is associated with an impaired oxygen supply. Retinal blood vessels contract in response to hyperoxia and dilate in response to hypoxia. This hypoxic vasodilation can be regulated by direct effects from the blood vessel wall or indirect effects from the surrounding tissue.^{2,17}

Winther et al. found that the hypoxia-induced vasorelaxation was present in porcine retinal arterioles with and without adhering retinal tissue.¹⁸ Delaey et al. provided evidence that the hypoxic vasodilation on bovine retinal arteries is largely regulated by an indirect effect from the retina. The induction of acute hypoxia in bovine retinal arteries without surrounding retinal tissue resulted in only a small relaxation, but when hypoxia was induced in the arteries with surrounding retinal tissue, it resulted in a large relaxation. Reoxygenation reversed this relaxation.¹⁰ This finding was confirmed on pigs, mice, and rats.^{6,7,18,19} Furthermore, it was discovered that direct contact between the retina and the blood vessel was not necessary for the hypoxic vasorelaxation, indicating the involvement of a diffusible factor.¹⁰

The potential involvement of different hypoxia-induced vasorelaxants was tested. NO, COX metabolites, pH, K^+ , adenosine, and excitatory amino acids (GABA, glutamate, aspartic acid, glycine, and taurine) could all be excluded.^{6,10} The hypoxic vasorelaxation was reduced when bovine retinal arteries were contracted with 120 mM K^+ KRB solution, which is in line with the RRF being involved in the hypoxic vasorelaxation. In addition, ATP, or actually the reduction of ATP, could be involved. Blocking the ATP production with iodoacetate or sodium cyanide produced a complete relaxation, which indicated that a reduction of ATP, as occurs during hypoxia, causes vasodilation.¹⁰

Kringelholz et al. reported that the hypoxia-induced relaxation by surrounding retinal tissue was mediated by prostaglandins and NO (Fig. 2).¹⁹ N^o-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a NOS inhibitor, could reduce the hypoxic relaxation, whereas Delaey et al. observed almost no inhibition by L-NA, also a NOS inhibitor.^{10,19} Also, other studies confirmed the finding that NO is involved in the hypoxic relaxation of retinal arteries.^{20,21} Furthermore, it was reported that this relaxation depends on inducible (iNOS) rather than nNOS, because the iNOS inhibitor 1400W could

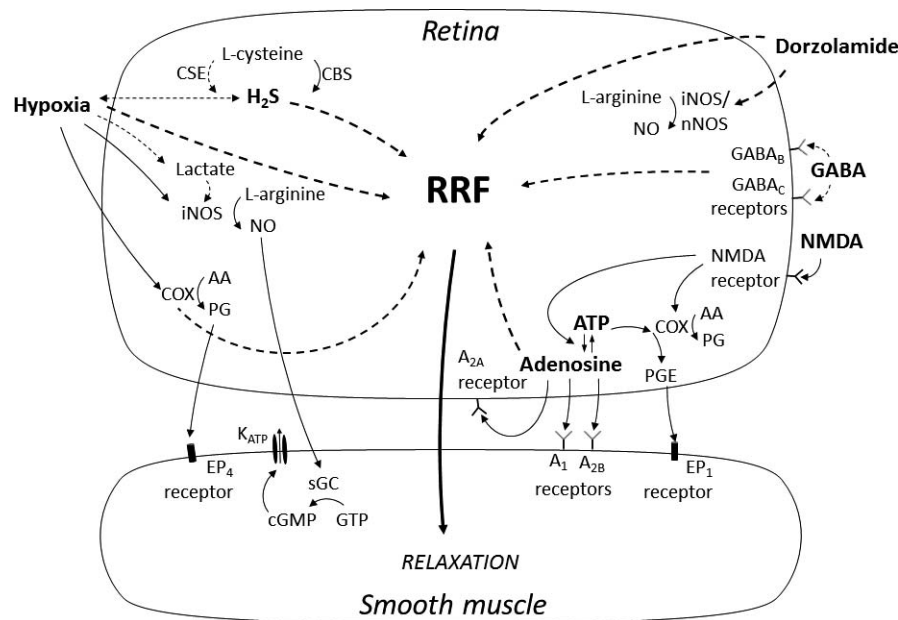


FIGURE 2. Overview of retina-dependent relaxation mechanisms of hypoxia, H_2S , adenosine, ATP, NMDA, GABA, and dorzolamide, of which the relaxation mechanisms may also be related to the RRF. Uncertain connections are represented by *dotted lines* and more confirmed connections by *solid lines*. Hypoxia induces relaxation by activating COX and iNOS (maybe via lactate) in the retina, which leads to the production of PGs from AA and NO from L-arginine. The PGs bind on the EP_4 receptor and NO on SGC, which converts GTP into cGMP, which then activates K_{ATP} . Furthermore, hypoxia-induced relaxation mechanisms may include the RRF, like the H_2S -induced relaxation, which may be linked to the hypoxia-induced relaxation. H_2S is formed in the retina by CBS and possibly CSE. Adenosine can induce relaxation by binding on A_1 and A_{2B} receptors on smooth muscle cells or on the A_{2A} receptor in the retina or by the RRF. Furthermore, adenosine can be transformed into ATP, which stimulates COX to produce PGE that binds on the EP_1 receptor. NMDA also activates COX to produce PG, but not PGE, and it causes hydrolysis of ATP to adenosine. GABA acid binds on the $GABA_B$ or $GABA_C$ receptor in the retina to induce relaxation, which may act via the RRF. Dorzolamide (a carbonic anhydrase inhibitor)-induced relaxation might also relax via the RRF, but also stimulates iNOS or nNOS in the retina, resulting in NO production.

also inhibit the relaxation, in contrast to the nNOS inhibitor 7-nitroindazole. The involvement of endothelial NO synthase (eNOS) could not be excluded.^{22,23} It has even been suggested that lactate formed during hypoxia could stimulate NOS and thus regulate the hypoxic relaxation (Fig. 2).¹¹ However, previous studies showed that L-lactate, which is released from retinal glial and neuronal cells during hypoxia, could only induce a relaxation at high concentrations.^{10,12,24} On the other hand, Hein et al. showed that L-lactate was able to produce a substantial relaxation at physiologic relevant concentrations.¹¹ Furthermore, this lactate-induced relaxation appeared to act via NOS, guanylyl cyclase, and K_{ATP} channel activation (Fig. 2).^{11,12}

Ibuprofen or diclofenac, COX inhibitors, also reduced the hypoxic relaxation, an observation that does not confirm the findings of Delaey et al.^{10,19,22} Interestingly, the lactate-induced relaxation could also be partially blocked by the COX inhibitor indomethacin.¹¹ Further investigation showed that the prostaglandin E_2 receptor 4 (EP_4) was involved and not EP_1 or EP_2 receptors (Fig. 2). Inhibiting iNOS and the EP_4 receptor could not abolish the hypoxia-induced relaxation completely, implying the involvement of other mechanisms in the hypoxia-induced relaxation of retinal arteries.²³

RETINAL RELAXING FACTOR, ADENOSINE, AND ATP

Besides hypoxia, adenosine-induced relaxations are stronger in the presence of perivascular retinal tissue.^{25,26} This suggests that adenosine might promote the release of the RRF.²⁶ Adenosine is formed by the hydrolysis of ATP, which also causes a relaxation of retinal arteries that is enhanced by the retinal tissue.²⁵ Maenhaut et al. reported that the enhancement

of the adenosine-induced relaxation by retinal tissue was larger than that of the ATP-induced one on bovine retinal arteries,²⁶ whereas Holmgaard et al. saw the opposite on porcine retinal arterioles, explaining this by ATP-induced ATP release in the retina.²⁵ This difference might be species related, because Maenhaut et al. reported that the vasorelaxing effect of adenosine was enlarged by using rat retina instead of bovine retina.²⁶ The adenosine-induced relaxation was independent of NOS, COX, and EET synthesis.^{25,26} It was reduced in presence of the 120 mM K^+ KRB solution and by using rat carotid arteries instead of bovine retinal arteries, which is in accordance with the characteristics of the RRF, suggesting an involvement of the RRF in the adenosine relaxation.²⁶ It has been reported that adenosine has both vasoconstrictive and vasorelaxing effects on porcine retinal arteries with perivascular tissue. It induces vasoconstriction by acting on A_3 receptors on the retinal artery and A_1 receptors on the perivascular tissue, whereas it induces relaxation by acting on the A_1 and A_{2B} receptors on the retinal artery and the A_{2A} receptors on the retinal tissue (Fig. 2).²⁷

The ATP-induced relaxation in the presence of the perivascular retina is, in contrast to the adenosine-induced one, mediated by COX, which produces prostaglandin E that acts on EP_1 receptors on the retinal artery. Prostaglandin E is produced by ATP but not by adenosine.²⁸

NMDA can induce the hydrolysis of ATP to adenosine in the retina by binding on its NMDA receptor.²⁹ Besides inducing vasorelaxation via adenosine, NMDA also produces COX products but not prostaglandin E. It is suggested that the RRF is related to these pathways (Fig. 2).²⁸

RETINAL RELAXING FACTOR AND GABA

The GABA-induced relaxation depends on the presence of perivascular retinal tissue.^{9,30,31} Hinds et al. proved that GABA was able to relax rat retinal arteries in the presence but not in the absence of perivascular retinal tissue.³¹ Bek et al. showed similar results, but remarkably GABA was only able to induce relaxation of porcine retina arterioles when the NMDA relaxation pathway was inhibited. Furthermore, the GABA-induced relaxation is mediated via GABA_C receptors in porcine retina,³⁰ whereas the GABA_B receptor is involved in rat retina (Fig. 2).³¹ This difference may be due to the use of different contractile substances (U46619 and DL-APV versus endothelin-1) or the use of different species (porcine versus rat retinal arterioles). However, the rat retinal arterioles could not contract with U46619 and DL-APV, in contrast to the porcine retinal arterioles, indicating that the contradictory results are due to species differences.³¹

RETINAL RELAXING FACTOR AND HYDROGEN SULFIDE

Hydrogen sulfide (H₂S) is endogenously synthesized from L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in a lot of tissues, including bovine eyes.³² The H₂S donor GYY4137 is able to induce a substantial vasorelaxation in porcine retinal arterioles and the H₂S donor NaHS in porcine and bovine retinal arteries.^{18,33} The NaHS-induced relaxation is reduced in the presence of 100 mM K⁺, just like the RRF-induced relaxation. Further research revealed that the H₂S relaxation was endothelium, NO, COX, guanylyl cyclase, and adenylyl cyclase independent, but that K_{ir} and K_v channels are involved. H₂S is probably formed by CBS and not by CSE in bovine retina, because the relaxation induced by L-cysteine could be blocked by a CBS inhibitor but not by a CSE inhibitor.³³ This is in contrast to the findings on porcine retinal arterioles where the CBS and CSE blockers both could inhibit the vasorelaxing influence of L-cysteine. The effect of the CBS and CSE blockers was larger in the presence of retinal tissue and during hypoxia. Therefore, it is suggested that H₂S partially mediates the RRF-induced and hypoxia-induced relaxation on retinal arteries (Fig. 2).¹⁸

RETINAL RELAXING FACTOR AND CARBONIC ANHYDRASE INHIBITORS

Carbonic anhydrases catalyze the association of carbon dioxide and water to carbonic acid, which dissociates spontaneously into bicarbonate and hydrogen ions. This reaction can be catalyzed by carbonic anhydrase in both directions.³⁴ Some inhibitors of carbonic anhydrases are known to relax bovine retinal arteries or porcine retinal arterioles.^{34,35} The relaxing effect of dorzolamide and acetazolamide, in contrast to methyl bromopyruvate and ethyl bromopyruvate, is reduced in the absence of perivascular retinal tissue, indicating that the retina plays a role in their relaxation.^{34,36} The relaxation is not due to inhibition of carbonic anhydrase, and the dorzolamide-induced relaxation is independent of the acidification in the extracellular and intracellular space of the retinal vascular smooth muscle cells. The dorzolamide-induced relaxation acts partially via NO. The origin of NO is as yet unknown, but it might be produced in the retina by nNOS or iNOS or in the vascular endothelium by eNOS. However, other factors of the retina must also be involved in this relaxation, and this might include the RRF (Fig. 2).³⁷

CELLULAR SOURCE OF THE RRF

Flickering light increases the retinal blood flow, indicating that, as in the brain, there exists a neurovascular coupling in the retina. This neurovascular coupling means that when the neural activity increases, in this case by flickering light, the higher demand for nutrients and oxygen is compensated by an increased blood flow through release of retinal vasodilators.^{38–41} The RRF could be one of these retinal vasodilators. Vasorelaxing substances can be released from two cell types of the retina: neurons and glial cells.³ Incubating bovine retina with tetrodotoxin, which inhibits neuronal activity, had no effect on the vasorelaxing effect of the retinal tissue, indicating that the RRF is rather released from retinal glial cells than from retinal neurons.⁴ Misfeldt et al. showed that the ATP-induced relaxation of porcine retinal arterioles, which is retina dependent, is associated with increased calcium activity in perivascular cells that are located external to the smooth muscle cells but internal to the glial cells.^{42,43} These perivascular cells are similar to pericytes, and it is known that pericytes, which are only present on smaller arteries without vascular smooth muscle cells, regulate the blood flow.⁴⁴ Further research is needed to find out if these perivascular cells might be the source of the RRF.

RETINAL RELAXING FACTOR IN RETINAL PATHOLOGY

There is not much known about the RRF in retinal pathology. Hyperglycemia, the hallmark of diabetes, causes macrovascular and microvascular damage, also in retinal vessels.⁴⁵ Agus et al. tested the retina-derived relaxation on carotid and mesenteric arteries of streptozotocin-induced diabetic rats. However, the retina-induced relaxations were similar when using tissues from diabetic and control animals.⁴⁶ The retina-derived relaxations were also evaluated in rats with hypertension induced by L-NAME, because hypertension also causes vascular damage in retinal arteries.^{47,48} The relaxing effect of the RRF was unaltered by hypertension.⁴⁷ Furthermore, the influence of glaucoma, which is characterized by an elevated intraocular pressure and damage of the optic nerve and surrounding retinal tissue, was studied on the RRF response.^{49,50} Glaucoma conditions were induced by elevating the intraocular pressure by cauterization of the episcleral veins. The RRF-induced relaxation was unaltered by glaucoma.⁴⁹ Thus far, no retinal pathology has been associated with an altered RRF response.

CONCLUSIONS

It is clear that the retinal tissue plays an important role in the regulation of the retinal blood flow by releasing relaxing factors from the retina; one of them might be the so-called RRF. The identity of the RRF is as yet elusive (Table), as is the cellular source of the RRF. Its vasorelaxing mechanism is being elucidated bit by bit: Rho kinase seems to be involved, as well as K_{ir} channels (Fig. 1). The RRF might also be involved in vasorelaxation pathways related to hypoxia, adenosine and ATP, hydrogen sulfide, NMDA, GABA, and dorzolamide (Fig. 2). Further research is required to reveal the identity and the mechanism of action of the RRF. This will unmask the physiologic role of the RRF in the regulation of the retinal blood flow.

Acknowledgments

Supported by a grant of the Special Investigation Fund of Ghent University (GOA 01G02410) and the Fund of Research in Ophthalmology (FRO).

Disclosure: **L. Vanden Daele**, None; **C. Boydens**, None; **B. Pauwels**, None; **J. Van de Voorde**, None

References

1. Delaey C, Van de Voorde J. Regulatory mechanisms in the retinal and choroidal circulation. *Ophthalmic Res.* 2000;32:249-256.
2. Maenhaut N, Boussery K, Delaey C, Van de Voorde J. Control of retinal arterial tone by a paracrine retinal relaxing factor. *Microcirculation.* 2007;14:39-48.
3. Pournaras CJ, Rungger-Brandle E, Riva CE, Hardarson H, Stefansson E. Regulation of retinal blood flow in health and disease. *Prog Retin Eye Res.* 2008;27:284-330.
4. Delaey C, Van de Voorde J. Retinal arterial tone is controlled by a retinal-derived relaxing factor. *Circ Res.* 1998;83:714-720.
5. Lee YC, Chang HH, Liu CH, et al. Methyl palmitate: a potent vasodilator released in the retina. *Invest Ophthalmol Vis Sci.* 2010;51:4746-4753.
6. Boussery K, Delaey C, Van de Voorde J. Rat retinal tissue releases a vasorelaxing factor. *Invest Ophthalmol Vis Sci.* 2002;43:3279-3286.
7. Boussery K, Franki AS, Delaey C, Van de Voorde J. A vasorelaxing factor is released from mouse retinal tissue. *Ophthalmic Res.* 2002;34:172-177.
8. Delaey C, Van de Voorde J. The effect of NO donors on bovine retinal small arteries and posterior ciliary arteries. *Invest Ophthalmol Vis Sci.* 1998;39:1642-1646.
9. Holmgaard K, Aalkjaer C, Lambert JDC, Hessellund A, Bek T. The relaxing effect of perivascular tissue on porcine retinal arterioles in vitro is mimicked by N-methyl-D-aspartate and is blocked by prostaglandin synthesis inhibition. *Acta Ophthalmol.* 2008;86:26-33.
10. Delaey C, Boussery K, Van de Voorde J. A retinal-derived relaxing factor mediates the hypoxic vasodilation of retinal arteries. *Invest Ophthalmol Vis Sci.* 2000;41:3555-3560.
11. Hein TW, Xu WJ, Kuo L. Dilation of retinal arterioles in response to lactate: role of nitric oxide, guanylyl cyclase, and ATP-sensitive potassium channels. *Invest Ophthalmol Vis Sci.* 2006;47:693-699.
12. Mendrinou E, Petropoulos IK, Mangioris G, Papadopoulou DN, Stangos AN, Pournaras CJ. Lactate-induced retinal arteriolar vasodilation implicates neuronal nitric oxide synthesis in minipigs. *Invest Ophthalmol Vis Sci.* 2008;49:5060-5066.
13. Boussery K, Delaey C, Van de Voorde J. Influence of adrenomedullin on tone of isolated bovine retinal arteries. *Invest Ophthalmol Vis Sci.* 2004;45:552-559.
14. Boussery K, Delaey C, Van de Voorde J. The vasorelaxing effect of CGRP and natriuretic peptides in isolated bovine retinal arteries. *Invest Ophthalmol Vis Sci.* 2005;46:1420-1427.
15. Takir S, Uydes-Dogan BS, Ozdemir O. Retina evokes biphasic relaxations in retinal artery unrelated to endothelium, K-V, K-ATP, K-Ca channels and methyl palmitate. *Microvasc Res.* 2011;81:295-302.
16. Takir S, Uydes-Dogan S, Ozdemir O. Retina derived relaxation is mediated by Kir channels and the inhibition of Ca²⁺ sensitization in isolated bovine retinal arteries. *Exp Eye Res.* 2015;132:240-248.
17. Cheng RW, Yusof F, Tsui E, et al. Relationship between retinal blood flow and arterial oxygen. *J Physiol Lond.* 2016;594:625-640.
18. Winther AK, Dalsgaard T, Hedegaard ER, Simonsen U. Involvement of hydrogen sulfide in perivascular and hypoxia-induced inhibition of endothelin contraction in porcine retinal arterioles. *Nitric Oxide Biol Chem.* 2015;50:1-9.
19. Kringelholz S, Holmgaard K, Bek T. Relaxation of porcine retinal arterioles during acute hypoxia in vitro depends on prostaglandin and NO synthesis in the perivascular retina. *Curr Eye Res.* 2013;38:965-971.
20. Nagaoka T, Sakamoto T, Mori F, Sato E, Yoshida A. The effect of nitric oxide on retinal blood flow during hypoxia in cats. *Invest Ophthalmol Vis Sci.* 2002;43:3037-3044.
21. Petersen L, Bek T. Diameter changes of retinal arterioles during acute hypoxia in vivo are modified by the inhibition of nitric oxide and prostaglandin synthesis. *Curr Eye Res.* 2015;40:737-743.
22. Kaya M, Petersen L, Bek T. Lack of effect of nitroglycerin on the diameter response of larger retinal arterioles in normal persons during hypoxia. *Graef Arch Clin Exp.* 2016;254:277-283.
23. Hansen PO, Kringelholz S, Simonsen U, Bek T. Hypoxia-induced relaxation of porcine retinal arterioles in vitro depends on inducible NO synthase and EP4 receptor stimulation in the perivascular retina. *Acta Ophthalmol.* 2015;93:457-463.
24. Brazitikos PD, Pournaras CJ, Munoz JL, Tsacopoulos M. Microinjection of L-lactate in the preretinal vitreous induces segmental vasodilation in the inner retina of miniature pigs. *Invest Ophthalmol Vis Sci.* 1993;34:1744-1752.
25. Holmgaard K, Aalkjaer C, Lambert JDC, Bek T. ATP-induced relaxation of porcine retinal arterioles depends on the perivascular retinal tissue and acts via an adenosine receptor. *Curr Eye Res.* 2007;32:353-359.
26. Maenhaut N, Boussery K, Delaey C, Van de Voorde J. Adenosine enhances the relaxing influence of retinal tissue. *Exp Eye Res.* 2009;88:67-78.
27. Riis-Vestergaard MJ, Misfeldt MW, Bek T. Dual effects of adenosine on the tone of porcine retinal arterioles in vitro. *Invest Ophthalmol Vis Sci.* 2014;55:1630-1636.
28. Holmgaard K, Bek T. ATP-induced relaxation of porcine retinal arterioles in vitro depends on prostaglandin E synthesized in the perivascular retinal tissue. *Invest Ophthalmol Vis Sci.* 2010;51:5168-5175.
29. Holmgaard K, Aalkjaer C, Lambert JDC, Bek T. N-methyl-D-aspartic acid causes relaxation of porcine retinal arterioles through an adenosine receptor-dependent mechanism. *Invest Ophthalmol Vis Sci.* 2008;49:4590-4594.
30. Bek T, Holmgaard K. GABA-induced relaxation of porcine retinal arterioles in vitro depends on inhibition from the perivascular retina and is mediated by GABA(C) receptors. *Invest Ophthalmol Vis Sci.* 2012;53:3309-3315.
31. Hinds K, Monaghan KP, Frolund B, McGeown JG, Curtis TM. GABAergic control of arteriolar diameter in the rat retina. *Invest Ophthalmol Vis Sci.* 2013;54:6798-6805.
32. Kulkarni M, Njie-Mbye YF, Okpobiri I, Zhao M, Opere CA, Ohia SE. Endogenous production of hydrogen sulfide in isolated bovine eye. *Neurochem Res.* 2011;36:1540-1555.
33. Takir S, Ortakoylu GZ, Toprak A, Uydes-Dogan BS. NaHS induces relaxation response in prostaglandin F-2 alpha precontracted bovine retinal arteries partially via K-v and K-ir channels. *Exp Eye Res.* 2015;132:190-197.
34. Kehler AK, Holmgaard K, Hessellund A, Aalkjaer C, Bek T. Variable involvement of the perivascular retinal tissue in carbonic anhydrase inhibitor-induced relaxation of porcine retinal arterioles in vitro. *Invest Ophthalmol Vis Sci.* 2007;48:4688-4693.
35. Josefsson A, Sigurdsson SB, Bang K, Eysteinnsson T. Dorzolamide induces vasodilatation in isolated pre-contracted bovine retinal arteries. *Exp Eye Res.* 2004;78:215-221.
36. Topping MS, Holmgaard K, Hessellund A, Aalkjaer C, Bek T. The vasodilating effect of acetazolamide and dorzolamide

- involves mechanisms other than carbonic anhydrase inhibition. *Invest Ophthalmol Vis Sci.* 2009;50:345–351.
37. El-Galaly A, Aalkjaer C, Kringelholm SK, Møller-Madsen MW, Bek T. Dorzolamide-induced relaxation of porcine retinal arterioles in vitro depends on nitric oxide but not on acidosis in vascular smooth muscle cells. *Exp Eye Res.* 2014;128:67–72.
 38. Kotliar KE, Vilser W, Nagel E, Lanzl IM. Retinal vessel reaction in response to chromatic flickering light. *Graefes Arch Clin Exp Ophthalmol.* 2004;42:377–392.
 39. Michelson G, Patzelt A, Harazny J. Flickering light increases retinal blood flow. *Retina.* 2002;22:336–343.
 40. Noonan JE, Lamoureux EL, Sarossy M. Neuronal activity-dependent regulation of retinal blood flow. *Clin Exp Ophthalmol.* 2015;43:673–682.
 41. Newman EA. Glial cell regulation of neuronal activity and blood flow in the retina by release of gliotransmitters. *Philos Trans R Soc Lond B Biol Sci.* 2015;370:20140195.
 42. Møller-Madsen MW, Aalkjaer C, Simonsen U, Bek T. Novel cellular bouton structure activated by ATP in the vascular wall of porcine retinal arterioles. *Invest Ophthalmol Vis Sci.* 2010;51:6681–6687.
 43. Møller-Madsen MW, Pedersen SMM, Bek T. Perivascular cells with pericyte characteristics are involved in ATP- and PGE(2)-induced relaxation of porcine retinal arterioles in vitro. *Invest Ophthalmol Vis Sci.* 2013;54:3258–3264.
 44. Trost A, Lange S, Schroedl F, et al. Brain and retinal pericytes: origin, function and role. *Front Cell Neurosci.* 2016;10:20.
 45. Watkinson S, Seewoodhary R. Ocular complications associated with diabetes mellitus. *Nurs Stand.* 2008;22:51–57; quiz 8, 60.
 46. Agus M, Takir S, Uydes-Dogan BS, Karaca C, Altug T, Ozdemir O. Retina derived relaxation is preserved in carotid and mesenteric arteries of diabetic rats. *Fund Clin Pharmacol.* 2008;22(Suppl 2):81.
 47. Gezerler F, Takir S, Ilkay Alp F, et al. Retina derived relaxations are maintained in carotid and mesenteric arteries of L-NAME induced hypertensive rats. *J Vasc Res.* 2009;46(Suppl 1):151.
 48. Lehmann MV, Schmieder RE. Remodeling of retinal small arteries in hypertension. *Am J Hypertens.* 2011;24:1267–1273.
 49. Takir S, Gurel-Gurevin E, Toprak A, Demirci-Tansel C, Uydes-Dogan BS. The elevation of intraocular pressure is associated with apoptosis and increased immunoreactivity for nitric oxide synthase in rat retina whereas the effectiveness of retina derived relaxing factor is unaffected. *Exp Eye Res.* 2016;145:401–411.
 50. Venkataraman ST, Flanagan JG, Hudson C. Vascular reactivity of optic nerve head and retinal blood vessels in glaucoma: a review. *Microcirculation.* 2010;17:568–581.