Published in final edited form as:
Mol Vis. ; 10: 512–520.

VEGF isoforms and their expression after a single episode of hypoxia or repeated fluctuations between hyperoxia and hypoxia: Relevance to clinical ROP

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Abstract

Purpose—Fluctuations in oxygen are associated with the development of severe retinopathy of prematurity (ROP) in humans. However, the causal relationships between oxygen variability and severe ROP remain unknown. We investigated whether isoforms of vascular endothelial growth factor (VEGF) were differentially stimulated by hypoxia and by repeated fluctuations between hypoxia and hyperoxia, and whether isoforms were differentially expressed in association with intravitreous neovascularization. We also determined whether pigment epithelium-derived factor (PEDF) was dysregulated by oxygen fluctuations perhaps contributing to a delay in normal retinal vascular development.

Methods—We used the 50/10 oxygen-induced retinopathy (50/10 OIR) model that exposes newborn rat pups to repeated cycles of 24 h of 50% oxygen alternating with 24 h of 10% oxygen to cause a condition similar to human ROP. Animals were euthanized at postnatal day 2 (P2; after one cycle of 50/10% oxygen), P7 (after 3.5 cycles of 50/10% oxygen), and P14 (after seven cycles of 50/10% oxygen). Room air raised control rat pups were also exposed to a single episode of 24 h of hypoxia at P7 and P14 and assayed immediately afterwards. Retinal VEGF isoforms and PEDF were measured by RT-PCR. Total VEGF protein was measured by ELISA.

Results—We found that repeated cycles of hyperoxia and hypoxia caused greater expression of VEGF protein compared to control than did a single cycle of hyperoxia and hypoxia. $VEGF_{164}$ mRNA had a greater fold change over control after repeated oxygen fluctuations than after a single episode of hypoxia. However, the other isoforms, $VEGF_{188}$ and $VEGF_{120}$, were expressed to a similar degree regardless of whether the stimulus was a single episode of hypoxia or repeated fluctuations in oxygen. $VEGF_{164}$ was the predominant isoform expressed at the time of maximal intravitreous neovascularization. Retinal PEDF expression was elevated in pups in the 50/10 OIR model compared to control at P7, immediately after 50% oxygen. PEDF expression in the experimental group was similar to control at P18, when intravitreous neovascularization occurred.

Conclusions—Repeated fluctuations in oxygen results in a greater expression of the pathologic isoform, $VEGF_{164}$, than does hypoxia alone. However, the other isoforms were upregulated to an equivalent degree over control by repeated fluctuations in oxygen or a single episode of hypoxia. Total VEGF protein was increased to a greater degree by repeated fluctuations in oxygen compared to a single cycle of oxygen. PEDF was increased over control early in the 50/10 OIR model and may play a role in the observed delay in retinal vascularization. These findings provide insight into the effect of repeated oxygen fluctuations on the development of severe ROP in preterm infants

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Vascular endothelial growth factor (VEGF) plays an essential role in blood vessel development in the embryo [1] and is required for retinal vascularization [2]. VEGF has been shown to have at least five human [3] and three murine isoforms [4] that develop from alternate splicing of a single gene. Based on the affinity for binding to heparan sulfate, the isoforms have been found to have soluble ($VEGF_{121/120}$ human/rodent analogs, respectively), cell-associated ($VEGF_{189/188}$), or both ($VEGF_{165/164}$) properties. Differential expression of these three VEGF isoforms in the mouse resulted in variations in retinal vascular, myocardial, and lung development, and suggested that different VEGF isoforms might have different cell and tissue effects [5–7]. There are a growing number of studies investigating the isoforms and their different roles in disease and it is now clear that there are isoform specific differences. $VEGF_{164}$ has been associated with pathologic intravitreous neovascularization [8] and has been found to be potently more pro-inflammatory than $VEGF_{121/120}$ [8,9]. $VEGF_{165/164}$ has been detected in human proliferative diabetic retinopathy [10] and was associated with a poor prognosis in certain tumors [11]. The cell-associated isoform, $VEGF_{188}$, is important in endothelial cell migration and adhesion whereas the other isoforms were not [12]. It too has been associated with poorer outcomes in certain tumors [13]. In mice expressing only $VEGF_{120}$, retinal vascular development was severely impaired [5], suggesting it alone is insufficient for normal vasculogenesis. Thus, VEGF isoforms have different tissue and organ effects in development and disease.

A number of studies support VEGF receptors as also having multiple and different cell and tissue effects on angiogenesis [8,10,14–16]. VEGF receptor 2 (VEGFR2) has been demonstrated to be upregulated in proliferative diabetic retinopathy [10,14], whereas soluble VEGF receptor 1 (R1) was not [10]. In vitro, VEGFR2 has been demonstrated as having a role in VEGF-induced proliferation and migration of endothelial cells [17]. The murine model of hyperoxia-induced retinopathy has also demonstrated upregulation of VEGFR2 [15], and, in a dog oxygen induced retinopathy model, when antibody against VEGFR2 was used, both intravitreous and intraretinal vascularization were reduced [18].

Pigment epithelium-derived factor (PEDF) is a potent angiogenic antagonist [19], upregulated by hyperoxia [19,20] and important in retinovascular development [21]. In rodents, PEDF was only detected after normal retinal vascularization was complete [19,20]; however, during hyperoxia-induced retinopathy, PEDF was detectable before retinal vascularization was complete [20].

Both clinically [22,23] and in animal models [24,25], severe retinopathy of prematurity (ROP) is associated with fluctuations in oxygen. However, the causal relationships that tie repeated fluctuations in oxygen to severe ROP remain unknown. We hypothesized that repeated variability in oxygen would present a greater stimulus for VEGF production than a single episode of hypoxia, and that VEGF isoforms would be differentially regulated by stresses that affect premature infants, i.e., hypoxia and/or repeated oxygen fluctuations. We also proposed that repeated fluctuations in oxygen would alter the expression of PEDF. Upregulation of PEDF after hyperoxia might impact normal retinal vascularization and, in part, account for delayed vascularization and the peripheral areas of avascularity seen in ROP. This study provides data on the differential expression of individual VEGF isoforms and PEDF under conditions of oxygen variability using a rat model of repeated cycles between hyperoxia and hypoxia. The data provide insight into how the differential expression of these two factors regulating angiogenesis might relate to events that occur in ROP.

METHODS

All animals were cared for in accordance with the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals).

Oxycycler

The Oxycycler (Biospherix, New York, NY) regulates the atmosphere inside an incubator by injecting either nitrogen or oxygen. Carbon dioxide in the cage was monitored and removed from the atmosphere by placing soda lime within the incubator. At the start of the experiment, female Sprague-Dawley rats (Charles River, Wilmington, MA) and their litters were placed into the incubator attached to the Oxycycler. The day of birth was designated P0 and animals were placed in the incubator at this time. Rats were fed ad libitum for the length of the experiment. Litters of <12 pups were supplemented from litters born on the same day to ensure all litters had at least 12 pups. Each experiment was repeated three times and to obtain enough tissue for protein and PCR analysis, retinas were pooled, on average three per sample.

Multiple cycles of hypoxia/hyperoxia (50/10 OIR model)

The 50/10 OIR model mimics the inspired oxygen extremes seen in human ROP [22]. Oxygen was cycled between 50% and 10% every 24 h for 14 days (50/10 OIR model) [25]. Animals kept longer than this were moved to room air until euthanasia. There were three experimental groups, animals euthanized at postnatal age 7 days (P7), P14, and P18. Age matched controls were room air raised.

Rat model single hypoxic exposure

Litters with their mothers were moved from room air into the incubator for 24 h at 10% oxygen. Animals were euthanized immediately upon removal to room air. There were two experimental groups, P7 and P14. Animals were placed in 10% oxygen on P7 or P14 and harvested 24 h later.

Tissue preparation for lectin stain

Retinal tissue was removed from all pups, and treated and stored as required for the specific analysis indicated below.

For visualization of the retinal vasculature, pups were anesthetized by intraperitoneal injection of ketamine (2.5 mg/kg) and xylazine (1 mg/kg). Paraformaldehyde (PFA) was then directly perfused (0.4 ml 0.5% PFA) into the left ventricle prior to euthanasia by intracardiac injection of Nembutal (80 mg/kg). Both eyes were enucleated and whole eyes were fixed in 2% PFA for 2 h before being washed in PBS (0.12 M NaCl, 0.02 M Na_2HPO_4 and 0.005 M KH_2PO_4, pH 7.4). The retinas were dissected using a modification of the method of Chan-Ling [26]. Briefly, under a dissecting microscope an incision was made at the limbus between the cornea and sclera. Scissors were then used to cut round the junction between the cornea and sclera until the cornea could be removed. The lens was gently removed, taking care not to remove the retina. The eyecup was transferred to PBS for further dissection. The retina was gently eased from the sclera using fine forceps, taking care to leave the ora serrata intact, which defines the anterior extent of the retina. The retina was then placed onto a microscope slide and flattened by making 4 incisions perpendicular to its outer edge. At this stage, as much vitreous as possible was removed using cellulose sponges and scissors.

Tissue preparation for RT-PCR and protein

Animals were euthanized with an overdose of pentobarbital (0.2 mg/ml). Both eyes were enucleated and the retinas were dissected under a dissecting microscope as described above, except that the ora serrata was removed. The retinal tissue was pooled and placed in RNAlater (Qiagen; Valencia, CA) for RT-PCR or in M-Per (Pierce; Rockford, IL) for protein analysis. All samples were frozen at −20 °C until analysis.

Lectin stain

Endothelial cells were visualized by incubation with *G. simplicifolia* (Bandeiraea) isolectin B4. The flattened, fixed, whole mounted retinas were made permeable in 70% v/v methanol (kept at −20 °C) for 20 min, and then in PBS/1% Triton X-100 for 30 min. The retinas were then incubated with FITC conjugated *G. simplicifolia* (Bandeiraea) isolectin B4 (Sigma, St. Louis, MO) at 5 μg/ml in PBS overnight at 4 °C. They were rinsed in PBS/1% Triton X-100 for 10 min, then in PBS for 10 min twice. The retinas were mounted in PBS:glycerol 2:1 and the coverslip sealed with nail varnish. Images were captured using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and digitally stored for later analysis.

Avascular areas

Digitized images of the total retinal and peripheral avascular areas were measured using the freeware program ImageTool, Version 3 (University of Texas, San Antonio, TX), and the avascular area was expressed as a percentage of the total retinal area.

Clock hours of neovascularization

Clock hours of neovascularization were estimated as described by Zhang et al [27]. Briefly, the whole mounted retina was divided into 12 segments based on a clock face and within each segment the presence of intravitreous neovascularization (ridges, clumps, sheets, or tufts of endothelial cells) was recorded.

Semi-quantitative RT-PCR

Samples were removed from RNAlater and RNA extracted using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). DNA contamination was removed by using DNA-*free* (Ambion; Austin, TX) and RNA quantity was determined spectrophotometrically. Reverse transcription was done using Retroscript Kit (Ambion). Briefly, 1 μg of RNA and 2 μl of random decamers were made up to a volume of 12 μl in nuclease free water. This was mixed, spun briefly and heated at 75 °C for 3 min. Then, 2 μl 10X RT buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, and 50 mM DTT), 4 μl dNTP (2.5 mM each), 10 U RNase inhibitor, and 100 U MMLV-reverse transcriptase were added. The reverse transcription reactions were incubated at 42 °C for 60 min and terminated at 92 °C for 10 min. Samples were frozen at this stage until PCR.

PCR was performed using specific primers to the VEGF isoforms (forward 5′-CCT CCG AAA CCA TGA ACT TTC TGC TC-3′ and reverse 5′-CAG CCT GGC TCA CCG CCT TGG CTT-3′; annealing temperature 60 °C) and PEDF (forward 5′-TCA CCA ACC CTG ACA TCC ACA G-3′ and reverse 5′-ACT GCC CCT TGA AGT AAG CCA C-3′; annealing temperature 58 °C). Products from the VEGF isoforms primers were 665 bp $VEGF_{188}$; 593 bp $VEGF_{164}$ and 461 bp $VEGF_{120}$. PEDF primers produced a single band at 312 bp.

The linear range of each sample was determined empirically by increasing the number of cycles and resolving the products on a 2% agarose gel (agarose from USB Corporation, Cleveland, OH). Sample reactions were 2.5 μl cDNA, 5 μl 10X PCR complete buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 2.5 μl dNTP mix (2.5 mM each), 1 μl primer mix (5 μM of each primer), 1 U superTaq polymerase (Ambion).

The control gene was 18S ribosomal RNA and was amplified using a QuantumRNA primer:competimer set (Ambion) yielding a band of 489 bp. As the 18S is far more abundant than most other RNA, 18S amplification was reduced by adding competimers which compete with 18S primer for binding. The competimers are primers modified at their 3′ ends to block extension by DNA polymerase.

The ratio of primer:competimer was quantified empirically by increasing the ratio at the predetermined number of cycles (see above). 2.5 μl of cDNA was added to 5 μl 10X PCR complete buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 2.5 μl dNTP mix (2.5 mM each), 1 μl primer:competimer mix, 1 U superTaq polymerase (Ambion). Samples were resolved on a 2% agarose gel and the 18S rRNA band that had an intensity matching that of the sample was selected for use in the relative-quantification.

Samples were done in triplicate. All PCR products were confirmed by gel extraction (Qiaquick Gel Extraction Kit, Qiagen) and sequence analysis (UNC-CH Genome Analysis Facility, Chapel Hill, NC). For quantification, the integrated optical density (IOD) of each band was captured using a UVP ChemiDoc System including a Chemi cooled CCD camera, PCI digitizing image acquisition board, EpiChemi II Dark-room with transilluminator, and LabWorks 4.0 Software. Data were then exported to an excel spreadsheet where values were expressed relative to the internal 18S control for each sample and means and standard deviations calculated.

Protein quantification

Tissue samples were thawed and subjected by centrifugation at 16,000x g for 10 min at 4 °C. The M-Per supernatant was removed and lysis buffer (0.15 M NaCl, 0.02 M Tris-HCl pH 8.0, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.01% proteinase inhibitor cocktail; [the inhibitor cocktail was from Sigma]) added to the sample. Samples were homogenized and centrifuged at 16,000x g for 10 min at 4 °C.

Total protein was quantified using a protein assay kit (Bio-Rad, Hercules, CA), which is a modification of the Lowry assay [28]. Supernatants were then assayed without dilution in duplicate using a commercially available ELISA kit for VEGF (R & D Systems, Minneapolis, MN), which recognizes the 120 and 164 isoforms.

Statistics

For protein, absolute values were obtained whereas for RNA a fold change was determined based on the value expressed relative to 18S RNA. Summary statistics are presented as mean (standard deviation) or median (interquartile range). Comparisons between groups were done using a t test.

RESULTS

The rat pup retina in the 50/10 OIR model showed delayed retinal vascularization resulting in peripheral avascularity and developed intravitreous neovascularization upon return to room air (Figure 1, Table 1). Pups in the experimental groups had lower weights at all stages than their age matched, room air raised controls. All groups had the same litter sizes and the pups spent equal time with their mothers. It is not known whether the effect of oxygen cycling is direct for the pups, or that mothers raised in the variable oxygen environments were affected and passed this on to their pups indirectly. However, our results are similar to those already published for this model [25] and for normal rat retinal vascular development [29].

VEGF isoform expression in normal retinovascular development

By P7, approximately 75% of the inner retina was vascularized and by P14 the inner capillary plexus completely covered the extent of the retina (Table 1, Figure 1). There was a twofold decrease in the expression of $VEGF_{120}$ from P7 to P14 (p=0.008, t test) and the expression was stable through P18 (Figure 2, room air (RA) controls), whereas $VEGF_{164}$, the predominant isoform expressed, remained stable over time points assayed. $VEGF_{188}$ had a stable to slightly increased expression over time.

A faint band also appeared at 145 bp from the PCR analysis. When the individual mRNA from the 120, 164 and 188 isoforms are mixed together and analyzed by PCR, this 145 bp fragment also appeared. Therefore, it is unlikely that this is an alternative splice variant of VEGF, but rather an artifact of the PCR analysis (personal communication, Dr. Magali Saint-Geniez, Schepens Eye Research Institute, Boston, MA).

Effect of hypoxia on VEGF isoform expression

The expression of $VEGF_{164}$, $VEGF_{120}$, and $VEGF_{188}$ was increased at P7 and P14 after a single episode of 24 h of hypoxia (10% oxygen) compared to respective control RA groups (Figure 3). $VEGF_{120}$ was expressed to a significantly greater extent at P7 than at P14, although the fold increase was similar at both time points compared to RA control. In contrast, $VEGF_{164}$ was expressed only to a slightly greater extent after 24 h of 10% oxygen at both time points. $VEGF_{188}$ had increased expression after hypoxia, more so at P7 than at P14, compared to respective controls (Figure 3). These data showed that 24 h of hypoxia caused upregulation of all VEGF isoforms, but mainly of $VEGF_{120}$ and $VEGF_{188}$.

Effect of repeated cycling in hyperoxia and hypoxia (50/10 OIR model) on VEGF isoform expression

After repeated cycling in the 50/10 OIR model, the development of the inner capillary plexus was slowed with approximately 50% of retina, becoming vascularized at P7 and 70% at P14 (Table 1). At P7, following 3.5 cycles and immediately after 24 h of 50% oxygen, $VEGF_{164}$ expression was significantly reduced compared to control (Figure 2), whereas $VEGF_{120}$ was reduced but not significantly. There was also little difference in the expression of the cell-associated isoform $VEGF_{188}$. At P14, after 7 fluctuations in oxygen and immediately after 24 h of 10% oxygen, there was a 1.7-fold increase in $VEGF_{120}$ and a 1.6-fold increase in $VEGF_{164}$ expression compared to respective controls (Figure 2). There was no change in $VEGF_{188}$ expression over control.

VEGF protein was measured by ELISA (mainly for $VEGF_{164}$ and some $VEGF_{120}$) and results were expressed relative to total protein for each sample. Repeated cycling (P14) caused a significant threefold increase in VEGF protein compared to P14 controls ($p<0.004$, t test) whereas a single cycle (P2) caused only a 1.5-fold increase compared to P2 controls ($p<0.03$, t test), even though both experimental groups of rat pups had just been exposed to 24 h of 10% O_2 (Figure 4), and P2 pups had greater percent retinal avascular areas than P14 pups (Table 1).

When rat pups exposed to single episodes of 24 h of 10% oxygen were compared to those exposed to repeated fluctuations in oxygen, at P14, immediately following 24 h of hypoxia in both groups (Figure 5), there was a greater fold increase in $VEGF_{164}$ expression after repeated cycles compared to a single episode of 10% oxygen ($p<0.02$, t test). This was not found to be true for the other isoforms that had equivalent increased fold expression after a single episode of hypoxia or after repeated cycles of hypoxia and hyperoxia. These data provide evidence that repeated fluctuations of hypoxia and hyperoxia causes a greater fold increase in expression of $VEGF_{164}$ than compared to a single episode of hypoxia.

PEDF expression in normal development and in 50/10 OIR model

Expression of PEDF was relatively stable in room air control eyes from P7 through P18 (Figure 6). In the 50/10 OIR model, animals at P7, immediately following 24 h of hyperoxia (50% oxygen), had a 1.5-fold increase in PEDF expression compared to control, however this was not significant. PEDF expression was similar to control at other time points tested, even during intravitreous neovascularization.

VEGF isoform expression and intravitreous neovascularization

In this OIR model, after 7 cycles of fluctuating hyperoxia and hypoxia, the pups are placed into room air and develop intravitreous neovascularization that peaks in extent at P18. In experimental animals at P18, we found that $VEGF_{164}$ remained elevated over control, whereas $VEGF_{120}$ and $VEGF_{188}$ had decreased to control levels (Figure 2).

DISCUSSION

Our data show that the VEGF isoforms are expressed to different degrees during normal retinovascular development and are differentially expressed after two different stresses: hypoxia, and repeated fluctuations between hyperoxia and hypoxia. We also show that PEDF is upregulated earlier (at P7), but was not significantly different to room air controls at any time point. We believe these data help in understanding the clinical findings observed after repeated fluctuations in oxygen in retinopathy of prematurity.

In normal development, $VEGF_{120}$ was expressed to a greater extent at early time points when the retinal capillary plexus had not completely covered the retinal surface and was expressed to a lesser degree at later time points. This was in accord with observations in mice that express only single VEGF isoforms as a result of targeted mutagenesis [7]. From histology of the retinas of these mice, $VEGF_{120}$ appeared important in initial retinal vessel growth, and not in later angiogenic processes of remodeling, maturation, and maintenance. These processes have been proposed to be facilitated by the cell-associated, heparin binding isoforms, $VEGF_{164}$ and $VEGF_{188}$ [7]. The expression of $VEGF_{164}$ appeared stable during the time points assayed in normal development and the expression of $VEGF_{188}$ was stable to modestly increased overtime. During development, $VEGF_{164}$ and $VEGF_{188}$ may be involved in further vascular development and in capillary remodeling, processes reported to occur from P6 to P12 [29].

$VEGF_{120}$ and $VEGF_{188}$ were upregulated by a single episode of hypoxia relative to their respective controls at the time points assayed. However, $VEGF_{164}$ was barely upregulated by a single episode of hypoxia. After repeated fluctuations in oxygen, $VEGF_{164}$ expression was substantially increased compared to RA control. VEGF protein (measuring $VEGF_{120}$ and $VEGF_{164}$) was also threefold greater than control after repeated oxygen fluctuations, and only 1.5-fold greater than control after exposure to a single cycle of hyperoxia and hypoxia. These data support the hypothesis that multiple fluctuations in oxygen and not just hypoxia alone cause increased expression of the pathologic isoform, $VEGF_{164}$. Other studies have shown that $VEGF_{164/165}$ was increased in pathologic intravitreous neovascularization [9,10,30] and our study supports those findings. In addition, individual episodes of hypoxia versus repeated fluctuations between hypoxia and hyperoxia may increase the expression of certain VEGF isoforms through different signaling pathways [31–34].

PEDF was upregulated with a 1.5-fold increase over control at P7 after 3.5 cycles of oxygen and remained comparable to controls values at P14 and P18. PEDF is a potent angiogenic inhibitor [35] and can inhibit VEGF-induced endothelial cell proliferation [36]. This early upregulation of PEDF, although not significant compared to control, may in part play a role in delayed retinal vascularization in rats and contribute to the persistent peripheral avascular retina associated with fluctuations in oxygen.

We found that VEGF isoforms were differentially expressed during normal retinovascular development in the rat. Repeated fluctuations between hyperoxia and hypoxia caused a greater fold increase in the expression of $VEGF_{164}$, than did a single episode of hypoxia. $VEGF_{164}$ was the only isoform that remained upregulated when intravitreous neovascularization was at its greatest extent. PEDF was upregulated early, although this was not significantly different from controls, and remained similar to control room air values through time points assayed in

the 50/10 OIR model. These experimental observations may have relevance to clinical findings that oxygen variability in premature infants is associated with the development of severe ROP [22,23].

Acknowledgements

We are grateful to Kathy Dorey, PhD, Department Biomedical Sciences, Florida Atlantic University, Boca Raton, FL for the use of her Oxycycler for these experiments. MEH is a member of the UNC Lineberger Comprehensive Cancer Center and the Carolina Cardiovascular Biology Center.

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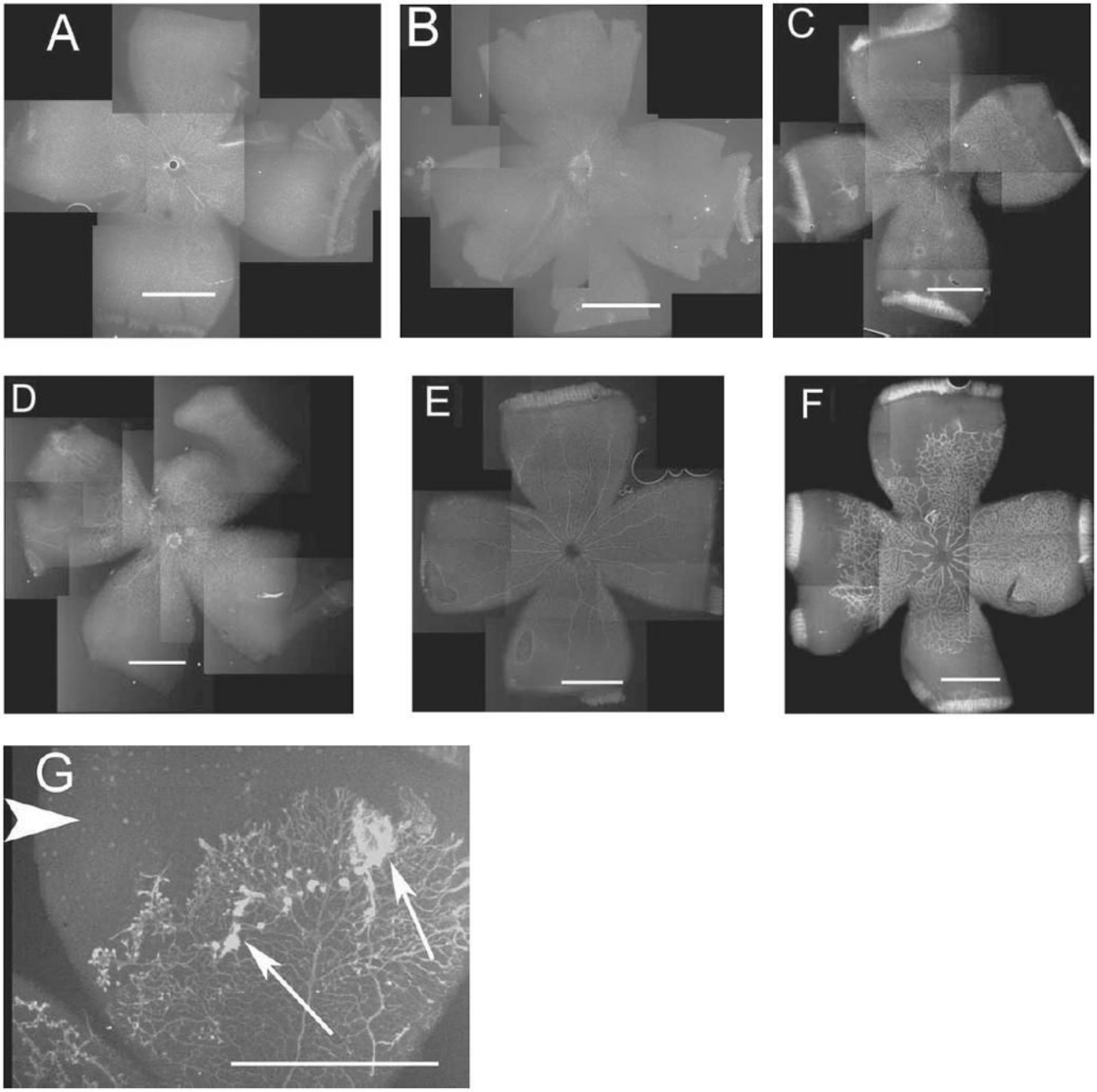

Figure 1.

Lectin stained retinas from the room air control and 50/10 OIR groups. Blood vessels are white. **A**: P2 room air (RA) controls. **B**: P2 50/10 OIR model, 24 h of 50% oxygen followed by 24 h of 10% oxygen. The retinal vasculature is not only retarded but is also visibly less complex than the control (**A**). **C**: P7 RA controls. **D**: P7 50/10 OIR model after 3.5 cycles of 24 h of 50% oxygen, 24 h of 10% oxygen. The retinal vasculature is still delayed compared to the RA control (**C**). **E**: P14 RA control. The retina is fully vascularized and this is similar to the appearance of the RA control retina at P18. **F**: P14 50/10 OIR after 7 cycles of 24 h of 50% oxygen, 24 h of 10% oxygen. The retinal vasculature has covered about the same area as the P7 RA control (**C**) but appears much less dense. **G**: P18 experimental after 7 cycles of 24 h of

50% oxygen, 24 h of 10% oxygen and then 4 days in room air. This image of one of the quadrants shows the persistent avascular area (arrowhead) and tufts of endothelial cells (arrows) from intravitreous neovascularization. Scale bars represent 1.5 mm. See Table 1 for a quantification of avascular areas.

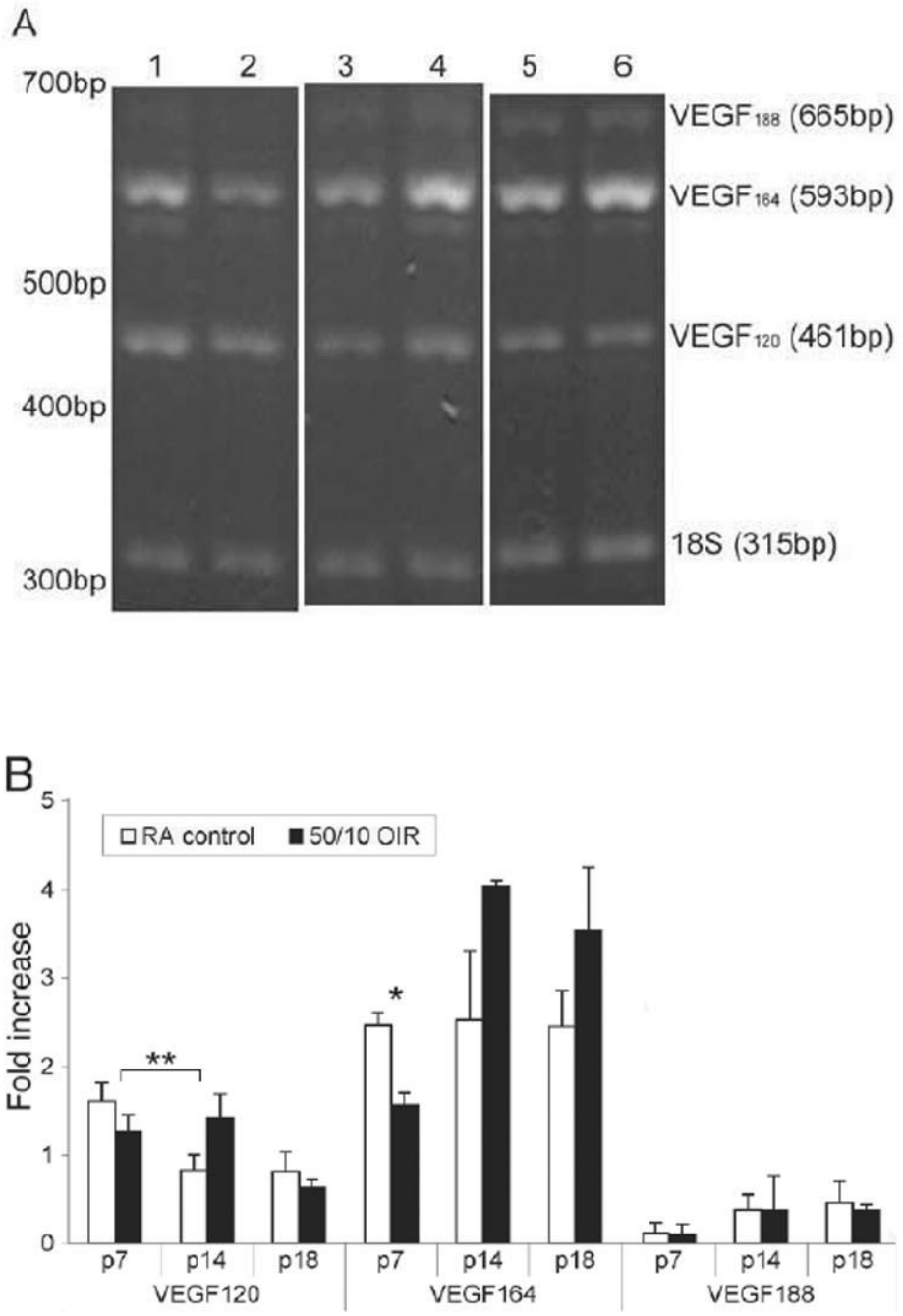

Figure 2.

Variable oxygen effect on VEGF isoform expression. **A**: RT-PCR analysis of VEGF mRNA from whole homogenized retina. P7 room air raised controls (lane 1); P7 50/10 OIR model that had 3.5 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (lane 2); P14 RA controls (lane 3); P14 50/10 OIR model that had 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (lane 4); P18 RA controls (lane 5); P18 50/10 OIR model that had 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen followed by 4 days in room air (lane 6). PCR was repeated three times on different experiments and this result is representative. **B**: Densitometry measurements using 18S RNA as the control gene. Samples were assayed in

triplicate and error bars are standard deviations. An asterisk (“*”) indicates $p<0.001$ compared to control, t test; double asterisks (“**”) indicate $p=0.008$, t test.

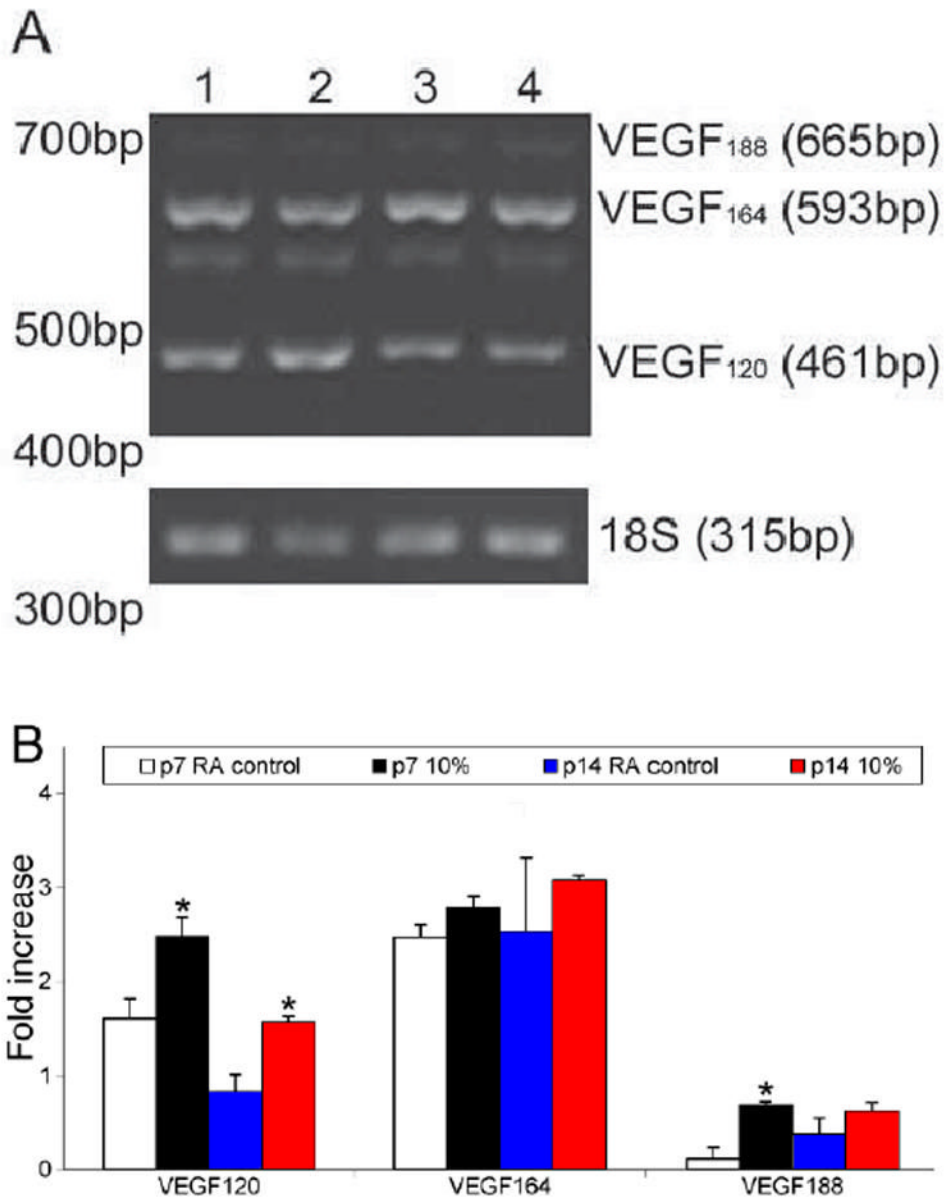

Figure 3.

Effect of a single episode of hypoxia on VEGF isoform expression. **A**: RT-PCR of VEGF isoform mRNAs in P7 (lane 1) and P14 (lane 3) control animals in room air (RA), and animals that had a single hypoxic episode of 24 h of 10% oxygen (10% O_2) at either P7 (lane 2) or P14 (lane 4). PCR was repeated three times on different experiments and this result is representative. **B**: Densitometry measurements using 18S RNA as the control gene. Samples were assayed in triplicate and error bars are standard deviations. An asterisk ("*") indicates $p<0.01$ compared to respective controls, t test.

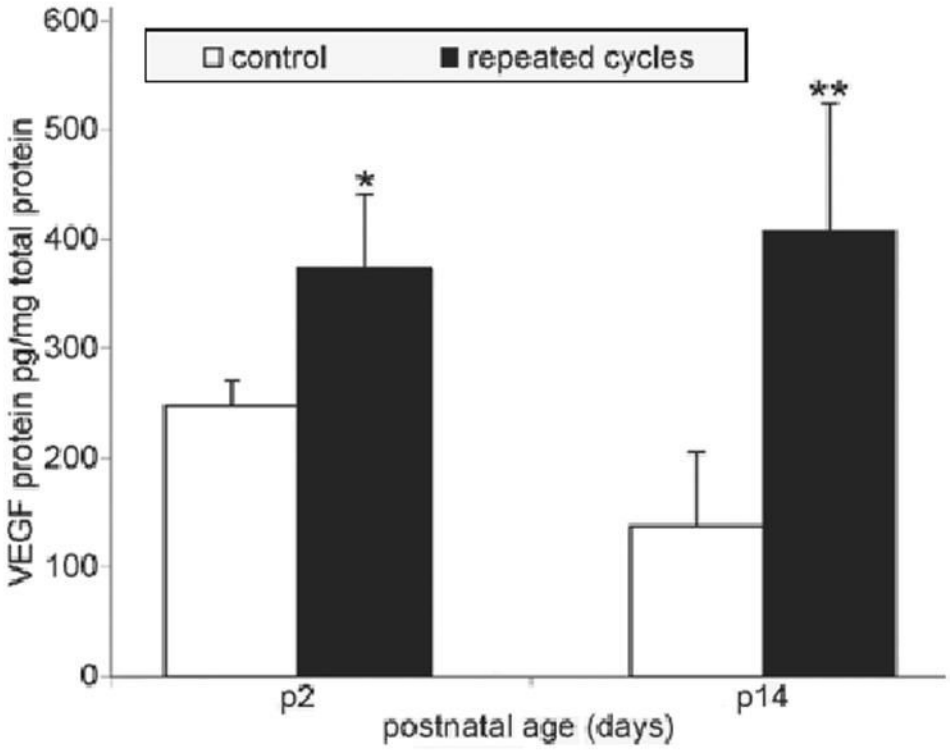

Figure 4.

VEGF protein in room air controls (RA) and in 50/10 OIR model. VEGF protein measured by ELISA in RA control animals and in 50/10 OIR model animals that had 1 cycle of 24 h of 50% oxygen followed by 24 h of 10% oxygen (P2) and 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (P14). Results are means of four samples; error bars are standard deviations. An asterisk ("*") indicates $p<0.03$ compared to control (t-test); double asterisks ("**") indicate $p<0.004$ compared to control (t-test).

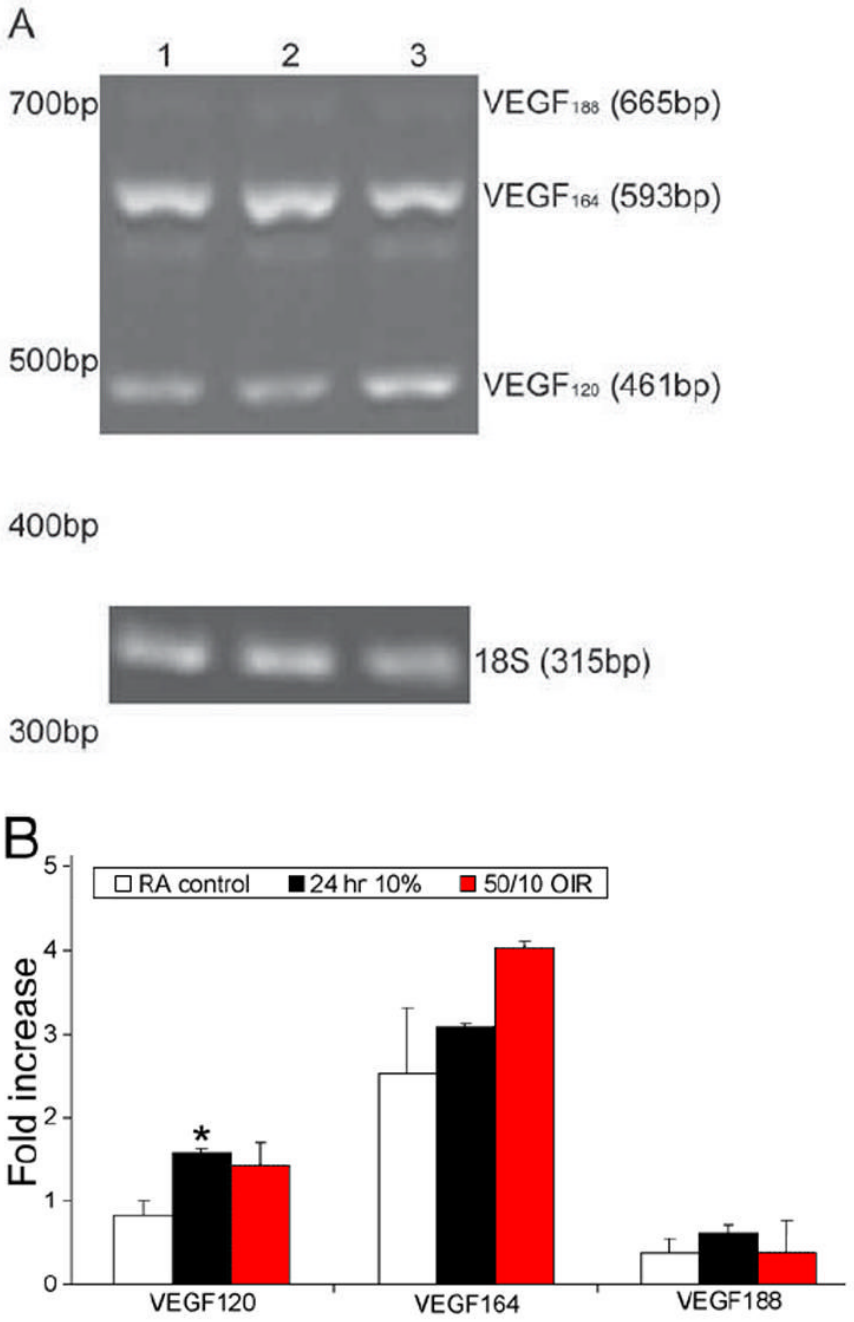

Figure 5.

Comparison of a single episode of hypoxia to continuous variable oxygen on the expression of VEGF isoforms. **A**: RT-PCR analysis of VEGF isoform mRNAs in P14 RA control (lane 1), P14 RA control after 24 h of 10% oxygen (lane 2), and P14 50/10 OIR model that had 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (lane 3). **B**: Densitometry measurements using 18S RNA as the control gene. PCR was repeated three times on different experiments and this result is representative. Samples were assayed in triplicate and error bars are standard deviations. An asterisk ("*") indicates $p<0.01$ compared to control (t test).

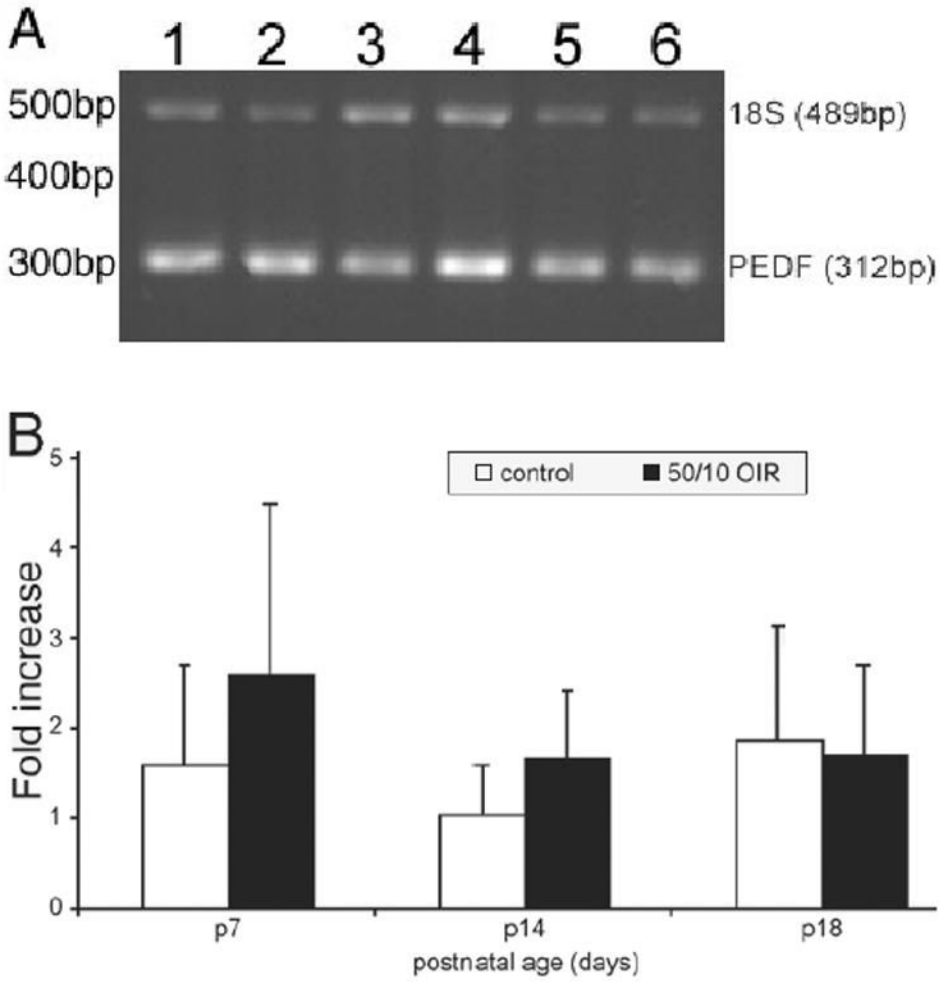

Figure 6.

Effect of variable oxygen on PEDF. **A**: RT-PCR analysis of PEDF mRNA from whole homogenized retina in RA control and in 50/10 OIR model. P7 RA control (lane 1); P7 50/10 OIR model that had 3.5 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (lane 2); P14 RA control (lane 3); P14 50/10 OIR model that had 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen (lane 4); P18 RA control (lane 5); P18 50/10 OIR model that had 7 cycles of 24 h of 50% oxygen followed by 24 h of 10% oxygen followed by 4 days in room air (lane 6). PCR was repeated four times on different experiments and this result is representative. **B**: Densitometry measurements using 18S RNA as the control gene. Samples were assayed in triplicate and error bars are standard deviations.

Table 1

The effect of oxygen fluctuations on retinal vascularization

	Total retinal area (sq. mm)		Avascular periphery (% of total retinal area)		Rat whole body weight (g)		Clock hours of neovascularization (median/ interquartile range)	
Postnatal age (days)	**RA**	**50/10**	**RA**	**50/10**	**RA**	**50/10**	**RA**	**50/10**
2	26.5 (1.0)	20.6 (1.9)	61.1 (5.0)	82.8 (2.9)	9.3 (0.2)	6.3 (0.4)	0	0
7	31.6 (1.1)	27.2 (3.0)	24.3 (3.8)	51.2 (5.0)	10.9 (0.5)	7.5 (0.3)	0	0
14	39.3 (0.4)	33.4 (3.4)	0	32.8 (10.6)	30.1 (2.9)	18.1 (1.2)	0	0
18	38.9 (1.5)	39.3 (1.8)	0	23.1 (6.2)	31.7 (1.4)	25.7 (1.7)	0	9.5 (9.5–10.3)

The table contains data from the retinal flatmount images on total retinal area, radial extent of vascularization, weights of the animals and the clock hours of pathological neovascularization for the different time points in controls and OIR animals. Values are means, and the standard deviation is given in parentheses below each mean, except where otherwise stated. Each time point of control or OIR animals is the combined data from 12 animals, 96 animals in total. Whole body weight refers to the weight of the animal at the end of the experiment.