Beta-Adrenoreceptor Agonism Influences Retinal Responses to Hypoxia in a Model of Retinopathy of Prematurity

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Purpose. In a mouse model of oxygen-induced retinopathy (OIR), a well-established model of retinopathy of prematurity (ROP), blocking beta-adrenoreceptors (β-ARs), and, in particular, β2-ARs, counteracts retinal responses to hypoxia. In the present work, we determined the effects of the β-AR agonist isoproterenol on retinal angiogenesis and β-AR signaling to better clarify the role of sympathetic transmission in ROP.

Methods. Isoproterenol was administered subcutaneously. Protein kinase A activity was determined by a colorimetric assay to assess drug effectiveness. Blood pressure and heart-to–body weight ratio were measured. Vascular endothelial growth factor (VEGF) and norepinephrine were measured with ELISA. Retinal neovascularization was assessed by CD31 immunohistochemistry. β-AR-coupled adenylyl cyclase (AC) activity was measured with a competition assay. β-ARs, G-protein-coupled receptor kinase (GRK)2, and β-arrestins were determined by Western blot. Association of β-arrestins with β2-ARs was assessed by immunoprecipitation.

Results. Isoproterenol-induced modulation of protein kinase A activity suggests that the drug was effective at the receptor level. Isoproterenol did not affect cardiovascular parameters, but decreased retinal levels of VEGF and reduced pathogenic neovascularization, likely through an influence on sympathetic transmission. In fact, isoproterenol downregulated β2-AR expression, recovered the hypoxia-induced increase in β-AR–coupled AC activity, and increased GRK2 and β-arrestins, which promote β-AR desensitization through the uncoupling of G-protein-coupled receptors from G proteins. Immunoprecipitation studies demonstrated that β-AR desensitization involved β2-ARs.

Conclusions. Our findings suggest that hypoxia-induced retinal neovascularization depends at least in part on increased sympathetic transmission, as reduction of sympathetic drive by agonist-induced β2-AR desensitization inhibits some of the hallmarks of OIR. (*Invest Ophthalmol Vis Sci.* 2012;53:2181–2192) DOI:10.1167/iovs.11-9408

Retinopathy of prematurity (ROP) is a serious threat to vision in preterm newborns and is the leading cause of blindness in the pediatric age. ROP progresses in two phases. Of them, the second phase of hypoxia-induced pathological hypervascularization occurs on the surface of the retina (preretinal neovascular tuft formation) and causes functional impairment and severe vision loss.[1,2]

Most recent therapeutic interventions against ROP have focused on the mechanisms and the factors leading to new vessel growth. Of them, vascular endothelial growth factor (VEGF) plays a major role in the pathogenic blood vessel formation that characterizes ROP.[2]

There is some evidence that angiogenesis is controlled by the adrenergic system through its regulation of proangiogenic factors.[3] For instance, in several cancer cell lines, either norepinephrine (NE) or the beta-adrenoreceptor (β-AR) agonist, isoproterenol, induces the upregulation of both VEGF and the hypoxia-inducible factor-1α, which is abolished by cell treatment with propranolol, a β-AR blocker.[4]

In several systems, hypoxia causes catecholaminergic overstimulation, which in turn alters signaling pathways associated with β-ARs.[5,6] In this respect, there are indications that distinct β-ARs are expressed in the rat retina.[7] In the mouse retina, β3-ARs are localized to the inner capillary network,[8] whereas β2-ARs are localized to several retinal cells, including Müller cells suggesting that β2-ARs play a role in regulating VEGF production by these cells.[9]

If one considers that β-AR stimulation upregulates VEGF[10,11] and that the second phase of ROP is supported by increased VEGF production,[2] then treatments intended to restore physiological sympathetic activity in the eye may lead to a reduction in the complications of ROP. Whether VEGF accumulation in ROP can be induced by β-AR stimulation, then β-AR blockers may be useful in ROP treatment.[12] This possibility is supported by the observation that propranolol reduces the growth of infantile capillary hemangiomas, the most common tumor of infancy,[3] and that hemangiomas are associated with ROP, suggesting a possible pathogenic relationship between the two diseases.[13]

Although the rat model of oxygen-induced retinopathy (OIR) produces a more humanlike pathology, the mouse model of OIR is the most widely used model to mimic ROP, as it very closely recapitulates the pathologic events that occur in ROP.[14] Using the mouse model of OIR, we have previously demonstrated the central role of sympathetic transmission in regulating the effect of hypoxia on retinal neovascularization.

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In fact, propranolol exerts beneficial effects on OIR, as its administration downregulates proangiogenic factors, ameliorates proangiogenic effects of hypoxia, and repairs blood-retinal barrier (BRB) breakdown.[8] In particular, β2-AR blockade is likely to be involved in the ameliorative effect of propranolol, as the selective β2-AR blocker, ICI 118,551, reduces the hypoxia-induced increase in retinal VEGF, decreases neovascular tuft formation, and recovers electroretinographic responses to hypoxia.[9] Together, these findings suggest that pathogenic angiogenesis depends at least in part on β2-AR activity and indicates that β2-AR blockade can effectively modulate angiogenic processes in the retina.

Results from studies aimed at investigating the role of noradrenergic transmission in diabetic retinopathy (DR) are not always consistent with what is found in OIR. For instance, β-AR activation with isoproterenol inhibits diabeticlike vascular changes in the rat retina, whereas loss of β-AR signaling seems to produce retinal alterations common to DR.[15–18] In addition, propranolol does not affect or even increases retinal levels of VEGF in DR,[15,17] whereas a decrease in VEGF has been observed after β-AR blockade in OIR.[8,9] These results, together, suggest that β-ARs may control angiogenic processes through diverse mechanisms in DR and OIR and that sympathetic transmission may be differently regulated in DR and OIR.

Additional information on the effects of β-AR agonism on retinal angiogenesis may help to clarify the role of sympathetic transmission in ROP. The aim of the present study was to investigate the effects of isoproterenol administration to OIR mice. Isoproterenol was subcutaneously administered according to previous studies in mice.[19,20] In this respect, subcutaneous delivery of noradrenergic drugs seems to be effective in influencing hypoxia-induced retinal neovascularization.[9] On the other hand, isoproterenol can induce cardiovascular alterations,[21] which, in turn, may influence retinal angiogenesis.[22]

In the present study, isoproterenol was first tested for the optimal dose for delivery using cellular signaling information, including determination of PKA activity in the retina. Then, we evaluated cardiovascular parameters following isoproterenol administration. Subsequently, the effects of isoproterenol on retinal VEGF and pathogenic neovascularization were investigated. Finally, we determined the effects of isoproterenol on sympathetic transmission, including determination of NE levels and β-AR signaling.

Materials and Methods

The PepTag nonradioactive PKA assay kit was from Promega (Madison, MI). The ELISA kit for the detection of VEGF was from R&D Systems (Minneapolis, MN). The ELISA kit for the detection of NE was purchased from IBL International (Hamburg, Germany). The primary antibody directed to CD31 was obtained from BD Pharmingen (San Diego, CA). The secondary antibody Alexa Fluor 488 was from Molecular Probes (Eugene, OR). Primary antibodies directed to β1-, β2-, β3-ARs, GRK2, and β-arrestin1/2, as well as the mouse anti-rabbit horseradish peroxidase-labeled secondary antibody and the rabbit anti-goat peroxidase-labeled secondary antibody, were from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyvinylidene difluoride (PVDF) membrane and protein G agarose were obtained from GE Healthcare (Piscataway, NJ). The enhanced chemiluminescence reagent was from Millipore (Billerica, MA). All other chemicals, including isoproterenol, were obtained from Sigma-Aldrich (St Louis, MO).

Animals

Procedures involving animals were carried out in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian guidelines for animal care (DL 116/92) and the European Communities Council Directive (86/609/EEC). Procedures were approved by the Ethical Committee in Animal Experiments of the University of Pisa. All efforts were made to reduce both animal suffering and the number of animals used. Two-month-old male and female mice (C57BL/6J strain) were originally purchased from Charles River Laboratories Italy (Calco, Italy) and were bred in our breeding colony. Experiments were performed on a total of 259 mouse pups of both sexes. Mice were euthanized at postnatal day (PD)17 (6 g body weight). Animals were kept in a regulated environment (23 ± 1°C, 50% ± 5% humidity) with a 12-hour light/dark cycle (lights on at 8 AM) with food and water ad libitum. In all experiments, mice were anesthetized with halothane (4%), killed by cervical dislocation, and the eyes were enucleated.

Model of Oxygen-Induced Retinopathy

In a typical model of OIR,[23] litters of mice pups with their nursing mothers were exposed in an infant incubator to high oxygen concentration (75% ± 2%) between PD7 and PD12, before return to room air between PD12 and PD17. Oxygen was checked twice daily with an oxygen analyzer (Pro-Custom Elettronica, Milano, Italy). Individual litters were reared in either oxygen or room air. The pharmacological treatment was performed in animals anesthetized by intraperitoneal injection of Avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water, 0.02 mL/g body weight). All experiments were performed at the same time of day to exclude possible circadian influences. The data were collected from both males and females and the results combined, as there was no apparent gender difference.

Pharmacological Treatment

Isoproterenol is a classical nonselective β-AR agonist with positive cardiac inotropic and chronotropic action, which may induce vasodilatory effects.[24] Isoproterenol clinical use is limited because of its cardiovascular side effects.[25] In basic research, isoproterenol is generally used in studies aimed at investigating β-AR control of cardiac activity, as systemic administration of isoproterenol at high doses may induce cardiac damage.[26] Recently, isoproterenol has been used to investigate the effects of β-AR activation on vascular abnormalities in the retina of diabetic rats.[16] In the present study, isoproterenol was given at low doses (0.1 and 0.5 mg kg^{-1} $dose^{-1}$) to reduce the incidence of cardiovascular side effects.[19,20] In addition, the mouse strain used in the present study has been reported to be resistant to isoproterenol-induced cardiac damage.[26] In OIR mice, isoproterenol, dissolved in sterile saline, was given two times a day subcutaneously from PD12 to PD16. Sham injections were performed with sterile saline.

Cardiovascular Parameters

Left ventricular (LV) pressure and heart-to–body weight ratio were evaluated in OIR mice that were either untreated ($n = 5$) or treated ($n = 6$) with 0.5 mg/kg isoproterenol. Mice were anesthetized with 1.5% isoflurane. Body temperature was maintained with a heating pad. Mean LV pressure was measured using a fluid-filled catheter (MLT 844; AD Instruments, Colorado Springs, CO) inserted from the right carotid artery.[27] The systolic LV pressure was calculated from the mean LV pressure, assuming that the LV diastolic pressure was zero.[28] Mice were then killed and weighed. Hearts were quickly excised, placed in ice-cold saline to eliminate the blood, and weighed.

PKA and Adenylyl Cyclase Activity

To measure PKA activity, 6 samples from 6 different mice, each containing 2 retinas from 2 different mice, were used for each experimental condition. To measure adenylyl cyclase (AC) activity, 6

samples from 6 different mice, each containing 6 retinas from 6 different mice, were used for each experimental condition.

PKA activity was measured with the PepTag nonradioactive PKA assay kit, as previously reported.[9] Briefly, retinas were sonicated in 25 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. Retinal cytosolic fractions were isolated by centrifugation at 14,000*g* for 10 minutes at 4°C. Protein concentration was determined using a fluorometer (Qubit; Invitrogen, Carlsbad, CA). PKA activity was measured at 30°C for 45 minutes using 15 μg protein per sample in a final volume of 25 μL containing 5 μL PepTag PKA reaction buffer, 2 μg PepTag A1 Peptide (L-R-R-A-S-L-G kemptide), and 1 μM cAMP. The reaction was stopped by placing the samples on a 95°C heating block for 10 minutes. Agarose gel electrophoresis (0.8%) was used to separate the phosphorylated (net charge −1) from nonphosphorylated (net charge +1) kemptide. The phosphorylated kemptide was excised from the gel and PKA activity was quantified spectrophotometrically (SmartSpec 3000; Bio-Rad, Hercules, CA).

AC activity was measured on membrane preparation, as previously reported.[29,30] Briefly, retinas were sonicated in 10 mM HEPES/Tris, pH 7.4, containing 10% sucrose. Homogenates were centrifuged at 1000*g* for 10 minutes at 4°C and supernatants (A) were stored. Pellets were resuspended, homogenized, centrifuged as described previously, and supernatants (B) were collected. Supernatants A and B were combined and centrifuged at 11,000*g* for 20 minutes. Pellets were resuspended in 30 mL buffer without sucrose and centrifuged at 27,000*g* for 10 minutes at 4°C. Finally, pellets were resuspended in the same buffer, supplemented with the protease inhibitors aprotinin (20 μg/mL) and leupeptin (20 μg/mL), and used immediately. Protein concentration was determined using a fluorometer (Qubit; Invitrogen). Aliquots of membrane preparation (50 μg membrane protein/aliquot) were preincubated for 20 minutes at 0°C in 75 mM HEPES/Tris, pH 7.4, containing 1 mM $MgSO_4$, 10 μM 3-isobutyl-1-methylxanthine, 500 μM guanosine triphosphate (GTP), 1 mM EGTA, and 100 μM isoproterenol[31] or 1 μM forskolin.[29,30] The reaction was then initiated by the addition of 500 μM ATP, carried out for 10 minutes at 30°C, and stopped by 2 minutes of boiling. After centrifugation, cAMP was measured on the clear supernatant, according to the method of Brown et al.[32]

For PKA activity, data were expressed as incorporated pmol phosphate/mg protein/min. For AC activity, data were expressed as pmol cAMP/mg protein/10 min. All experiments were run in duplicate. After statistical analysis, data from the different experiments were plotted and averaged on the same graph.

Enzyme-Linked Immunosorbent Assay

To measure VEGF levels, 6 samples from 6 different mice, each containing 2 retinas from 2 different mice, were used for each experimental condition. To measure NE levels, 6 samples from 12 different mice, each containing 4 retinas from 4 different mice, were used for each experimental condition. VEGF and NE levels were measured using commercially available kits in line with previous works.[8,9,33] Briefly, retinas were sonicated in 200 μL buffer A (10 mM Tris-HCl, pH 7.6, containing 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulphonyl fluoride, 1 μM peptistatin, 10 μg/mL leupeptin, and 2 μg/mL aprotinin) for VEGF measurements or in 330 μL of a solution containing 0.1 M HCl and 1 mM EDTA for NE measurements. Retinal homogenates were centrifuged at 22,000*g* for 15 minutes at 4°C. Protein concentration was determined using a fluorometer (Qubit; Invitrogen). The ELISA plates were evaluated spectrophotometrically (Microplate Reader 680 XR; Bio-Rad). Data were expressed as pg VEGF/mg protein or pmol NE/mg retina. All experiments were run in duplicate. After statistical analysis, data from the different experiments were plotted and averaged in the same graph.

Immunohistochemistry and Quantitative Analysis

Immunohistochemistry on retinal whole mounts, as well as quantitative analysis of preretinal neovascular tuft formation, vascular and avascular areas were performed in line with previous works.[8,9,34] Dissected retinas were immersion-fixed for 1.5 hours in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C, transferred to 25% sucrose in 0.1 M PB, and stored at 4°C. To visualize blood vessels, retinal whole mounts were rinsed in 0.1 M PB and incubated for 72 hours at 4°C in the CD31 rat monoclonal antibody diluted (1:50) in 0.5% Triton X-100 containing 0.1 M PB. After incubation, the whole mounts were rinsed in 0.1 M PB and incubated for 48 hours at 4°C in Alexa Fluor 488 (1:200) in 0.1 M PB. Finally, they were rinsed in 0.1 M PB, mounted on gelatin-coated glass slides, and cover-slipped with a 0.1 M PB-glycerine mixture. Immunofluorescent materials were observed with confocal microscopy (Laser Scanning Microscope Radiance Plus; Bio-Rad). Electronic images from the confocal microscope were processed using image-editing software (Adobe Photoshop 7.0; Adobe Systems, Inc., Mountain View, CA).

To perform quantitative analysis, overlapping stacks of confocal optical sections were acquired with a ×10 objective and a detector resolution of 1024 × 1024 pixels. With image-editing software (Adobe Photoshop 7.0), each individual image was converted to 2 × 2 inches with 600-pixel/inch resolution and whole retina montages were created by software (LaserSharp Radiance Plus; Bio-Rad) based on retinal landmarks, such as the optic disc and major vessels. Subsequent quantification was performed on these montages, in which preretinal neovascular tufts were clearly distinguishable from the underlying intraretinal vascular plexus focusing just above the inner limiting membrane. Quantitative analysis was performed in the entire retina. In each whole mount, the total area of preretinal neovascular tufts and the extent of the avascular area were measured (in pixels) using the freehand selection tool of an image-editing software (ImageJ; National Institutes of Health, Bethesda, MD) and were expressed as the percentage of the respective average calculated in the corresponding hypoxic retinas. For each experimental condition, quantitative data originated from 6 retinas from 6 different mice. After statistical analysis, averaged data were plotted on the same graph.

Western Blot Analysis

To perform Western blot experiments, 6 samples from 6 different mice, each containing 2 retinas from 2 different mice, were used for each experimental condition in agreement with previously published protocols.[8,35] Retinal samples were sonicated in 150 μL buffer A and centrifuged at 22,000*g* for 30 minutes at 4°C. The supernatants, containing cytosolic proteins, were used to detect GRK2 and β-arrestins. Pellets were resuspended in 20 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 4 mg/mL n-dodecyl-β-maltoside, 1 mM phenylmethylsulphonyl fluoride, 1 μM peptistatin, 10 μg/mL leupeptin, and 2 μg/mL aprotinin and centrifuged at 22,000*g* for 30 minutes at 4°C. The supernatants, containing membrane proteins, were used to detect β-ARs. Protein concentration was determined using a fluorometer (Qubit; Invitrogen). Aliquots of each sample containing equal amounts of protein (40 μg) were subjected to SDS-PAGE. β-Actin was used as the loading control. The gels were transblotted onto PVDF membrane using a transfer system (TransBlot Turbo transfer system; Bio-Rad) and the blots were blocked in 3% skim milk for 1 hour at room temperature. Blots were then incubated overnight at 4°C with mouse monoclonal antibodies directed to GRK2 (1:100) and β-arrestin1/2 (1:100), or rabbit polyclonal antibodies directed to β1-ARs (1:100) and β2-ARs (1:200), or a goat polyclonal antibody directed to β3-ARs (1:200). The same membrane was reblotted with a mouse monoclonal antibody directed to β-actin (1:10,000) as loading control. Finally, blots were incubated for 1 hour at room temperature with a rabbit anti-mouse horseradish peroxidase–labeled secondary antibody (1:25,000), a mouse anti-rabbit horseradish peroxidase–labeled secondary antibody (1:5000), or a rabbit anti-goat peroxidase-labeled secondary antibody (1:5000) and developed with the enhanced chemiluminescence reagent. Images were acquired (Chemidoc XRS+; Bio-Rad) and the optical density of the bands was evaluated (Image Lab 3.0 software; Bio-Rad). The data were

normalized to the level of β-actin. All experiments were run in duplicate. After statistical analysis, data from the different experiments were plotted and averaged on the same graph.

β2-AR Immunoprecipitation

Immunoprecipitation was performed on samples used for Western blot. To evaluate β-arrestin association to β2-ARs, immunoprecipitation of β2-ARs was performed following a previously published protocol.[36] Aliquots of membrane proteins (100 μg) were incubated with 30 μL of protein G agarose for 1 hour at 4°C to exclude nonspecific binding. Then, samples were incubated with anti-β2-AR antibody overnight, followed by incubation with protein G agarose for 2 hours at 4°C. After washing steps and denaturation at 100°C for 5 minutes, proteins were separated by SDS-PAGE and transblotted on PVDF membrane. Blots were then developed with β-arrestin1/2 antibody, as reported in the Western blot section. The data were normalized to the level of β2-ARs. All experiments were run in duplicate. After statistical analysis, data from the different experiments were plotted and averaged in the same graph.

Statistics

All data were analyzed by the Shapiro-Wilk test to verify their normal distribution. Statistical significance was evaluated using ANOVA followed by Newman-Keuls Multiple Comparison post-test. The results are expressed as mean ± SE of the indicated n values (Prism 4; GraphPad Software, San Diego, CA). Differences with $P < 0.05$ were considered significant.

Results

PKA Activity

To determine the optimal dose for drug administration, mice were treated with increasing concentrations of isoproterenol two times a day between PD7 and PD12. The doses investigated were 0.1 and 0.5 mg kg^{-1}. Measurement of PKA activity was used as a biomarker that isoproterenol was reaching the retina and eliciting a normal cellular response according to a previous study in the retina of diabetic rats.[16] In all experiments, no effects were observed after vehicle treatment (not shown). As shown in Figure 1, hypoxia decreased PKA activity by approximately 48% ($P < 0.001$ versus normoxic) in line with previous results in the retina of OIR mice.[9] Isoproterenol at 0.1 mg kg^{-1} did not influence hypoxic levels of PKA activity, whereas isoproterenol reduced them by approximately 18% ($P < 0.01$ versus hypoxic) at 0.5 mg kg^{-1}. For all subsequent experiments, isoproterenol at 0.5 mg kg^{-1} was used.

Cardiovascular Parameters

To evaluate whether isoproterenol subcutaneously administered at 0.5 mg kg^{-1} may induce alterations in cardiovascular parameters, we measured systolic LV pressure and heart-to–body weight ratio in OIR mice either untreated or treated with isoproterenol. As shown in Table 1, neither systolic LV pressure nor heart-to–body weight ratio were affected by isoproterenol.

VEGF Levels and Pathogenic Vascularization

In agreement with previous results,[8,9] hypoxic retinas displayed an approximately 311% increase in VEGF ($P < 0.001$ versus normoxic; Fig. 2). Isoproterenol decreased hypoxic levels of VEGF by approximately 27% ($P < 0.001$ versus hypoxic). To investigate the effects of isoproterenol on

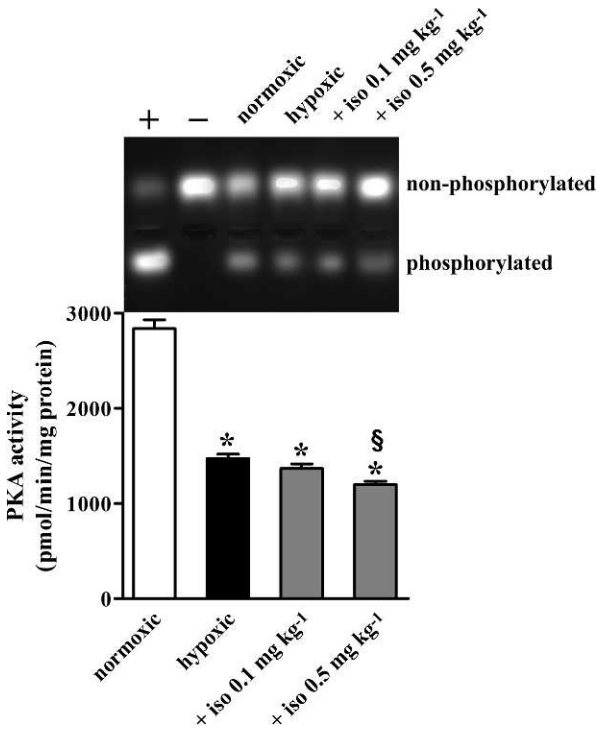

Figure 1. Measurements of PKA activity after isoproterenol. Mice were exposed to room air or to 75% ± 2% oxygen from PD7 to PD12. Mice exposed to oxygen were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. PKA activity was evaluated in the retina of normoxic, hypoxic, and isoproterenol-treated mice. Representative agarose gel containing phosphorylated and nonphosphorylated kemptide bands is shown. The positive control (+) was provided by the manufacturer. In the negative control (–), PKA activity was measured in the absence of the retinal cytosol fraction. PKA activity was quantified spectrophotometrically from bands excised from the gel. PKA activity was decreased by hypoxia (*$P <$ 0.001 versus normoxic; ANOVA). Isoproterenol at 0.1 mg kg^{-1} dose^{-1} did not affect hypoxic levels of PKA activity which in contrast were reduced by isoproterenol at 0.5 mg kg^{-1} dose^{-1} ($^{\S}P < 0.01$ versus hypoxic; ANOVA). Each column represents the mean ± SE of data from 6 independent samples, each containing 2 retinas from 2 different mice. iso, isoproterenol.

angiogenesis, we performed CD31 immunohistochemistry in the retinas of OIR mice, as CD31 is a convenient endothelial cell marker to visualize and quantify the pathogenic neovascular tuft formation.[9,34] Figure 3 is representative of the vascular pattern in the retina of normoxic (Fig. 3A) and OIR mice untreated (Fig. 3B) or isoproterenol treated (Fig. 3C). In OIR mice, CD31 immunohistochemistry showed regression of large areas of the vascular network in the center of the retina, leaving only the major vessels and practically no capillary network. In contrast, the midperipheral region of the retina showed excessive regrowth of CD31-positive superficial vessels leading to preretinal neovascular tufts, with most of them forming at the border between the vascularized peripheral and the obliterated central regions. No effects were observed after vehicle treatment (not shown). As shown in Figure 3C, isoproterenol drastically reduced the vessel tuft area but did not influence the extent of the avascular area. Changes in the vessel tuft area after isoproterenol had no preferential regional distribution. Quantitative analysis of the entire retina confirmed our qualitative observation and revealed that retinas of mice treated with isoproterenol had a significantly reduced neovascular tuft area (approximately 52%, $P < 0.001$ versus hypoxic; Fig. 3D).

Table 1. Cardiovascular Parameters After Isoproterenol

Parameter	Untreated Mice ($n = 5$)	Isoproterenol-treated Mice ($n = 6$)
Systolic LV pressure, mm Hg	73 ± 9	78 ± 12
Body weight, mg	6075 ± 843	5895 ± 775
Heart weight, mg	61 ± 4	67 ± 6
Heart-to–body weight ratio	0.010 ± 0.001	0.011 ± 0.001

Mice were exposed to 75% ± 2% oxygen from PD7 to PD12 and were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. Data are expressed as mean ± SE. *n*, number of mice; LV, left ventricular.

Levels of NE

Stress conditions, including hypoxia, can alter catecholaminergic neurotransmission, resulting in catecholamine dysregulation. For instance, hypoxia has been reported to influence NE levels in the rat brain,[37,38] although no information is available in the retina. NE levels in normoxic retinas were in the range of those reported in rat and bovine retinas.[39,40] As shown in Figure 4A, hypoxia increased NE levels by approximately 90% ($P < 0.01$ versus normoxic), indicating that hypoxic stress may enhance sympathetic transmission in the retina. Hypoxic levels of NE were not influenced by either vehicle treatment (not shown) or isoproterenol.

β-AR Signaling: β-AR Expression, AC Activity, GRK2, and β-Arrestins

To determine whether changes in β-AR signaling may contribute to reduced levels of VEGF and decreased pathogenic neovascularization in response to isoproterenol, we first evaluated the expression of plasma membrane β-ARs. In agreement with previous results,[8] Western blot analysis demonstrated that hypoxia did not influence β1- and β2-ARs, whose levels were similar to those measured in control retinas (Figs. 4B, 4C, respectively), but increased β3-ARs by approximately 112% ($P < 0.01$ versus normoxic; Fig. 4D). β-AR levels were not influenced by vehicle treatment (not shown). Isoproterenol did not influence levels of β1- and β3-ARs (Figs. 4B, 4D, respectively), whereas it reduced β2-AR levels by approximately 37% ($P < 0.01$ versus hypoxic; Fig. 4C), suggesting that the effect of isoproterenol on angiogenic processes involves a decrease of available β2-ARs on cell membranes.

Receptor downregulation is a key component of agonist-stimulated G-protein coupled receptor (GPCR) desensitization.[41] To investigate whether isoproterenol may influence coupling efficiency between cell surface β-ARs and the intracellular signaling machinery, we measured AC activity on cell membrane preparations after preincubation with two different AC activators: isoproterenol, acting at β-ARs, or forskolin, bypassing the receptors. In line with previous results,[29] hypoxia increased AC activity in the retina. Either isoproterenol- or forskolin-stimulated AC activities were unaffected by vehicle administration (not shown), whereas they were increased by hypoxia by approximately 59% and approximately 37%, respectively ($P < 0.001$ versus normoxic; Figs. 5A, 5B). Isoproterenol reduced this increase by approximately 34% and approximately 29%, respectively ($P < 0.001$ versus hypoxic).

GPCR desensitization is regulated by receptor phosphorylation. In particular, several kinases, including the GPCR kinases (GRKs), promote β-AR desensitization,[41] creating a binding site for β-arrestin1 and β-arrestin2. After binding to phosphorylated receptors, β-arrestins inhibit the function of the GPCRs, including β-ARs, by preventing their association with the G proteins.[42] To evaluate whether isoproterenol influences the expression of proteins involved in β-AR desensitization, we measured the expression of GRK2, which is the prototype GRK for β-ARs.[43] In addition, we evaluated the expression of β-arrestin1 and β-arrestin2, which are known to be expressed in the retina.[44] As shown in Figure 6, hypoxia did not affect GRK2 (Fig. 6A) and β-arrestins (Fig. 6B). Levels of GRK2, β-arrestin1, and β-arrestin2 were not influenced by vehicle treatment (not shown), whereas levels were increased by isoproterenol (approximately 101%, approximately 39%, and approximately 176%, $P < 0.001$ versus hypoxic, respectively) indicating β-AR desensitization. To evaluate whether β-AR desensitization involves β2-ARs, retinal extracts were immunoprecipitated with the β2-AR antibody and developed using antibodies against β-arrestin1/2. As shown in Figure 6C, a band corresponding to β-arrestin2 was evident in the immunoprecipitate, whereas only a faint band corresponding to β-arrestin1 could be observed. Semiquantitative analysis showed that hypoxia did not affect the recruitment of β-arrestin2 to β2-ARs, which, in contrast, was increased by approximately 460% ($P < 0.001$ versus hypoxic) after isoproterenol.

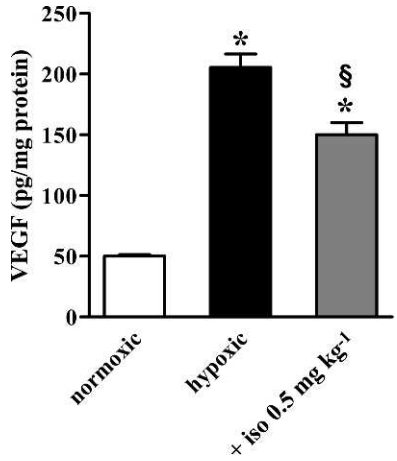

Figure 2. Levels of VEGF protein after isoproterenol. Mice were exposed to room air or to 75% ± 2% oxygen from PD7 to PD12. Mice exposed to oxygen were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. VEGF was evaluated by ELISA in the retina of normoxic, hypoxic and isoproterenol-treated mice. VEGF levels were increased by hypoxia (*$P < 0.001$ versus normoxic; ANOVA). Isoproterenol at 0.5 mg kg^{-1} $dose^{-1}$ reduced hypoxic levels of VEGF (§$P < 0.001$ versus hypoxic; ANOVA). Each column represents the mean ± SE of data from 6 independent samples, each containing 2 retinas from 2 different mice. iso, isoproterenol.

Discussion

Most recent therapeutic interventions against ROP have focused on the mechanisms and the factors leading to new vessel growth. In this respect, much attention has been paid to the study of the role of the noradrenergic system in hypoxic/ischemic retinal diseases. This study demonstrates that the β-AR agonist isoproterenol inhibits hypoxia-induced angiogenic processes presumably through reduced β2-AR signaling.

Effectiveness of Isoproterenol

After interactions with their ligands, β-ARs couple to G-proteins to modulate AC activity, which in turn affects cAMP levels and PKA. Thus, measurement of PKA activity can be

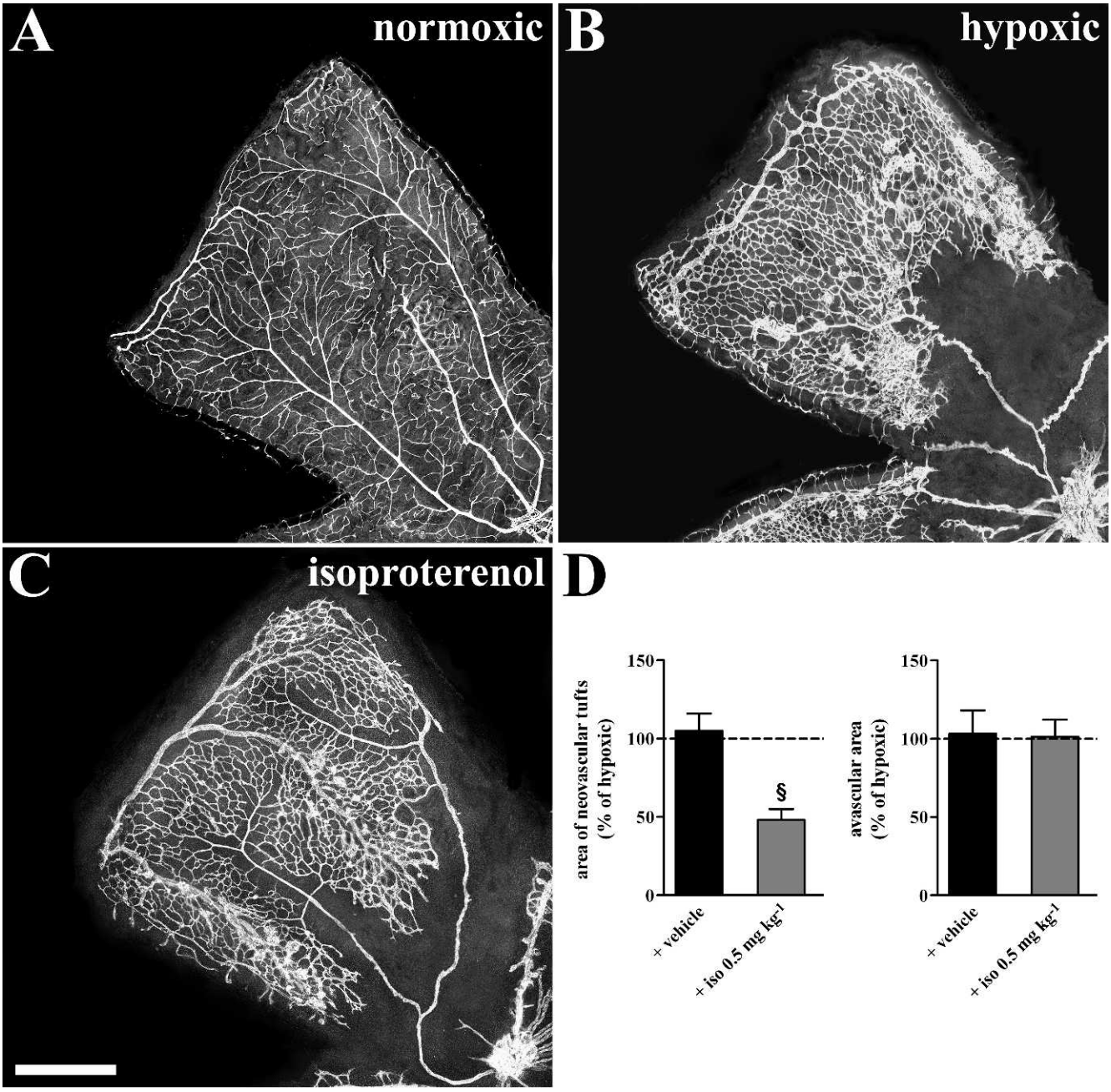

Figure 3. Flat-mounted retinas immunolabeled with a rat monoclonal antibody directed to CD31. Mice exposed to room air (**A**) or to 75% ± 2% oxygen from PD7 to PD12 (**B**, **C**), untreated (**B**) or treated with isoproterenol (**C**) from PD12 to PD16. Retinas were explanted at PD17. Hyperoxia followed by normoxia for 5 days produced the central loss of blood vessels and the formation of tufts. Isoproterenol at 0.5 mg kg^{-1} $dose^{-1}$ reduced drastically the vessel tuft area, but did not influence the extent of the avascular area. Scale bar, 400 μm. The extent of the neovascular tuft area and the avascular area were quantitatively evaluated (**D**) ($^{\S}P < 0.001$ versus hypoxic; ANOVA). iso, isoproterenol. Each column represents the mean ± SE of data from 6 retinas.

used as a biomarker of the effectiveness of β-AR ligands as demonstrated in the retina of rodents after topical application of isoproterenol or subcutaneous administration of β-AR antagonists.[9,16] As shown by the present results, hypoxia reduces PKA activity, suggesting that downregulation of PKA activity is involved in mechanisms by which retina adapts to hypoxia. This finding is in agreement with previous results in OIR mice,[3] although there are indications that PKA activity may be either increased or decreased by hypoxia in other experimental models.[45,46] The additional finding that isoproterenol reduces hypoxic levels of PKA activity indicates its effectiveness at the retinal level. PKA activity reduction after isoproterenol is in line with the result that PKA activity increases after β2-AR blockade in OIR,[9] but in contrast with the result that PKA activity increases after isoproterenol in DR.[16] Different drug dosages, administration routes, or experimental models may explain this discrepancy. In particular, in OIR, isoproterenol is administered during the proliferative phase of neovascularization, which is a rare occurrence in DR.[47] The isoproterenol concentrations used here are in line with those reported in previous studies in mice.[19,20] In particular, the finding that isoproterenol at 0.5 mg kg^{-1} reduces hypoxic

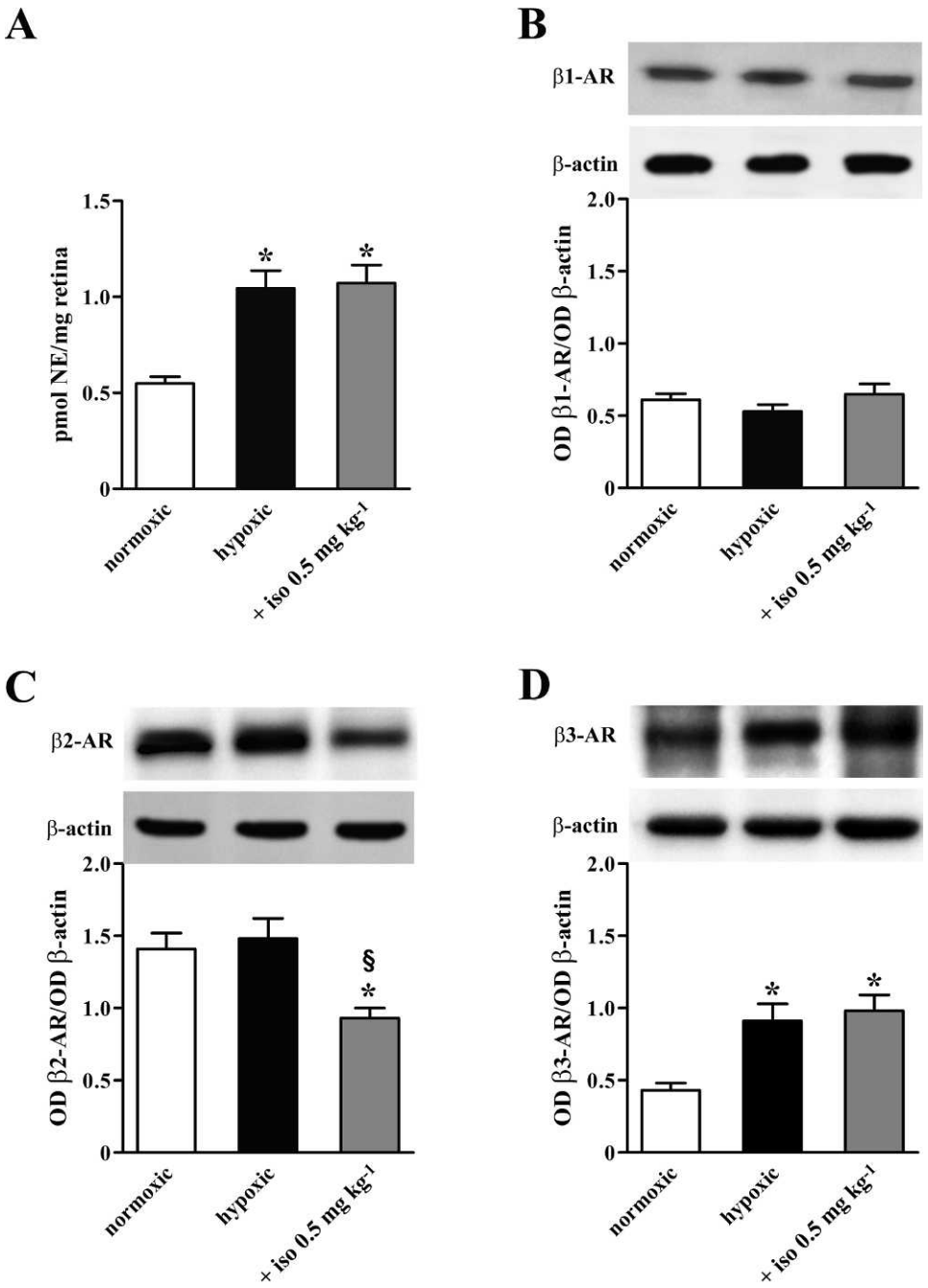

Figure 4. Measurements of endogenous NE levels and β-AR expression after isoproterenol. Mice were exposed to room air or to 75% ± 2% oxygen from PD7 to PD12. Mice exposed to oxygen were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. NE levels and β-AR expression were evaluated in the retina of normoxic, hypoxic and isoproterenol-treated mice. NE levels were evaluated by ELISA (**A**). Hypoxia increased NE levels (*$P < 0.01$ versus normoxic; ANOVA), which are not affected by isoproterenol. The expression of β1- (**B**), β2- (**C**), and β3- (**D**) ARs was evaluated by Western blot and densitometric analysis. Hypoxia did not affect β1- and β2-ARs, whereas increased β3-ARs (*$P < 0.01$ versus normoxic; ANOVA). Isoproterenol at 0.5 mg kg^{-1} dose^{-1} did not affect β1- and β3-ARs, whereas decreased β2-ARs ($^{\S}P < 0.01$ versus hypoxic; ANOVA). β-AR expression was relative to the loading control β-actin. Each column represents the mean ± SE of data from 6 independent samples, each containing 4 retinas from 4 different mice (measurement of NE levels) or 2 retinas from 2 different mice (measurement of β-AR expression). iso, isoproterenol.

levels of PKA, whereas at 0.1 mg kg^{-1} it does not, indicates that isoproterenol dose-dependently affects PKA activity and suggests that 0.5 mg kg^{-1} is the minimal effective dose reaching the retina and affecting β-AR–mediated signaling.

It is difficult to conjecture about the isoproterenol concentration that actually reaches the retina. In preliminary experiments, we found that subcutaneous administration of 20 mg kg^{-1} propranolol results in a retinal concentration of 16.22 ± 1.62 μg g^{-1} (unpublished results, 2011). In this respect, the ability of propranolol, a high lipophilic drug, to cross the blood-brain barrier (BBB) is about 15-fold higher than that of isoproterenol, a hydrophilic drug.[48] Considering that retinal vessels are sensibly more permeable than brain vessels,[49] it is possible to hypothesize that 0.5 mg kg^{-1} isoproterenol may

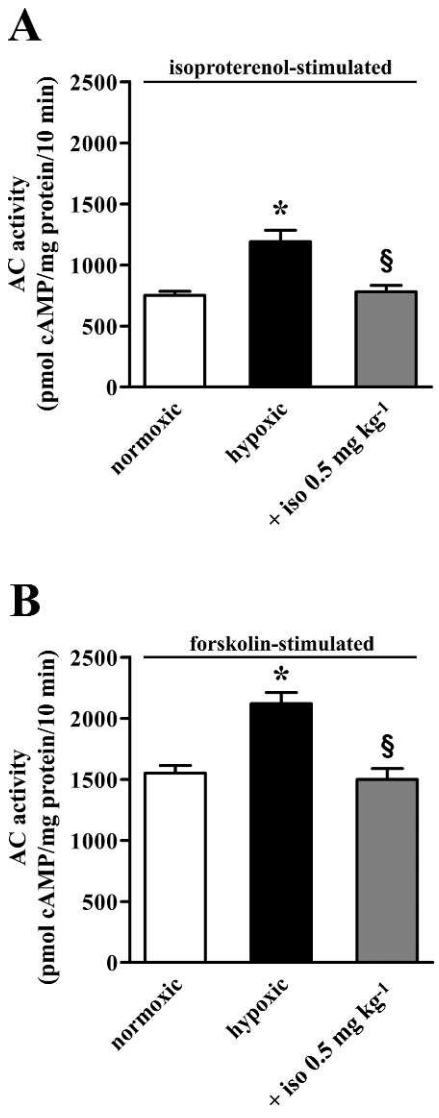

Figure 5. Measurements of AC activity after isoproterenol. Mice were exposed to room air or to 75% ± 2% oxygen from PD7 to PD12. Mice exposed to oxygen were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. AC activity was evaluated in the retina of normoxic, hypoxic and isoproterenol-treated mice. Membrane preparations were preincubated for 10 minutes with 100 μM isoproterenol (**A**) or 1 μM forskolin (**B**). Hypoxia increased both isoproterenol- and forskolin-stimulated AC activities ($^{*}P < 0.001$ versus normoxic; ANOVA). Isoproterenol at 0.5 mg kg^{-1} $dose^{-1}$ reduced this increase ($^{\S}P < 0.01$ versus hypoxic; ANOVA). Each column represents the mean ± SE of data from 6 independent samples, each containing 6 retinas from 6 different mice. iso, isoproterenol.

result in a retinal concentration at least 100-fold lower than that of propranolol. We can also expect that isoproterenol delivery to the retina may be facilitated by hypoxia, which stimulates the production of cytokines that are known to increase vascular permeability. For instance, tumor necrosis factor-α increases the penetration of chemotherapeutic drugs in several tumor models by increasing the permeability of tumor vasculature.[50] In the retina, hypoxia may enhance drug penetration by promoting BRB leakage, which becomes about five- to ninefold higher than in normoxia.[51,52] For instance, in hypoxia, 20 mg kg^{-1} propranolol results in a retinal concentration of 20.02 ± 3.21 μg g^{-1}, which is about 25% higher than in normoxia (unpublished results, 2011). As another example, the brain concentration of the hydrophilic β1-AR blocker atenolol, which penetrates the BBB in a limited amount, is markedly increased by osmotic opening of the BBB.[53]

As shown by the present results, sufficient isoproterenol penetrates the BRB to achieve retinal levels adequate to elicit biological effects. In this respect, the hydrophilic β2-AR agonist, terbutaline, with limited ability to cross the BBB, can be effective on β-ARs in the rabbit brain.[54] In addition, xamoterol, a hydrophilic partial β1-agonist, seems to reach the hippocampus and the basolateral amygdala to impair memory retrieval in the rat.[55]

Effects of Isoproterenol on VEGF and Pathogenic Neovascularization

Previous results indicate that the noradrenergic system plays an important role in retinal angiogenesis, although their interpretation remains to be clarified. For instance, in human choroidal endothelial cells, isoproterenol increases growth factors that are active during vascular remodeling.[56] In addition, previous results demonstrate that β-AR blockade with either propranolol or the selective β2-AR blocker ICI 118,551 reduces retinal angiogenesis in OIR mice and that ICI 118,551 recovers electroretinographic responses to hypoxia,[8,9] indicating that β-AR antagonism is effective to counteract pathogenic vascularization and restore visual function. Unexpectedly, the present results show that isoproterenol reduces retinal levels of VEGF and neovascular tuft formation, suggesting an angiostatic effect of β-AR agonism on pathogenic neovascularization. A decrease in neovascular tuft formation without effects on intraretinal physiologic vascularization has been observed in the mouse model of OIR after treatment with ICI 118,551[9] or other antiangiogenic compounds.[34] In line with the present results, isoproterenol inhibits vascular remodeling that occurs in the retina of diabetic rats and ameliorates the diabetes-induced changes in the electroretinogram (ERG).[16] In line with these findings, in nondiabetic rats, propranolol induces retinal alterations common to DR and produces a dysfunctional ERG.[15] In addition, loss of sympathetic innervation or lack of NE in rodents causes retinal dysfunctions similar to those characteristic of DR.[18,57] Recently, in rat Müller cells, β-AR agonism has been shown to prevent the glucose-induced changes in insulin signaling.[58]

The possibility that isoproterenol may induce cardiovascular changes, which, indirectly, affect retinal neovascularization can be excluded by the finding that isoproterenol concentration used in the present study does not seem to affect cardiovascular parameters. In line with this finding, there are previous results indicating that 0.5 mg kg^{-1} isoproterenol given systemically does not appear to change systolic blood pressure in rodents.[19,59]

Effects of Isoproterenol on Sympathetic Transmission

The finding that isoproterenol ameliorates angiogenesis-associated events raises concerns about the interpretation of previous results demonstrating that β-AR blockade with propranolol, and specifically with ICI 118,551, is effective against pathogenic angiogenesis in the retinas of OIR mice.[8,9] The simplest interpretation is that isoproterenol affects the sympathetic transmission in the retina, thus masking the direct effects of β-AR activation. Our result that hypoxia almost doubles NE levels in the retina is in line with previous findings in the rat brain[37,38] and suggests that increased levels of NE may, in turn, overstimulate β-ARs and potentially activate signaling pathways by acting as a proangiogenic switch. In this

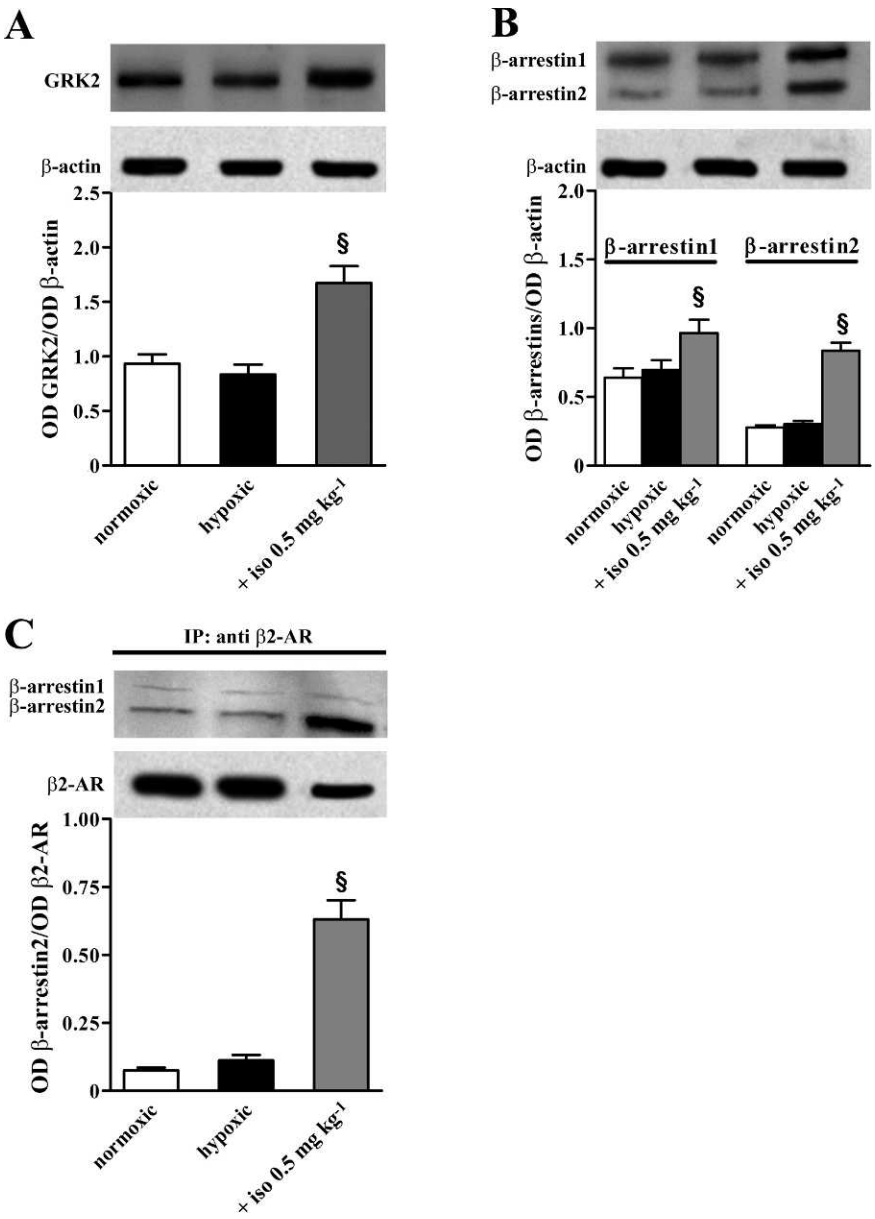

Figure 6. Measurements of GRK2 or β-arrestin expression and evaluation of β-arrestin interaction with β2-ARs after isoproterenol. Mice were exposed to room air or to 75% ± 2% oxygen from PD7 to PD12. Mice exposed to oxygen were untreated or treated with isoproterenol from PD12 to PD16. Retinas were explanted at PD17. Measurements were performed in the retina of normoxic, hypoxic and isoproterenol-treated hypoxic mice. GRK2 (**A**) and β-arrestin (**B**) expression was evaluated by Western blot and densitometric analysis. Hypoxia did not affect GRK2 and β-arrestins. Isoproterenol at 0.5 mg kg^{-1} $dose^{-1}$ increased GRK2 and β-arrestins ($^{\S}P < 0.001$ versus hypoxic; ANOVA). Protein expression was relative to the loading control β-actin. Interaction of β-arrestins with β2-ARs (**C**) was evaluated by immunoprecipitation with the β2-AR antibody on membrane proteins. Hypoxia did not affect the interaction between β-arrestins and β2-ARs, whereas isoproterenol at 0.5 mg kg^{-1} $dose^{-1}$ increased the recruitment of β-arrestin2 to β2-ARs ($^{\S}P < 0.001$ versus hypoxic; ANOVA). β-arrestin2 expression was normalized to the level of β2-ARs. Each column represents the mean ± SE of data from 6 independent samples, each containing 2 retinas from 2 different mice. iso, isoproterenol.

respect, alterations of sympathetic transmission are known to contribute to the development of vascular abnormalities in DR.[16,18,57] These findings, together, indicate a crucial role of the sympathetic system in regulating retinal angiogenesis and suggest that drugs that can restore sympathetic homeostasis can effectively ameliorate vascular dysfunctions. Our result that isoproterenol does not affect hypoxic levels of NE seems to exclude that retinal responses to isoproterenol may involve compensatory regulation of ligand levels.

As also shown by the present results, isoproterenol reduces levels of membrane-associated β2-ARs without affecting those of β1- and β3-ARs, indicating that β2-ARs are downregulated by prolonged agonist exposure. These results demonstrate that β2-AR signaling plays a central role in mediating the effects of isoproterenol and suggests that decreased receptor functional activity may be responsible for isoproterenol ameliorative effects on angiogenic processes. β2-AR downregulation after isoproterenol has been described in several experimental models[60–62] and appears to be a key component of prolonged agonist-induced receptor desensitization, a protective mechanism against excessive sympathetic transmission that initiates with the phosphorylation of multiple serine residues by

kinases.[63] That β-ARs are desensitized by isoproterenol is supported by the finding that isoproterenol reduces AC activity, indicating a decrease in β-AR coupling mechanisms. Previous studies have demonstrated that 0.5 mg kg^{-1} isoproterenol is in the range of concentrations that can effectively desensitize β-ARs. For instance, 0.5 mg kg^{-1} isoproterenol reduces β-AR density in the rat pineal gland.[64] In addition, 0.18 mg kg^{-1} isoproterenol is effective in causing β-AR desensitization in the dog heart,[65] whereas a dose of 0.72 mg kg^{-1} markedly desensitizes β2-ARs in the rat heart.[66] The finding that β2-ARs are more efficiently coupled to AC than β1- and β3-ARs[67] suggests an involvement of β2-ARs in the isoproterenol-induced desensitization process. In this respect, hypoxia has been demonstrated to increase the sensitivity of β2-ARs to desensitization in rat alveolar epithelial cells.[68]

Uncoupling between GPCRs and G proteins depends on receptor phosphorylation, which can be induced by several kinases, including GRKs.[41] Of them, GRK2 is the prototype GRK for β-AR desensitization[43] and is expressed in the mouse retina.[69] In addition, GRK2 is involved in hypoxic/ischemic damage[70] and plays a major role in agonist-induced phosphorylation of β2-ARs.[35,71] As shown by the present results, isoproterenol increases GRK2 expression, suggesting that β-AR agonism may promote GRK2-mediated β2-AR phosphorylation and, in turn, β2-AR uncoupling from G proteins.

GPCR desensitization involves the coordinated action of GRKs and β-arrestins. In fact, GRK-mediated phosphorylation of GPCRs creates a receptor conformation that allows high-affinity binding by β-arrestin1 and β-arrestin2.[41,42] They are two distinct members of the four-member family of arrestins and are both expressed in the retina.[44] β-arrestin1 and β-arrestin2 display different efficiency to bind β-ARs and desensitize them. In fact, they regulate the desensitization of β1- and β2-ARs, but not β3-ARs and, in particular, they play a major role in β2-AR desensitization,[72] with β-arrestin2 more efficiently involved than β-arrestin1.[73] Our finding that β-arrestin2 recruitment to β2-ARs is increased by isoproterenol, indicates that isoproterenol effects in the retina involve β2-AR desensitization. On the other hand, the increased expression of β-arrestins induced by isoproterenol may be independent of β2-AR desensitization and may reflect systemic effects of isoproterenol. For instance, in rat cardiomyocytes, isoproterenol induces the production of angiotensin II (AT-II),[74] which, in turn, promotes the recruitment of β-arrestins to AT-II receptors, as demonstrated in human kidney cells.[75] In this respect, the components of the renin-angiotensin system, which is classically recognized for its role in the control of systemic blood pressure, are expressed by retinal cells and play a central role in retinal angiogenesis.[22] On the other hand, the finding that isoproterenol does not affect blood pressure in OIR mice seems to exclude an involvement of AT-II in the isoproterenol-induced increase in β-arrestin expression.

Conclusion

In summary, the present results suggest that pathogenic angiogenesis, a key change in many hypoxic/ischemic vision-threatening retinal diseases, depends at least in part on increased sympathetic β-adrenergic transmission, and that reduction in β2-AR signaling may counteract hypoxia-induced angiogenic processes. Although extrapolation of these findings to the human situation of ROP is difficult, we suggest that maintaining β2-AR signaling homeostasis is crucial to counteract vascular abnormalities and that sympathetic β-adrenergic transmission may represent a target for therapeutic intervention against vascular abnormalities.

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