

# Beta3-adrenergic receptors modulate vascular endothelial growth factor release in response to hypoxia through the nitric oxide pathway in mouse retinal explants

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**Abstract** Beta-adrenergic receptors ( $\beta$ -ARs) play a role in angiogenic processes that characterize neovascularization-associated retinal diseases, but the role of  $\beta$ 3-ARs has not been disclosed yet. We used *ex vivo* retinal explants to investigate the role of  $\beta$ 3-ARs in regulating vascular endothelial growth factor (VEGF) release associated with hypoxia. Whether nitric oxide (NO) mediates  $\beta$ 3-AR regulation of VEGF release was also investigated.  $\beta$ 3-AR activation was obtained using BRL 37344, whereas SR59230A, L-748,337, or specific siRNAs were used to block  $\beta$ 3-ARs. Pharmacological approaches were used to interfere with the NO pathway. Western blot was used to determine  $\beta$ -AR levels. Enzyme-linked immunosorbent assay was used to measure VEGF release. NO production was assessed by a colorimetric assay. We found that hypoxia upregulates  $\beta$ 3-ARs. In addition, we observed that  $\beta$ 3-AR activation with BRL 37344 increases VEGF release in response to hypoxia. Either  $\beta$ 3-AR blocker or  $\beta$ 3-AR silencing downregulates drastically hypoxic levels of VEGF. With experiments using NO synthase (NOS) blockade with L-NAME, NOS activation with fluvastatin or NO supplementation with SNAP, we demonstrated that  $\beta$ 3-ARs and VEGF are functionally coupled via the NO pathway. In summary, the data presented here support the assumption that  $\beta$ 3-ARs are involved in the

regulation of angiogenic responses to hypoxia through the NO signalling, a key pathway in hypoxic/ischemic diseases. Although extrapolation of these data to the human situation is difficult, these findings may help to explore the possible role of  $\beta$ 3-ARs in vascularization-associated disorders.

**Keywords** Angiogenesis · Retinal explants ·  $\beta$ 3-AR agonists/antagonists · RNA silencing · NO–VEGF relationships

## Introduction

Angiogenesis is a multistep process, controlled by opposing regulatory factors, which plays a crucial role in several ocular diseases (Sapieha et al. 2010). In the retina, hypoxia is considered to be one of the key factors to trigger angiogenesis by inducing vascular endothelial growth factor (VEGF; Arden and Sivaprasad 2011). In this respect, anti-VEGF therapy has become a mainstay of managing vascularization-associated diseases in the retina, although some limitations including recurrence of neovascularization and a number of side effects may be taken into account (Tremolada et al. 2012). In this respect, new pharmacological strategies to counteract retinal diseases characterized by angiogenesis are urgently needed.

Recently, the beta-adrenergic system has been shown to interfere with angiogenesis-dependent diseases (Pérez-Sayáns et al. 2010; Storch and Hoeger 2010). For instance, in C57BL/6J mice with oxygen-induced retinopathy (OIR), a well-established model of retinopathy of prematurity (Chen and Smith 2007), we have demonstrated that  $\beta$ -AR blockade with propranolol downregulates VEGF and reduces proangiogenic effects of hypoxia (Ristori et al. 2011). We have also found that  $\beta$ 2-AR blockade with ICI 118,551 or  $\beta$ 2-AR desensitization after prolonged isoproterenol administration reduces

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VEGF upregulation and retinal neovascularization in response to hypoxia (Dal Monte et al. 2012; Martini et al. 2011).  $\beta$ 3-AR blockade does not seem to affect angiogenic response to hypoxia in the OIR model (Martini et al. 2011); however, several findings are indicative of a role of  $\beta$ 3-ARs in retinal angiogenesis. Indeed,  $\beta$ 3-ARs are localized to blood vessels in rodent retinas (Chen et al. 2012; Mori et al. 2010; Ristori et al. 2011), they are upregulated by hypoxia in OIR mice (Chen et al. 2012; Ristori et al. 2011), and  $\beta$ 3-AR activation induces markers of angiogenesis in human retinal and choroidal endothelial cells (Steinle et al. 2005; 2003).

A possible role of  $\beta$ 3-ARs in retinal angiogenesis may involve nitric oxide (NO). Although no data demonstrating NO as a downstream effector of  $\beta$ 3-AR are available in the retina, increasing evidence indicates that  $\beta$ 3-ARs promote NO synthesis in different experimental models. For instance,  $\beta$ 3-AR agonism activates NO signaling cascade in mouse cardiac myocytes (Cawley et al. 2011) and in rat aortic rings (Ignarro et al. 2002) and stimulates lipolysis by inducing NO production in rat adipocytes (Hodis et al. 2011). There is also accumulating evidence that NO is an important modulator of the expression of endogenous angiogenic factors such as VEGF (Ziche and Morbidelli 2009). In this respect, NO is known to be implicated in pathogenic retinal angiogenesis (Wilkinson-Berka 2004), and inhibiting NO production results in decreased VEGF levels and attenuated retinal neovascularization (He et al. 2007; Zhang et al. 2009).

In the present study, we used *ex vivo* mouse retinal explants to investigate whether (1)  $\beta$ 3-ARs regulate VEGF release in response to hypoxia and (2) NO is involved in mediating this regulation. Although the *ex vivo* model does not exactly recapitulate *in vivo* homeostasis, it does permit direct retinal manipulation, allows greater control of retinal environment, and provides better efficiency of compounds (Curatola et al. 2005; Kaempfer et al. 2008). In addition, mouse retinal explants are a well-established model to study angiogenesis-related events (DeNiro et al. 2009; Martini et al. 2011; Mei et al. 2012).

## Materials and methods

### Materials

Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were obtained from Lonza (Basel, Switzerland). Millicell-CM culture inserts (0.4 mm  $\times$  30 mm) and the enhanced chemiluminescence reagent were from Millipore (Billerica, MA, USA). The  $\beta$ 3-AR agonist BRL 37344 (( $\pm$ )-(R\*,R\*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium hydrate), the  $\beta$ 3-AR antagonist SR59230A (3-(2-ethylphenoxy)-1-[[1(S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt), the

nitric oxide synthase (NOS) inhibitor L-NAME (N $\omega$ -nitro-L-arginine methyl ester hydrochloride), the NOS activator fluvastatin (( $\pm$ )-(3R\*,5S\*,6E)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid sodium salt hydrate), the NO donor SNAP (S-nitroso-N-acetyl-DL-penicillamine), the primary monoclonal antibody directed to  $\beta$ -actin and the rabbit anti-mouse horseradish peroxidase (HRP)-labeled secondary antibody were from Sigma-Aldrich (St. Louis, MO, USA). The  $\beta$ 3-AR antagonist L-748,337 was purchased from Tocris Bioscience (Bristol, UK). The protease inhibitor cocktail Complete was obtained from Roche Applied Science (Indianapolis, IN, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare (Piscataway, NJ). The primary mouse monoclonal antibody directed to mitogen-activated protein kinase 1 (MAPK1), primary rabbit polyclonal antibodies directed to  $\beta$ 1- or  $\beta$ 2-ARs, primary goat polyclonal antibody directed to  $\beta$ 3-ARs, the mouse anti-rabbit HRP-labeled secondary antibody, and the rabbit anti-goat HRP-labeled secondary antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The enzyme-linked immunosorbent assay (ELISA) kit for the detection of VEGF was from R&D Systems (Minneapolis, MN, USA). The RNeasy Mini Kit, the QuantiTect Reverse Transcription Kit, pre-designed Flexitube siRNAs directed to  $\beta$ 3-ARs, a non-silencing siRNA, a positive control siRNA directed to mitogen-activated protein kinase 1 and the HiPerfect transfection reagent were purchased from Qiagen (Valencia, CA, USA). The iQ Sybr Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The colorimetric assay kit for the detection of NO production was from Enzo Life Sciences (Plymouth Meeting, PA, USA).

### Animals

Adult C57BL/6J mice (Charles River Laboratories Italia, Calco, Italy) at 6–8 weeks after birth were used. 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. Experiments were performed on a total of 141 mice of both sexes. In all experiments, mice were anesthetized with halothane (4 %) and killed by cervical dislocation, and the eyes were enucleated.

### Retinal explants

Retinal explants were cultured according to Mei et al. (2012). Briefly, enucleated eyes were immediately transferred to DMEM containing 10 % FCS plus Fungizone (1.25  $\mu$ g/mL).

The choroid, sclera, cornea, and iris were removed from the globe under a dissecting microscope. The remaining retina, vitreous, and lens were incubated in a dish with fresh DMEM for 30–60 min at 37 °C. Then, retinal pigment epithelium was gently teased away from the retina using fine forceps. The isolated retina was transferred onto a culture insert with enough fresh DMEM to barely cover the explant. The retina was cut at 3, 6, 9, and 12 o'clock to flatten the whole mount and placed photoreceptor-side down onto the center of the insert. The medium was removed from the insert leaving only a moist film covering the tissue. The edges of the retina were flattened by light brushing. Inserts were placed in six-well tissue culture plates containing 1 mL of DMEM with 10 % FCS plus Fungizone (1.25 µg/mL) without or with drugs or siRNAs. Retinal explants were cultured in either normoxia (20 % O<sub>2</sub>, 5 % CO<sub>2</sub>, 37 °C) or hypoxia (1 % O<sub>2</sub>, 5 % CO<sub>2</sub>, 37 °C) in line with a previous study (Mei et al. 2012). Hypoxic conditions were achieved using an incubator (MiniGalaxy A; RS Biotech, Irvine, Scotland, UK) flushed with 5 % (v/v) CO<sub>2</sub> and N<sub>2</sub> to reduce O<sub>2</sub> level. The O<sub>2</sub> concentration was monitored and maintained at 1±0.1 %. Retinal explants were incubated in hypoxia for 24 h (Mei et al. 2012). Drugs were added to the medium at the onset of hypoxia. In the silencing experiments, siRNAs were added to the medium 24 h before the onset of hypoxia (Mei et al. 2012).

#### β<sub>3</sub>-AR agonists and antagonists

BRL 37344 is an established β<sub>3</sub>-AR agonist (Vrydag and Michel 2007) which has been previously used in mice (Aragón et al. 2011). BRL 37344 agonistic effects vary in different species. For instance, its EC<sub>50</sub> toward the lipolytic response in white adipocytes is in the nanomolar range in rat, while it is in the micromolar range in human (Carpéné et al. 1999). In addition, BRL 37344 has been found to be effective at 10 µM in the human isolated internal anal sphincter model (Ballester et al. 2010) and in human retinal endothelial cells (Steinle et al. 2003). Based on these data, we used BRL 37344 at 1, 3, and 10 µM.

SR59230A is the most widely used β<sub>3</sub>-AR antagonist (Vrydag and Michel 2007), and it has been previously used in mice (Martini et al. 2011). Its IC<sub>50</sub> toward the lipolytic response in rat white adipocytes is in the micromolar range, with maximal inhibitory effects at 10 µM (Carpéné et al. 1999). Similar values for IC<sub>50</sub> and maximal inhibitory effects have been reported in the human detrusor strip model (Kanie et al. 2012). Based on these data, we used SR59230A at 1, 3, and 10 µM.

L-748,337 is one of the very few antagonists with high selectivity for β<sub>3</sub>-ARs (Wuest et al. 2009). Its IC<sub>50</sub> toward cAMP production in CHO cells transfected with β<sub>3</sub>-ARs is in the nanomolar range, with maximal inhibitory effects in the micromolar range (Candelore et al. 1999). In addition, in the

murine isolated urinary bladder, L-748,337 at 10 µM has been reported to inhibit the effect of fenoterol, a β<sub>2</sub>-AR agonist which also displays an agonistic activity at β<sub>3</sub>-ARs (Palea et al. 2012). Based on these data, we used L-748,337 at 1, 3, and 10 µM.

BRL 37344 was dissolved in phosphate buffer (PB), while SR59230A and L-748,337 were dissolved in DMSO (final concentration 0.1 %). Control experiments were performed with 0.1 % DMSO in PB (vehicle).

#### Pharmacology of the NO pathway

NOS inhibition was obtained using 1 mM L-NAME as previously reported (Nakagawa et al. 2006). NOS activation was induced using 1 µM fluvastatin, a concentration known to activate both endothelial and inducible NOS (Chen et al. 2000; Meda et al. 2010). To increase NO production, we also used a different approach bypassing NOS activation with NO supplementation. To this aim, we used 2 mM SNAP, a stable analogous of endogenous S-nitrosothiols which acts as NO donor, as previously reported (West et al. 2006). L-NAME and fluvastatin were dissolved in PB, while SNAP was dissolved in DMSO (final concentration 0.1 %).

#### Western blot analysis

Western blot analysis was performed using antibodies directed against MAPK1, β<sub>1</sub>-, β<sub>2</sub>-, β<sub>3</sub>-ARs, and β-actin in line with Dal Monte et al. (2012). Retinas were homogenized in 10 mM Tris-HCl, pH 7.6, containing 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, and protease inhibitor cocktail, and centrifuged at 22,000×g for 30 min at 4 °C. The supernatant containing cytosolic proteins was used to detect MAPK1 (not shown). The pellet was 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 and protease inhibitor cocktail and centrifuged at 22,000×g for 30 min at 4 °C. The supernatant containing membrane proteins were used to detect β<sub>1</sub>-, β<sub>2</sub>-, and β<sub>3</sub>-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. The gels were transblotted onto PVDF membrane. PVDF membranes were blocked in 5 % skim milk for 1 h at room temperature and then incubated overnight at 4 °C with a primary mouse monoclonal antibody directed to MAPK1 (1:100 dilution), primary rabbit polyclonal antibodies directed to β<sub>1</sub>- (1:200 dilution) or β<sub>2</sub>-ARs (1:200 dilution) or a primary goat polyclonal antibody directed to β<sub>3</sub>-ARs (1:200 dilution). The same PVDF membrane was reblotted with a primary mouse monoclonal antibody directed to β-actin (1:2,500 dilution) as loading control. Finally, PVDF membranes were incubated for 1 h at room temperature with rabbit anti-mouse (1:25,000), mouse anti-rabbit (1:1,000), or rabbit anti-goat (1:5,000) HRP-labeled

secondary antibodies, as appropriate, and developed with the enhanced chemiluminescence reagent. Images were acquired (Chemidoc XRS+; Bio-Rad Laboratories). The OD of the bands was evaluated (Image Lab 3.0 software; Bio-Rad Laboratories). The data were normalized to the level of  $\beta$ -actin as loading control. For each experimental condition, six samples from six different mice were used. Each sample refers to the protein extracted from one retina. All experiments were run in duplicate. After statistics, data were averaged and plotted in the same graph.

#### Enzyme-linked immunosorbent assay

VEGF release was measured in the culture medium using a commercially available ELISA kit. VEGF release was measured spectrophotometrically (Microplate Reader 680 XR; Bio-Rad Laboratories) at 450 nm (correction wavelength set at 570 nm). For each experimental condition, six samples from six different mice were used. Each sample refers to the culture medium of one retina. Data were collected as picograms VEGF per milliliter culture medium. All experiments were run in duplicate. After statistical analysis, data were averaged and plotted in the same graph.

#### siRNA transfection

In preliminary experiments, four different pre-designed siRNAs targeting  $\beta$ 3-AR gene, each at three different concentrations (5, 15, and 50 nM), were used to identify the siRNA which gave the maximum reduction in the expression of  $\beta$ 3-AR at the lowest concentration (Online Resource 1). A non-silencing siRNA was used as negative control together with mock control. siRNA transfection was performed using the transfection reagent. For each experimental condition, six samples from six different mice, each containing one retina, were used. In order to evaluate transfection efficiency, Western blot was used to measure MAPK1 expression after transfection with MAPK1-positive control siRNA. After MAPK1 silencing, MAPK1 expression was reduced by ~85 % as compared with untransfected, mock-transfected, or non-silencing-transfected explants (not shown). The success of transfection with  $\beta$ 3-AR-siRNAs was demonstrated by Quantitative real-time RT-PCR (qPCR) (Online Resource 1, panel a). The silencing efficiency was confirmed by Western blot (Online Resource 1, panel b). After these preliminary experiments, we used  $\beta$ 3-AR-siRNA4 at 50 nM. After transfection with  $\beta$ 3-AR-siRNA4 or non-silencing siRNA, retinal explants were cultured for 24 h in normoxia followed by 24 h hypoxia.

#### RNA isolation and cDNA preparation

From each retina, total RNA was extracted (RNeasy Mini Kit; Qiagen), purified, resuspended in RNase-free water and

quantified using a fluorometer (Qubit; Invitrogen, Milan, Italy). First-strand cDNA was generated from 1  $\mu$ g of total RNA (QuantiTect Reverse Transcription Kit; Qiagen).

#### Quantitative real-time RT-PCR

qPCR was performed using published primers for  $\beta$ 3-ARs (Ristori et al. 2011). qPCR was performed using SYBR Green on a detection system (MiniOpticon Two-Color; Bio-Rad Laboratories). Polymerase chain reaction (PCR) reactions included 1  $\mu$ L of cDNA, 300 nM of each primer, 7.5  $\mu$ L of mastermix (iQ Sybr Green Supermix; Bio-Rad Laboratories), and RNase-free water to a final volume of 15  $\mu$ L. The target gene was run concurrently with the ribosomal protein L13A gene (Rpl13a), which encodes a ribosomal protein that is a component of the 60S subunit (Mazumder et al. 2003). Rpl13a is known to be a stable housekeeping gene in OIR mice and mouse retinal explants (Dal Monte et al. 2009, 2010; Martini et al. 2011; Mei et al. 2012; Ristori et al. 2011). Negative control PCR reactions without any template cDNA were performed. The qPCR was performed with hot-start denaturation step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, and 58 °C for 20 s. The fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. Primers were initially used to generate a standard curve over a large dynamic range of cDNA quantity, which allows calculation of the amplification efficiency for the primer pair (not shown). Amplification efficiency was close to 100 % (Opticon Monitor 3 software; Bio-Rad Laboratories). After amplification, first derivative melting curve analysis was used to confirm that the signal corresponded to a unique amplicon (not shown). PCR products were analyzed by nucleic acid staining on a gel stain (GelStar) containing 3 % agarose to verify the correct product size (not shown). After performing qPCR, the results were analyzed using the comparative cycle threshold method (Livak and Schmittgen 2001). Changes in mRNA expression were relative to the hypoxic levels after normalization to Rpl13a. For each experimental condition, six samples from six different mice were used. Each sample refers to the mRNA extracted from one retina. All reactions were run in triplicate. After statistical analysis, data were averaged and plotted in the same graph.

#### Colorimetric assay

Total amount of NO (micrometer) was assessed by the Griess reaction using a colorimetric assay kit that detects nitrite, a stable reaction product of NO (Kaur et al. 2009). Measurements were performed according to the manufacturer's instructions on the supernatants containing the cytosolic proteins used in Western blot. OD was measured spectrophotometrically (Microplate

Reader 680 XR; Bio-Rad Laboratories) at 540 nm. For each experimental condition, six samples from six different mice were used. Each sample refers to the protein extracted from one retina. All experiments were run in duplicate. After statistics, data were averaged in the same graph.

### Statistics

Statistical significance was evaluated using unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple-comparison post-test as appropriate. The results were expressed as means  $\pm$  SEM of the indicated *n* values. Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze data. Differences with  $P < 0.05$  were considered statistically significant.

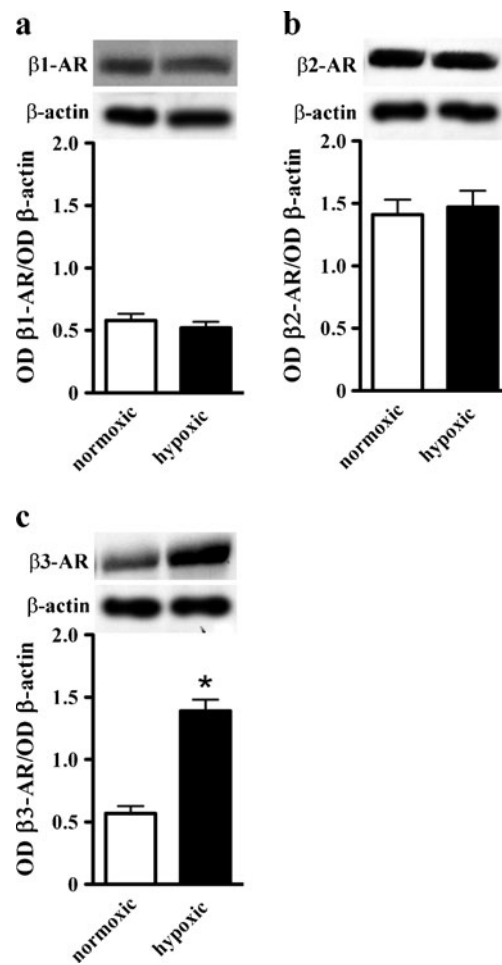
## Results

### Effects of $\beta 3$ -AR agonism/antagonism or $\beta 3$ -AR silencing

In a preliminary set of experiments, we confirmed that, similar to what was found in the retina of the OIR model (Ristori et al. 2011),  $\beta 3$ -ARs were upregulated by hypoxia, whereas  $\beta 1$ - or  $\beta 2$ -ARs were unaffected (Fig. 1).

$\beta 3$ -AR agonists/antagonists were then used in order to investigate whether  $\beta 3$ -ARs play a role in regulating VEGF release in response to hypoxia. As shown in Fig. 2, VEGF release was increased by  $\sim 4$ -fold by hypoxia (\*\* $P < 0.001$  versus normoxic). No effects were observed after vehicle treatment (not shown).  $\beta 3$ -AR agonists/antagonists dose-dependently affected hypoxia-induced VEGF release. BRL 37344 did not affect VEGF release at 1  $\mu\text{M}$ , while significantly increased VEGF release at 3 or 10  $\mu\text{M}$  ( $\sim 26\%$ ,  $^{\S}P < 0.05$  and  $\sim 46\%$ ,  $^{\S\S}P < 0.001$  versus hypoxic, respectively). SR59230A did not influence VEGF release at 1 or 3  $\mu\text{M}$ , while it significantly reduced VEGF release at 10  $\mu\text{M}$  ( $\sim 54\%$ ,  $^{\S\S}P < 0.001$  versus hypoxic). L-748,337 significantly reduced VEGF release by  $\sim 28\%$  at 1  $\mu\text{M}$  ( $^{\S}P < 0.05$  versus hypoxic),  $\sim 51\%$  at 3  $\mu\text{M}$  ( $^{\S\S}P < 0.001$  versus hypoxic), and  $\sim 74\%$  at 10  $\mu\text{M}$  ( $^{\S\S}P < 0.001$  versus hypoxic), respectively. After SR59230A at 10  $\mu\text{M}$ , VEGF release was  $\sim 140\%$  higher than in normoxia ( $^*P < 0.05$  versus normoxic), whereas after L-748,337 at 10  $\mu\text{M}$ , VEGF release did not differ significantly from that measured in normoxia.

Specificity of  $\beta 3$ -AR pharmacological activation/blockade was confirmed by experiments with  $\beta 3$ -AR silencing. As shown in Fig. 3a, after  $\beta 3$ -AR-siRNA4 at 50 nM, VEGF release was significantly lower than in hypoxia ( $\sim 52\%$ ,  $^{\S}P < 0.001$  versus hypoxic) but  $\sim 135\%$  higher than in normoxia ( $^*P < 0.05$  versus normoxic). In addition, the effects of BRL 37344, SR59230A, or L-748,337 (10  $\mu\text{M}$ ) on VEGF



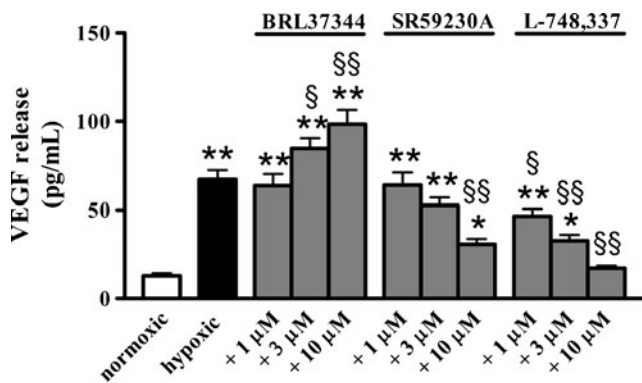
**Fig. 1** Hypoxia regulation of  $\beta 3$ -ARs. The expression of  $\beta 1$ - (a),  $\beta 2$ - (b), and  $\beta 3$ -ARs (c) was evaluated by Western blot and densitometric analysis in normoxic and hypoxic retinal explants. Protein expression was relative to the loading control  $\beta$ -actin. Each column represents the mean  $\pm$  SEM of data from six independent samples, each containing one retina.  $^*P < 0.001$  versus normoxic (unpaired Student's *t* test)

release were prevented by  $\beta 3$ -AR silencing (Fig. 3b). Effects of BRL 37344, SR59230A, or L-748,337 on VEGF release were not influenced by non-silencing siRNA (not shown).

### Role of NO signalling in the $\beta 3$ -AR-induced regulation of VEGF release

On the basis of evidence supporting NO as a downstream effector of  $\beta 3$ -ARs (Hodis et al. 2011; Ignarro et al. 2002; Massion et al. 2004) and of the reported regulation of VEGF expression by NO (Ziche and Morbidelli 2009), we investigated whether NO signaling participates in the  $\beta 3$ -AR-induced regulation of VEGF release.

We first determined whether NO production in response to hypoxia may be influenced by  $\beta 3$ -AR activation, blockade, or silencing. No effects were observed after vehicle treatment or non-silencing siRNA (not shown). As shown



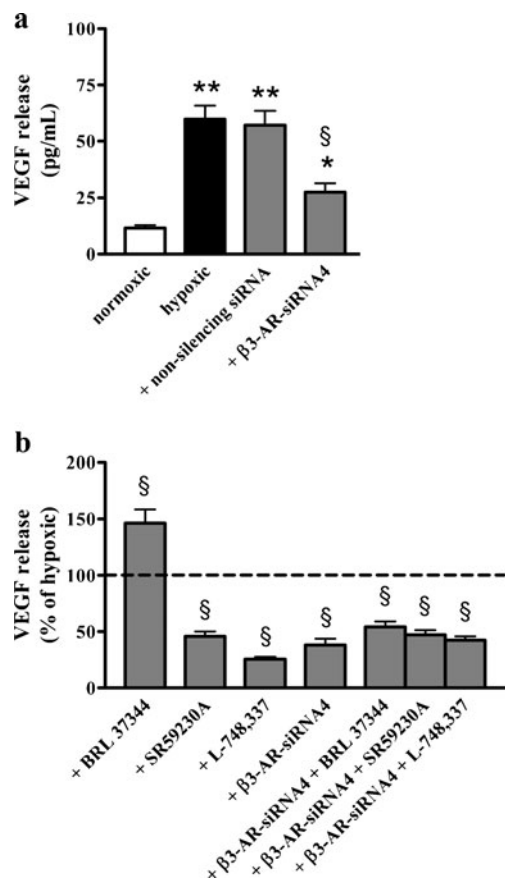
**Fig. 2** VEGF release after BRL 37344, SR59230A, or L-748,337. VEGF release was evaluated by ELISA in normoxic and in hypoxic retinal explants either untreated or treated with BRL 37344, SR59230A, or L-748,337. All drugs were used at 1, 3, or 10  $\mu$ M. Each column represents the mean  $\pm$  SEM of data from six independent samples, each containing one retina. \* $P$ <0.05 and \*\* $P$ <0.001 versus normoxic; § $P$ <0.05 and §§ $P$ <0.001 versus hypoxic (ANOVA followed by Newman–Keuls multiple-comparison post-test)

in Fig. 4, hypoxia significantly increased NO levels (205 %, \*\*\* $P$ <0.001 versus normoxic). BRL 37344 at 10  $\mu$ M increased hypoxic levels of NO by ~25 % (§ $P$ <0.05 versus hypoxic), while SR59230A and L-748,337 at 10  $\mu$ M or  $\beta$ 3-AR-siRNA4 at 50 nM significantly reduced hypoxic levels of NO (~31 %, §§ $P$ <0.01, ~45 % and ~53 %, §§§ $P$ <0.001 versus hypoxic, respectively).

In a second set of experiments, we interfered with NO signaling in order to establish whether  $\beta$ 3-AR-induced regulation of VEGF release in response to hypoxia occurs through NO. To this aim, we used 1 mM L-NAME in combination with BRL 37344 at 10  $\mu$ M in order to determine whether NOS inhibition prevents VEGF upregulation in response to  $\beta$ 3-AR agonism. Fluvastatin at 1  $\mu$ M or SNAP at 2 mM was also used in combination with SR59230 and L-748,337 at 10  $\mu$ M or  $\beta$ 3-AR-siRNA4 at 50 nM in order to determine whether NOS activation or NO supplementation prevents VEGF downregulation in response to  $\beta$ 3-AR antagonism/silencing. As shown in Fig. 5, L-NAME prevented BRL 37344-induced increase of VEGF. In addition, effects of SR59230, L-748,337, or  $\beta$ 3-AR-siRNA4 were abolished by fluvastatin or SNAP. In these experimental conditions, VEGF levels did not differ from those measured after BRL 37344 alone.

## Discussion

In this study, we report a novel role for  $\beta$ 3-ARs as endogenous regulators of angiogenesis-associated events in the retina. The fact that VEGF release is modulated by  $\beta$ 3-ARs indicates that these receptors may participate in maintaining VEGF homeostasis and that  $\beta$ 3-AR blockade may be effective in counteracting the damage produced by a proangiogenic environment. The additional finding that  $\beta$ 3-AR effects on VEGF release



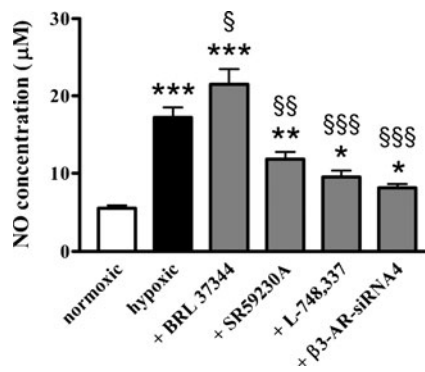
**Fig. 3** VEGF release after  $\beta$ 3-AR silencing and effects of BRL 37344, SR59230A, or L-748,337 on VEGF release after  $\beta$ 3-AR silencing. **a** VEGF release was evaluated by ELISA in normoxic and hypoxic retinal explants either untreated or treated with non-silencing siRNA or 50 nM  $\beta$ 3-AR-siRNA4. **b** VEGF release was evaluated by ELISA in hypoxic retinal explants treated with  $\beta$ 3-AR agonists/antagonists at 10  $\mu$ M either in the absence or in the presence of 50 nM  $\beta$ 3-AR-siRNA4. Each column represents the mean  $\pm$  SEM of data from six independent samples, each containing one retina. \* $P$ <0.05 and \*\* $P$ <0.001 versus normoxic; § $P$ <0.001 versus hypoxic (ANOVA followed by Newman–Keuls multiple-comparison post-test)

may be regulated by NOS activation, NOS inhibition, or NO supplementation demonstrates that NO is one of the functional links between  $\beta$ 3-ARs and VEGF in hypoxic retinas.

## $\beta$ 3-AR role in regulating VEGF release

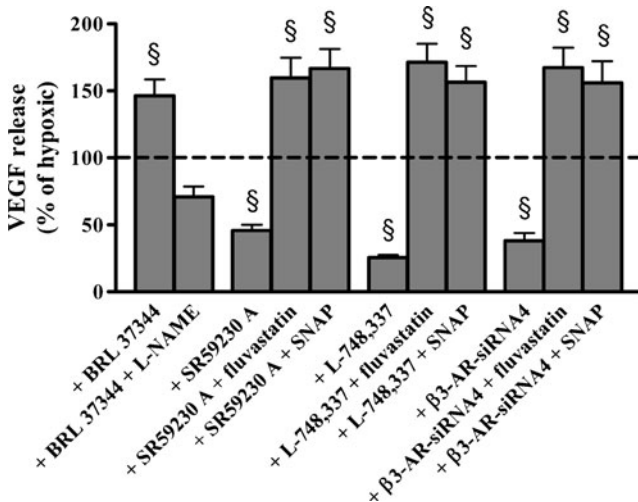
Recently, the  $\beta$ 3-AR has emerged as a potential target for the treatment of ischemic disorders because of its upregulation in response to hypoxia and its resistance to desensitization (Dessy and Balligand 2010).

As shown by present results,  $\beta$ 3-ARs regulate VEGF release in hypoxic retinas. In fact, BRL 37344 increases VEGF release suggesting that activation of  $\beta$ 3-ARs may constitute an important part of the retinal response to hypoxia. In line with our present results, BRL 37344 induces VEGF expression in mouse adipocytes (Fredriksson et al. 2000) and causes



**Fig. 4** NO production after BRL 37344, SR59230A, L-748,337, or β3-AR-siRNA4. NO production was evaluated by a colorimetric assay in normoxic and hypoxic retinal explants either untreated or treated with β3-AR agonists/antagonists at 10 μM or 50 nM β3-AR-siRNA4. Each column represents the mean ± SEM of data from six independent samples, each containing one retina. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus normoxic; § $P < 0.05$ , §§ $P < 0.01$ , and §§§ $P < 0.001$  versus hypoxic (ANOVA followed by Newman–Keuls multiple-comparison post-test)

proliferation of human retinal endothelial cells (Steinle et al. 2003) indicating β3-ARs as a possible regulatory switch in retinal angiogenesis. Some issues have been reported in the use of BRL 37344. For instance, studies in the human heart support the idea that BRL 37344 may have an intrinsic activity at β1- or β2-ARs (Pott et al. 2003). In addition, BRL 37344 may have effects unrelated to β-ARs that may occur at similar



**Fig. 5** Effects of NOS inhibition after BRL 37344 and effects of NOS activation or NO supplementation after SR59230A, L-748,337, or β3-AR-siRNA4 on VEGF release. VEGF release was evaluated by ELISA in hypoxic retinal explants treated with 10 μM BRL 37344 either in the absence or in the presence of 1 mM L-NAME or with 10 μM SR59230A, 10 μM L-748,337, or 50 nM β3-AR-siRNA4 either in the absence or in the presence of 1 μM fluvastatin or 2 mM SNAP. Each column represents the mean ± SEM of data from six independent samples, each containing one retina. § $P < 0.001$  versus hypoxic (ANOVA followed by Newman–Keuls multiple-comparison post-test)

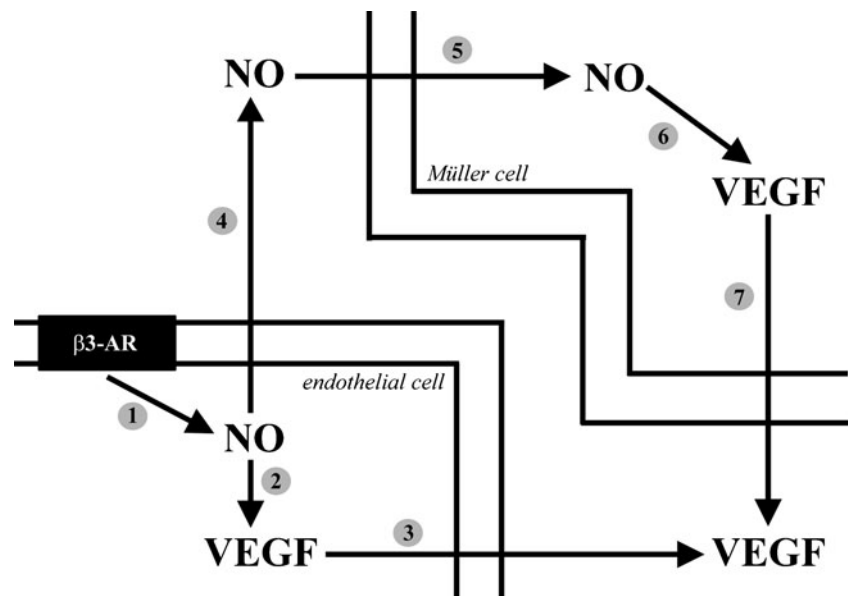
concentrations as those used for the activation of β3-ARs (Kubota et al. 2002; Leblais et al. 2005). On the other hand, our finding that β3-AR knockdown with selective siRNAs prevents the BRL 37344-mediated VEGF upregulation in hypoxic conditions supports the selectivity of BRL 37344 in our experimental setting and excludes that BRL 37344 can exert an off-target effect.

The fact that β3-AR antagonists prevent VEGF upregulation in response to hypoxia indicates that blocking β3-AR signaling may affect angiogenesis-related events. In this respect, SR59230A, a widely used β3-AR antagonist, may lead in some cases to misleading conclusions. For instance, SR59230A may also act as partial agonist at β3-ARs (Vrydag and Michel 2007) or it may not suppress the receptor constitutive activity, which is typical of β3-ARs (Perrone and Scilimati 2011). On the other hand, the fact that L-748,337, a β3-AR antagonist belonging to a different chemical class than SR59230A, prevents VEGF upregulation supports the selectivity of β3-AR blockade and demonstrates that interfering with β3-AR signaling is effective in attenuating retinal angiogenesis. The additional finding that β3-AR silencing reproduces the effects of β3-AR antagonism on VEGF release also supports the selectivity of β3-AR blockers. It is noteworthy that L-748,337 has potency greater than SR59230A in decreasing VEGF. This is in line with the finding that L-748,337 prevents vasodilation of rat retinal arterioles with a potency of one order of magnitude higher than SR59230A (Mori et al. 2010).

The fact that β3-ARs participate in VEGF regulation is consistent with the finding that β3-ARs are localized to retinal blood vessels and are upregulated by hypoxia (Chen et al. 2012; Ristori et al. 2011) indicating the possibility that β3-ARs may modulate VEGF release by endothelial cells. There is evidence that, in the retina, endothelial cells express and release VEGF (Tugues et al. 2011), although a major source of retinal VEGF is represented by Müller cells, particularly under hypoxic-ischemic conditions (Kaur et al. 2008). Thus, β3-AR localization to endothelial cells may not necessarily be linked to the regulation of VEGF release. In this respect, we have previously demonstrated that β2-ARs localized to Müller cells play an important role in regulating retinal VEGF in OIR (Martini et al. 2011).

The observed potent effect of β3-AR blockade on VEGF release in retinal explants would suggest an equally potent action of β3-AR blockade against pathologic angiogenesis. However, it has been observed recently that SR59230A does not affect either VEGF release or retinal neovascularization in the OIR model (Martini et al. 2011). This can be explained by assuming that, in OIR, systemic administration of SR59230A does not provide the retina with a sufficient drug concentration to interfere with VEGF release. As shown by our results, SR 59230A concentrations lower than 10 μM do not affect VEGF release in retinal explants.

**Fig. 6** A schematic diagram of the possible pathway by which  $\beta 3$ -ARs regulate VEGF release through NO.  $\beta 3$ -ARs expressed by endothelial cells modulate the production of NO (1) which, in turn, regulate VEGF release by the same endothelial cells (2, 3) or, since NO is a diffusible molecule (4, 5) by surrounding cells, including Müller cells (6, 7)



Another possibility is that different post-receptor mechanisms may be activated in retinal explants and OIR. For instance,  $\beta 3$ -ARs may hetero-oligomerize with  $\beta 2$ -ARs, generating a  $\beta$ -adrenergic signaling unit with functional properties distinct from those of  $\beta 2$ - and  $\beta 3$ -ARs (Breit et al. 2004). Since the formation of hetero-oligomers between membrane receptors is influenced by oxidative stress caused by hypoxia–ischemia (van der Wijk et al. 2005; Li et al. 2012), we cannot exclude that differences in oxygen tension in the two models may be responsible of the different  $\beta 3$ -AR function in retinal explants and OIR. In fact, in retinal explants, hypoxia corresponds to 1 % oxygen, whereas in OIR hypoxia corresponds to atmospheric oxygen tension when mice are returned to normoxia after hyperoxia.

#### $\beta 3$ -AR coupling to NO signalling

As shown by the present results,  $\beta 3$ -ARs regulate NO production. This result is consistent with previous experimental evidence demonstrating that  $\beta 3$ -ARs are coupled to NO pathway in the ischemic heart (Dessy and Balligand 2010), in human coronary microarteries (Dessy et al. 2004), in rat thoracic aorta (Trochu et al. 1999), and in rat retinal vessels (Mori et al. 2010).

Our results also show that modulating the NO pathway affects the  $\beta 3$ -AR-induced VEGF release indicating that  $\beta 3$ -AR-induced regulation of VEGF response to hypoxia is mediated, at least in part, by NO signaling. In this respect, complex but not completely elucidated relationships between NO and VEGF have been reported. In fact, NO may act upstream of VEGF, regulating its expression (Dulak and Józkowicz 2003). For instance, inhibition of the inducible isoform of NOS reduces VEGF levels in OIR (He et al. 2007; Zhang et al. 2009). Conversely, VEGF may act as an upstream regulator

of NO (Wilkinson-Berka 2004). For instance, a VEGF trap reduces the diabetes-related NO increase in the rat retina (Joussen et al. 2002). This complexity may reflect overlapping and complementary functions between NO and VEGF pathways, suggesting their relationships as a nodal point in the control of angiogenesis-related retinal diseases.

Our evidence that  $\beta 3$ -ARs regulate VEGF release through NO suggests possible pathways by which  $\beta 3$ -AR agonists/antagonists may modulate VEGF. In fact, we may hypothesize that  $\beta 3$ -ARs, expressed by VEGF-containing endothelial cells, modulate VEGF release acting through NO both in the same endothelial cells and, since NO is a diffusible molecule (Bryan et al. 2009), on surrounding cells, including Müller cells (Fig. 6).

#### Conclusions and clinical implications

Overall, the present results suggest that VEGF accumulation, a key factor in many hypoxic–ischemic vision-threatening retinal diseases, depends at least in part on  $\beta 3$ -AR activity, suggesting  $\beta 3$ -AR blockers as attractive therapeutic tools. Although extrapolation of these data to the human situation is difficult, these results may help to explore the possible role of  $\beta 3$ -ARs in vascularization-associated disorders. In this respect, the fact that  $\beta 3$ -ARs are upregulated by hypoxia and that they influence retinal VEGF through the involvement of NO pathway suggests that blocking this signaling pathway may be effective in reducing VEGF-stimulated retinal angiogenesis.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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