Vasorelaxing Effect of Resveratrol on Bovine Retinal Arteries

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Purpose. Resveratrol is a red wine polyphenol that causes vasorelaxation, which could be of interest in the treatment or prevention of eye diseases with an impaired blood flow. In this study, the vasorelaxant capacity of resveratrol (cis and trans) on bovine retinal arteries, its vasorelaxing mechanism, and its influence on the relaxation induced by the retinal relaxing factor (RRF) were examined.

Methods. Isolated bovine retinal arteries were mounted into wire myographs for isometric tension measurements. Concentration-response curves of cis- and trans-resveratrol and concentration-response curves of resveratrol in the absence or presence of the endothelium or different inhibitors were constructed. Relaxations elicited by the RRF with and without resveratrol incubation were also compared.

Results. Both resveratrol isomers caused a similar strong concentration-dependent relaxation. Removal of the endothelium or blocking endothelium-dependent pathways did not change the relaxation. Also, K^+ channel blockers did not reduce the relaxation, except the 120 mM K^+ Krebs Ringer bicarbonate solution. Phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate blocked the relaxation partially and so did the inhibition of heme oxygenase-1. Blocking adenylyl cyclase, AMP-activated protein kinase, estrogen receptors, sirtuin 1, or sarco/endoplasmic reticulum Ca^{2+} ATPase did not have an effect. The relaxation caused by the RRF was not altered by resveratrol incubation.

Conclusions. Cis- and trans-resveratrol relax bovine retinal arteries similarly and concentration dependently. The main relaxation mechanism remains unclear, but K^+ channels, carbon monoxide, and the myosin phosphatase pathway may be involved. Resveratrol does not have an influence on the RRF.

Keywords: resveratrol, retinal artery, vasorelaxation

In an era with huge interest in healthy food and nutraceuticals, much attention is given to resveratrol, a polyphenolic compound present in the skin of grapes and thus in red wine.[1–3] Resveratrol is also found in other foods, such as cranberries and peanuts. However, it is best absorbed from the wine matrix. The interest in resveratrol has exploded since resveratrol was advanced as being responsible for the cardiovascular benefit of moderate red wine consumption.[2] This hypothesis originates from the so-called French paradox, the lower incidence of cardiovascular diseases in the French population despite their higher intake of dietary saturated fat.[1,4] The potential beneficial effects of resveratrol are explained by many studies showing that resveratrol has vasorelaxant, antioxidant, antiapoptotic, antitumorigenic, antiangiogenic, and anti-inflammatory properties.[5]

These properties are also of interest for diseases of the eye. Many studies have already focused on the potential effect of resveratrol on eye diseases. It has been proven that resveratrol causes a decrease in the expression of glaucoma markers as a result of its antioxidant, anti-inflammatory, and antiapoptotic properties.[6] Resveratrol also reduces diabetic retinopathy[7] and apoptosis and/or oxidative stress in models of (diabetic) cataract.[8–10] Furthermore, resveratrol protects retinal pigment epithelial cells against oxidative stress, which is involved in the pathogenesis of age-related macular degeneration.[11] Resveratrol also inhibits the tumor growth of uveal melanoma and retinoblastoma and the neovascularization in animal models of retinopathy of prematurity and macula telangiectasia.[12–15]

Resveratrol is also well known for its vasorelaxing influence. However, studies on its effect on retinal circulation are scarce despite the fact that the vasorelaxant activity of resveratrol would be beneficial to treat or prevent eye diseases associated with an impaired blood flow, such as glaucoma, age-related macular degeneration, and diabetic retinopathy.[5] As yet only one study has reported that resveratrol relaxes retinal arteries; porcine retinal arterioles are relaxed by resveratrol, and this occurs through both an endothelium-dependent (nitric oxide [NO]-mediated) and endothelium-independent (large-conductance Ca^{2+}-activated K^+ channels-mediated) mechanism.[16]

The aim of the present study was to investigate whether resveratrol also relaxes retinal arteries from another species, namely bovine retinal arteries; potential differences between cis- and trans-resveratrol; the potential mechanisms involved in the vasorelaxing action of resveratrol; and a potential influence of resveratrol on the continuously released retinal relaxing factor (RRF).[17–19]

Methods

Tissue Preparation

Bovine eyes were obtained from the local slaughterhouse and were transported and stored in cold Krebs Ringer bicarbon-

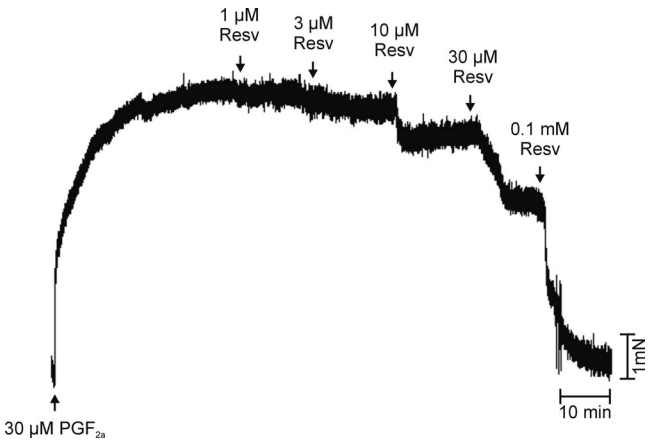

FIGURE 1. Original tracing showing the relaxation curve of (trans-) resveratrol (Resv) on 30 µM $PGF_{2\alpha}$ contracted bovine retinal arteries.

ate (KRB) solution. Extraocular muscles and connective tissue were cut off the eye. The eyecup was then cut open and the anterior segment and the vitreous were removed. Under a dissection microscope, the part of the retinal artery between the optic disc and the first branching of the artery was gently detached with its surrounding retinal tissue from the choroid.

The retinal arterial segments were mounted into wire myographs (constructed by the technical department of the research unit) for isometric tension measurements containing 10 mL KRB solution (37°C, pH 7.4, and bubbled with 95% O_2–5% CO_2). Two stainless steel wires of 40 µm diameter were guided through the lumen of the vessel. One wire was connected to a force-displacement transducer and the other to a micrometer. Before guiding the wires through the lumen of the artery, the adhering retinal tissue was carefully removed. The femoral arteries were dissected from Swiss mice (8–12 weeks, from Janvier, Saint-Berthevin, France) and cleaned from its surrounding tissue after guiding the first wire through the lumen of the artery.

The arterial segments were allowed to equilibrate for 30 minutes in the KRB solution before their normalization. During the normalization process, the passive wall tension–internal circumference characteristics of the vessels were determined. The internal circumference of the vessels was then set to 90% of the internal circumference of the vessels at a transmural pressure of 100 mmHg.

At the start of each experiment, the arteries were three times contracted by adding 120 mM K^+ KRB solution and 30 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the organ bath. Next, 0.1 mM acetylcholine (Ach) was added to a retinal artery contracted with 30 µM $PGF_{2\alpha}$ to test the functionality of the endothelium.[17,19]

Experimental Protocols

To test the potential difference in relaxation between cis- and trans-resveratrol, concentration-response curves of cis- and trans-resveratrol (1–100 µM) were performed on the same $PGF_{2\alpha}$-contracted bovine retinal arteries. To investigate the relaxation mechanism, the concentration-response curves of resveratrol were performed on arteries with or without endothelium or in the presence or absence of blockers of certain pathways. The influence of resveratrol on the RRF response was tested by comparing the relaxing effect, elicited by placing retinal tissue on the precontracted artery with and without incubation of the retina and artery with resveratrol and performed on the same artery with the same retina. Because placing a piece of bovine retina on bovine retinal arteries caused 100% relaxation and so a possible enlargement of the relaxing effect could not be detected, mouse retinas and mice femoral arteries were used instead, as previously described.[20]

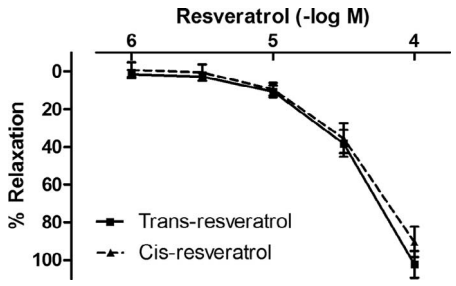

FIGURE 2. Percentage relaxation of bovine retinal arteries caused by trans- and cis-resveratrol (1–100 µM); $n = 4$. $^*P < 0.05$.

Drugs and Chemicals

The KRB solution contained the following components (mM): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2, and EDTA 0.026 in H_2O. The 120 mM K^+ and 30 mM K^+ KRB solutions were made by equimolar replacement of NaCl by KCl. (Trans-) resveratrol, Ach, tetraethylammonium (TEA), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, 4-aminopyridine (4-AP), glibenclamide, 1H-[1,2, 4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), phorbol 12-myristate 13-acetate (PMA), compound C, zinc protoporphyrin IX (ZnPP), fulvestrant (ICI 182780), cyclopiazonic acid (CPA), niacinamide, PD98059, SQ 22,536, and EX-527 were obtained from Sigma-Aldrich (St. Louis, MO, USA), $PGF_{2\alpha}$ from Zoetis (Florham Park, NJ, USA), phorbol 12,13-dibyturate (PDBu) from Axon MedChem (Groningen, The Netherlands), cis-resveratrol from Cayman (Ann Harbor, MI, USA) and papaverine from Sterop (Brussels, Belgium). All stock solutions were made in DMSO, except those of $PGF_{2\alpha}$, Ach, TEA, 4-AP, L-NAME, niacinamide, and papaverine, which were made in water, indomethacin was made in ethanol, and ZnPP was made in 0.1M NaOH.

Data Analysis

The data were computed as mean ± SEM and evaluated statistically using a Wilcoxon test. Two groups of data were considered significantly different if $P < 0.05$. Relaxations are expressed in percent decrease of the preexisting tone elicited by $PGF_{2\alpha}$ (n = number of preparations tested from all different animals).

Results

Effect of Trans- and Cis-Resveratrol

Resveratrol relaxed bovine retinal arteries clearly concentration dependently (Fig. 1). A rapid and almost complete relaxation was reached at the concentration of 0.1 mM (trans-resveratrol: 102.27% ± 7.09; cis-resveratrol: 90.44% ± 8.08). The EC_{50} of trans- and cis-resveratrol was, respectively, 31.67 µM ± 9.64 and 39.54 µM ± 8.19 (Fig. 2). Because there was no significant difference detected, further experiments were performed using only trans-resveratrol.

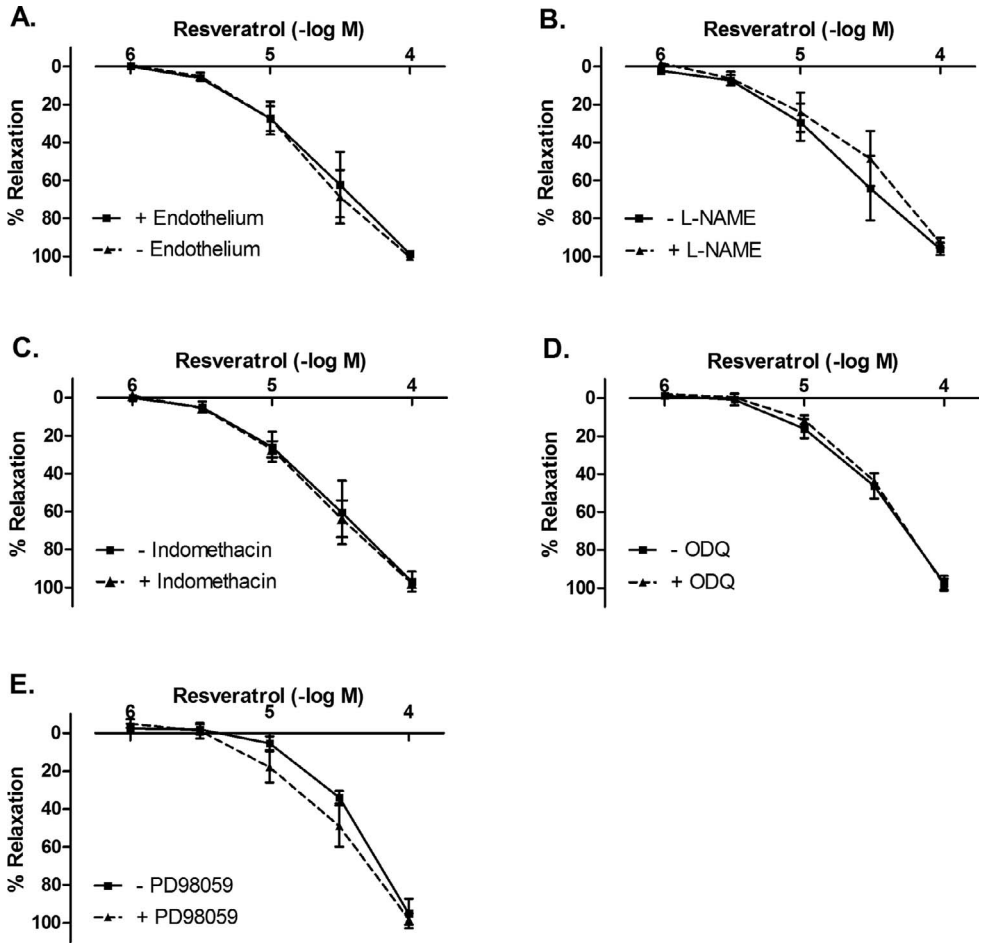

FIGURE 3. Percentage relaxation of bovine retinal arteries caused by resveratrol (1–100 μM) in the presence or absence of the endothelium (**A**), L-NAME (0.1 mM) (**B**), indomethacin (10 μM) (**C**), ODQ (10 μM) (**D**), PD98059 (10 μM) (**E**); $n = 4$. $^*P < 0.05$.

Role of Endothelium and NO

Removal of the endothelium, performed by rubbing a hair through the lumen and considered successful if 0.1 mM acetylcholine did not induce a relaxation, did not reduce the relaxation (Fig. 3A). Also, the incubation with the NO-synthase inhibitor L-NAME (0.1 mM, 10 minutes), the soluble guanylyl cyclase (sGC) inhibitor ODQ (10 μM, 20 minutes), the cyclooxygenase (COX) inhibitor indomethacin (10 μM, 20 minutes), or the mitogen-activated protein kinases / extracellular signal-regulated kinases (MAPK/ERK) inhibitor PD98059 (10 μM, 20 minutes) did not change the relaxation (Fig. 3B–E).

Role of K^+ Channels

The 120 mM K^+ KRB solution (20 minutes) inhibited the resveratrol relaxation slightly. However, a significant difference was detected at the concentration of 0.1 mM resveratrol (97.02% ± 3.08 vs. 82.88% ± 4.74) (Fig. 4A). The nonselective K^+ channel blocker TEA (10 mM, 20 minutes), the voltage-gated K^+ channel blocker 4-AP (2 mM, 20 minutes), and the ATP-sensitive K^+ channel blocker glibenclamide (10 μM, 20 minutes) did not reduce the relaxation (Fig. 4B–D). The relaxation in the presence of glibenclamide was even somewhat increased, probably a result of the impaired precontractile tone.

Role of Protein Kinase C

Both protein kinase C (PKC) activators PMA (10 μM, 20 minutes) and PDBu (1 μM, 20 minutes) decreased the relaxation caused by resveratrol (Fig. 5A, 5B). The presence of PMA caused a small but significant increased relaxation in response to 3 μM of resveratrol, but a large significant decreased relaxation in response to 100 μM of resveratrol (89.51% ± 5.40 vs. 60.45% ± 5.03). PDBu significantly reduced the relaxation at resveratrol concentrations of 1 μM, 30 μM, and 100 μM (81.31% ± 7.16 vs. 48.23% ± 7.21 at 100 μM resveratrol).

Role of Other Mediators

The adenylyl cyclase (AC) blocker SQ 22,536 (0.1 mM, 20 minutes) and the AMP-activated protein kinase (AMPK) blocker compound C (10 μM, 20 minutes) did not reduce the resveratrol-induced relaxation (Fig. 6A, 6B). In addition, neither did the estrogen receptor blocker fulvestrant (0.1 mM, 20 minutes), nor the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker CPA (10 μM, 20 minutes), nor the sirtuin 1 blockers EX-527 (5 μM, 15 minutes) and niacinamide (10 mM, 20 minutes) (results not shown) reduce the resveratrol relaxation (Fig. 6C–E). The incubation with fulvestrant reduced the contractile tone, explaining the larger relaxation percentages. ZnPP (10 μM, 60 minutes), a blocker of heme oxygenase-

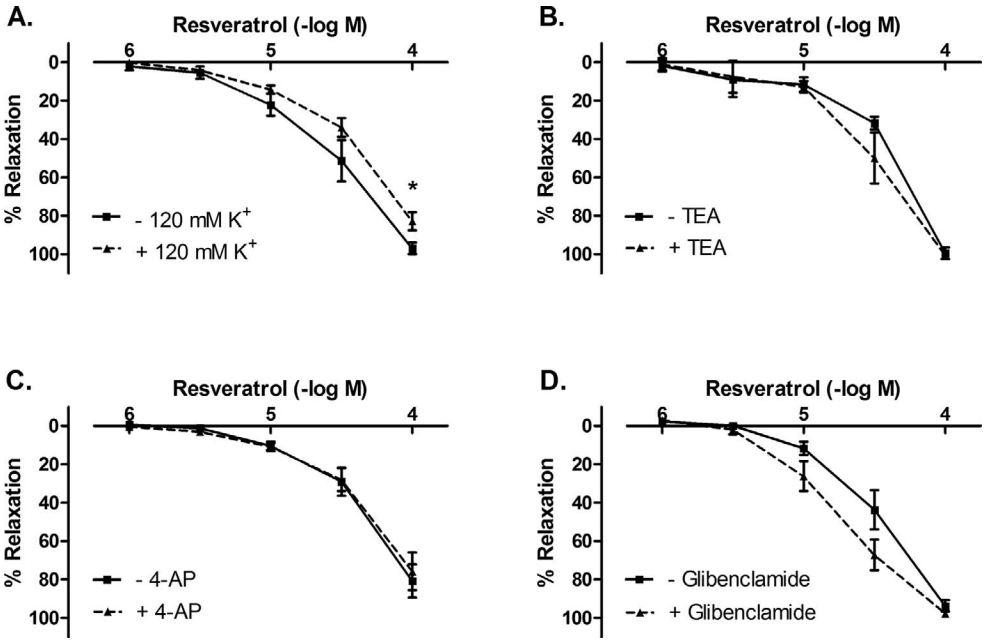

FIGURE 4. Percentage relaxation of bovine retinal arteries caused by resveratrol (1–100 μM) in the absence or presence of 120 mM K^+ KRB solution (**A**), TEA (10 mM) (**B**), 4-AP (2 mM) (**C**), glibenclamide (10 μM) (**D**); $n = 4$ to 6. $^*P < 0.05$.

1 (HO-1), did cause a significant reduction at resveratrol concentrations of 3 μM, 10 μM, and 100 μM (95.50% ± 1.22 vs. 78.37% ± 8.48 at 100 μM resveratrol) (Fig. 6F).

Influence of Resveratrol on the RRF

The relaxation of mice femoral arteries caused by placing a mouse retina on top of the femoral artery (as previously described[20]) was similar before and after incubating the retina and femoral artery with resveratrol (0.1 mM, 15 minutes) (24.91% ± 2.35 and 24.40% ± 4.91, respectively) (Fig. 7).

DISCUSSION

Our study demonstrates for the first time that resveratrol has a very prominent relaxing influence on bovine retinal arteries. Relaxation starts at a concentration of 3 μM and is almost complete at a concentration of 0.1 mM, similar to the relaxation on porcine retinal arterioles.[16] Retinal arteries have approximately the same sensitivity to resveratrol as porcine and sheep coronary arteries and rat aortas because their EC_{50} is similar.[21–23] The EC_{50} turns out to be higher for human internal mammary arteries and lower for rat abdominal aortas and rat mesenteric arteries.[24–26]

Resveratrol exists as both cis- and trans-isomers. Cis-resveratrol is found only in small amounts in grapes because trans-resveratrol is the more stable natural form. However, trans-resveratrol can be transformed into the cis-isomer by ultraviolet irradiation or yeast isomerases during fermentation, with the result that cis-resveratrol is present in wine at variable concentrations.[5,27–29] Differences in effectivity of the isomers have been reported. Several studies provided evidence that the antioxidant capacity of trans-resveratrol is the strongest, whereas other studies showed a similar effect.[27,30,31] Furthermore, trans-resveratrol has been shown to be a stronger inhibitor of platelet aggregation, angiogenesis, and tumor growth in vivo than cis-resveratrol, and only trans-resveratrol is able to inhibit vascular inflammation.[32–34] The present study shows that both isomers have a similar vasorelaxing effect, at least in bovine retinal arteries. To the best of our knowledge, isomer sensitivity has not yet been studied on other blood vessels.

Many studies have shown that the vasorelaxing effect of resveratrol depends at least partially on the presence of endothelium and the formation of NO.[16,21,25] On porcine retinal arteries, the relaxation of resveratrol is partially mediated by NO released from the endothelium.[16] However, our study provides no evidence for that in bovine retinal arteries. Removal of the endothelium, blocking NO-synthase with L-NAME or blocking COX with indomethacin, does not

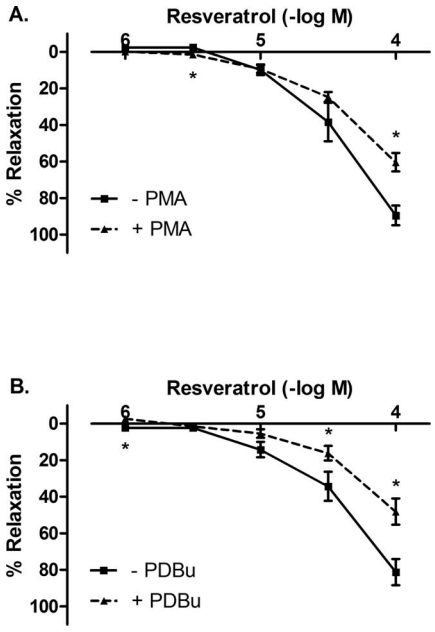

FIGURE 5. Percentage relaxation of bovine retinal arteries caused by resveratrol (1–100 μM) in the absence or presence of PMA (10 μM) (**A**), PDBu (1 μM) (**B**); $n = 5$ to 6. $^*P < 0.05$.

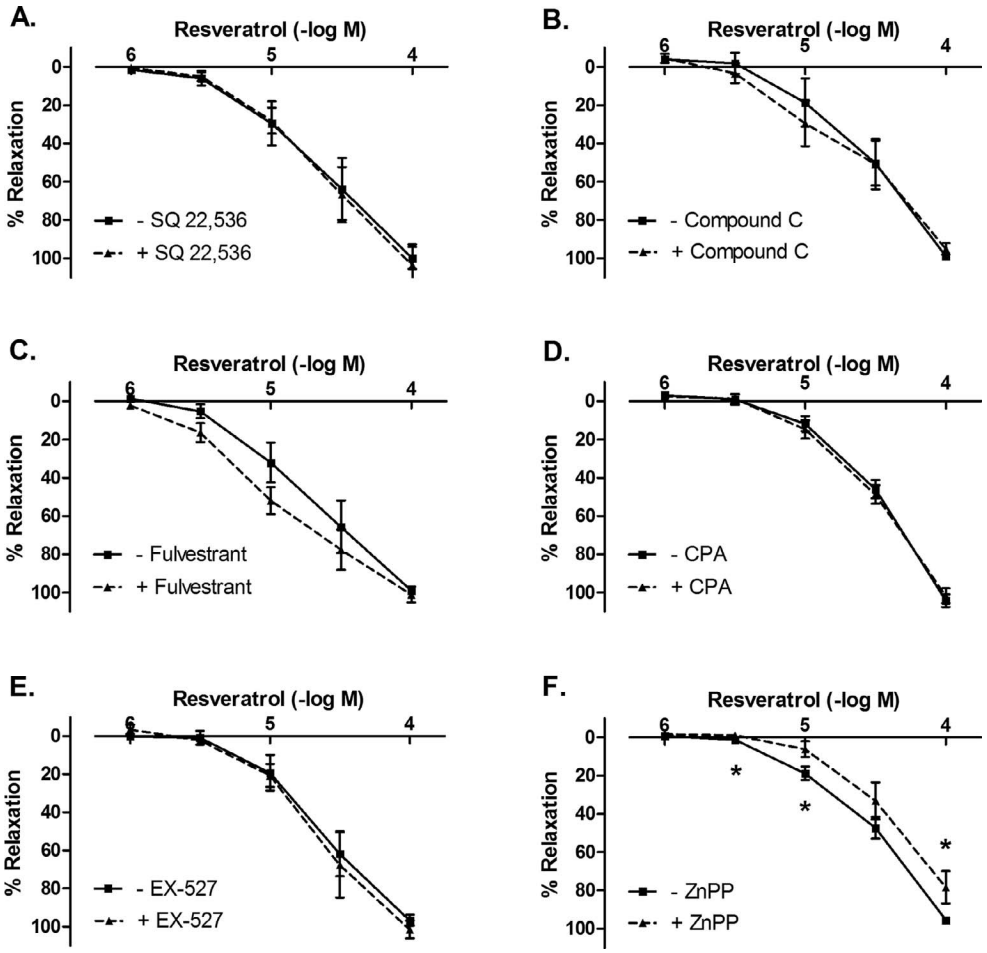

FIGURE 6. Percentage relaxation of bovine retinal arteries caused by resveratrol (1–100 μM) in the absence or presence of SQ 22,536 (0.1 mM) (**A**), compound C (10 μM) (**B**), fulvestrant (0.1 mM) (**C**), CPA (10 μM) (**D**), EX-527 (5 μM) (**E**), ZnPP (10 μM) (**F**); $n = 4$ to 6; $^*P < 0.05$.

alter the relaxation in response to resveratrol. However, it should also be noted that on other blood vessels L-NAME failed to block the resveratrol relaxation[35,36] and COX has not yet been reported to be involved in the relaxation caused by resveratrol.[16,21,25,35,37] It has also been revealed that the release of NO in porcine retinal arteries is activated by the MAPK/ERK pathway and that NO activates then sGC.[16] Also, these mediators do not seem to be involved in the relaxation on bovine retinal arteries.

The endothelium-independent pathway of resveratrol on porcine retinal arterioles is mediated by large-conductance Ca^{2+}-activated K^+ channels, the nonselective K^+ channel blocker TEA blocked the relaxation significantly.[16] This is not seen in bovine retinal arteries. Incubation with 120 mM K^+ KRB solution reduced the relaxation caused by 0.1 mM resveratrol minimally, indicating only a small involvement of K^+ channels. In other tissues, resveratrol relaxations could be partially blocked by glibenclamide or 4-AP, even when TEA failed to block the relaxation.[26,38] However, this was not the case with bovine retinal arteries. All used K^+ blockers failed to block the relaxation of resveratrol. This is in line with what has been reported on mouse corpus cavernosum.[35]

Resveratrol has also been reported to form cGMP through particulate or membrane-bound guanylyl cyclase (pGC) and not through sGC.[22] Because pGC is blocked by PKC, the PKC activators PMA and PDBu were used to inhibit pGC.[39] Both reduced the relaxation of resveratrol significantly, with PDBu having the strongest effect. From this it could be concluded that resveratrol induces relaxation partially via pGC. However, earlier research on bovine retinal arteries showed very little relaxation in response to cGMP.[40] Therefore, it is unlikely that pGC plays a substantial role in the strong resveratrol relaxation. It should be mentioned that the activation of PKC also has other effects besides blocking pGC, such as inactivating myosin phosphatase (as does Rho-kinase), which then can no longer dephosphorylate the light chain of myosin to induce relaxation.[41] Therefore, an interaction of resveratrol with the myosin phosphatase pathway could be involved in the relaxation effect of resveratrol. Rho-kinase inhibition has previously been reported to make a major contribution to the resveratrol-induced vasorelaxation.[42] We tried to test this influence by blocking Rho-kinase with Y-27632 dihydrochlo-

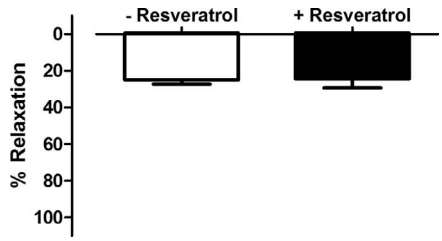

FIGURE 7. Percentage relaxation of mice femoral arteries caused by the RRF in the absence or presence of resveratrol (0.1 mM); $n = 4$. $^*P < 0.05$.

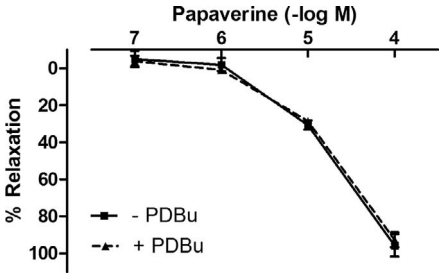

FIGURE 8. Percentage relaxation of bovine retinal arteries caused by papaverine (0.1–100 μM) in the absence or presence of PDBu (1 μM).

ride, but the incubation caused loss of contractile tone, which could be explained by the fact that Y-27632 dihydrochoride is a strong dilator of $PGF_{2\alpha}$-contracted retinal arteries.[43] To be sure that the inhibition of the resveratrol relaxation by PMA or PDBu is not aspecific, experiments were carried out with papaverine, a strong dilator of bovine retinal arteries.[40] Relaxations in response to papaverine were not affected by PDBu (Fig. 8), proving that PDBu blocks the resveratrol relaxation in a specific way.

The potential involvement of cAMP was also verified, although resveratrol activates rather cGMP than cAMP.[22] Indeed, the resveratrol relaxation was not changed by inhibiting AC with SQ 22,536. Furthermore, it has been described that resveratrol interacts with SERCA, AMPK, sirtuin 1, and the estrogen receptor,[44–48] but none of these seem to be involved in the relaxation as respectively, CPA, compound C, EX-527, niacinamide, and fulvestrant did not alter the resveratrol relaxation. It has been described that resveratrol activates HO-1,[49] which can be confirmed because the relaxation was a little, but significantly, reduced by ZnPP, a HO-1 blocker. HO-1 catalyzes the degradation of heme, which results in the production of CO.[50]

So the relaxation mechanisms of resveratrol on porcine retinal arterioles seem to differ from those on bovine retinal arteries.[16] However, it should be mentioned that besides species and caliber differences, the experimental setup was also different, which might have influenced the results. In our study, the arteries were mounted into a wire myograph for isometric force measurements, in the other study the arteries were cannulated for isobaric diameter measurements. The arteries in the wire mygraph show only a passive tension, so a vasoconstrictor has to be added first to see a vasorelaxing effect. The cannulated arteries in the pressure myograph have already a certain tone, probably a result of myogenic autoregulatory mechanisms,[51] so a preconstrictor is not needed to detect vasorelaxing effects.

It has been shown that the presence of retinal tissue influences the tone of retinal arteries by releasing a relaxing factor (so called retinal relaxing factor, RRF).[17,18] The identity of the RRF is still a matter of debate, but Takir et al. investigated the vasorelaxing mechanism of the RRF. An induction of relaxation by activating inward rectifier K^+ channels and an inhibition of contraction by inhibiting the Ca^{2+} sensitization pathway probably via the Rho-kinase pathway was suggested.[43] Changes in the RRF pathway could affect the retinal blood flow. Therefore, in bioassay experiments we also investigated whether resveratrol influences the relaxing effect of RRF elicited by placing mouse retinal tissue on a precontracted mouse femoral artery. However, the RRF-induced relaxing effect was not influenced by incubation with resveratrol.

In conclusion, trans- and cis-resveratrol relax bovine retinal arteries similarly and clearly concentration dependently. The relaxation mechanism of resveratrol on these arteries is still unclear and differs from that on porcine retinal arteries. There might be a small involvement of K^+ channels, CO, and the myosin phosphatase pathway. Further mechanisms explaining the complete relaxation caused by resveratrol remain to be discovered. In addition, resveratrol does not seem to influence the relaxing influence of the RRF.

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