Pentoxifylline Inhibition of Vasculogenesis in the Neonatal Rat Retina

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Purpose. The zeta isozyme of protein kinase C (PKC) is essential for activation of the transcription factor nuclear factor (NF)κB and transcription of vascular endothelial growth factor (VEGF). This study examined the antiangiogenic potential of an existing drug, pentoxifylline (PTX), which inhibits PKC-dependent activation of NFκB and is reported to prevent hypoxia-induced expression of VEGF.

Methods. Neovascularization was induced by maintaining neonatal rats for 10 full days in 80% oxygen, interrupted daily by 30 minutes in room air followed by a progressive return to 80% oxygen. On experimental day 11, they were placed in room air until they were killed on day 17. Daily intraperitoneal injections of PTX in saline (25 or 75 mg/kg per day), or saline alone, were administered from day 6 through day 16. Retinal neovascularization was scored, and avascular areas (AVAs) were measured in ADPase stained retinas.

Results. PTX inhibited radial extension of retinal vessels, causing increases in AVA of 65% ($P < 0.01$) and 33% ($P < 0.15$) at the lower and upper doses, respectively. A significant increase in mean neovascular score was seen at the lower dose ($P < 0.0001$), but analysis of variance indicated that neovascularization was strongly and positively influenced by the AVA ($P < 0.0001$) and only weakly stimulated by PTX ($P < 0.05$).

Conclusions. Systemic PTX significantly inhibited VEGF-mediated retinal vasculogenesis, but was not effective in reducing neovascularization in the oxygen-exposed neonatal rat. (*Invest Ophthalmol Vis Sci.* 2000;41:2774–2778)

In the developing rat retina, hypoxia-induced expression of vascular endothelial growth factor–vascular permeability factor (VEGF) in the avascular peripheral retina[1] drives vasculogenesis (de novo formation of new vessels from angioblasts,[2] which may proliferate before their assembly into tubes[3]). VEGF also induces neovascularization (growth of new vessels in aberrant patterns) through angiogenesis (growth from existing vessels),[4] and incorporation of bone marrow–derived endothelial cell progenitors (vasculogenesis).[5] Experimentally induced neovascularization has been spatially and temporally correlated with elevated expression of VEGF in the retina and/or vitreous.[6–8]

Hypoxia-induced transcription of VEGF is mimicked by exposure to cobalt, which induces formation of reactive oxygen species and activation of nuclear factor (NF)κB.[9] Activation of NFκB is critically dependent on the atypical zeta isozyme of protein kinase C (PKC_ζ) in fibroblasts[10] and endothelial cells.[11] Recent evidence indicates that PKC_ζ plays a decisive role in the transcription of VEGF,[12] and VEGF-stimulated endothelial cell proliferation.[13] Pentoxifylline (PTX), a methylxanthine derivative that inhibits the PKC-dependent activation of NFκB,[14,15] also suppresses hypoxia-induced expression of VEGF.[16] Nonspecific PKC inhibitors and inhibition of NFκB suppress neovascularization.[17,18] The purpose of these experiments was to determine whether systemic PTX inhibits neovascularization in a rat model of retinopathy of prematurity. PTX was of particular interest because, it was reported to increase retinal and choroidal blood flow in patients with diabetes and age-related macular degeneration.[19–21] Moreover, PTX had suppressed neovascularization in rabbit corneas injected with oxidized lipids[22] and was reported to reduce neovascularization in 27 patients with retinal hemorrhages.[23]

Data from the current study demonstrate that PTX effectively inhibited vasculogenesis in the oxygen-exposed neonatal rat retina, causing significantly increased avascular areas (AVAs). Neovascular responses to the enlarged AVAs were mildly stimulated by PTX.

Materials and Methods

Rat Model of Ischemic Retinopathy

Neovascularization is reproducibly observed in neonatal rats placed for 10 full days in elevated oxygen interrupted daily by an episode of relative hypoxia in room air and then transferred on day 11 to room air for 7 additional days.[24] Oxygen exposure reduces VEGF production[25] and impairs development of reti-

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TABLE 1. Criteria for Scoring Neovascular Intensity

Quadrant Score	Characteristics of Neovascularization
0	None observed
1	Less than five glomerular buds
2	Five or more buds or a frond
3	Short ridge*
4	Long ridge*

* Short and long ridges were defined as those that extended less than, or at least halfway, across the ridge.

nal vessels.[24] When the 11-day-old rats are placed in a room air environment, the hypoxic consequences of the enlarged peripheral AVA stimulate expression of VEGF.[7] Both the incidence and the intensity of the neovascular response are highly correlated with the area of peripheral avascular retina[7,24] and with the duration of the hypoxic episodes.[24] Almost 90% of the neovascularization was observed in the inferior quadrant, which has the largest AVA. The superior quadrant, with little AVA, has negligible neovascularization.[24]

Experimental Protocol

Animals were treated in accordance with a protocol approved by the institutional Animal Care and Use Committee and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For each experiment, neonates from six albino Sprague–Dawley rats with timed pregnancy (Charles River, Kingston, NY) were divided into three equal litters in oxygen (10–15 pups, depending on the number of pups born) and three smaller litters in room air. Mothers were rotated between room air and oxygen every 2 days. From days 6 through 16, room air– and oxygen-exposed animals were given intraperitoneal injections of either PTX (25 or 75 mg/kg; prepared fresh daily) or phosphate-buffered saline vehicle (0.137 M, pH 7.4; Boehringer–Mannheim, Indianapolis, IN). The oxygen-exposed animals received five treatments in oxygen (days 6 through 10) and six in room air (days 11 through 16) before they were killed on day 17. The doses of PTX used in these experiments were within the range for rats in the literature and varied by a factor of 3. The results of three independent experiments are presented. Eighty-one oxygen-exposed animals were treated with 25 mg PTX/kg ($n = 19$), 75 mg PTX/kg ($n = 29$), or saline ($n = 33$). All animals were treated, killed, processed, and analyzed in parallel. One retina from every animal was processed, and every retina was included in the analysis. Unless otherwise indicated, all chemicals were from Sigma (St. Louis, MO).

Retina Processing

Enucleated eyes were fixed 24 to 48 hours in 4% paraformaldehyde (Polysciences, Warrington, PA) in cacodylate buffer (0.1 M, pH7.2). After the retina was freed from vitreous and the eyecup, the retinal vessels were revealed by adenosine diphosphatase (ADPase) histochemistry, scored, and flatmounted as previously described.[24]

Neovascular Scores and Image Analysis

Neovascularization, recognized as structures that are abnormal in architecture and intensely stained with ADPase, included intravitreal knots of capillaries (glomerular buds), fan-shaped vascular fronds, and thickened vascular ridges that elevated the retinal surface and ran parallel to the ciliary body (perpendicular to the radial arteries and veins). The intensity of neovascularization in each retina was determined as the total of individual quadrant scores, using a minor modification of previously published criteria (Table 1).[7]

Avascular Area

Digital images of the mounted ADPase-stained retinas were acquired using a CCD video camera (Hamamatsu CCD; Dage–MTI, Michigan City, IN). The peripheral avascular retina of every quadrant in one eye from each animal was traced (Fig. 1) and measured in square millimeters by computer (NIH Image software; National Institutes of Health, Bethesda, MD; installed on a Power Tower 250; Power Computing, Round Rock, TX). Significant interexperimental variation ($P < 0.0001$; analysis of variance [ANOVA]) made it necessary to compare areas of treated animals with controls from the same experiment. To determine the effect of treatment on AVA (and for this analysis only) each measurement of area was expressed as a percentage of the mean area in the vehicle-injected, oxygen-exposed control samples from the same experiment. This corrected measurement of area was designated as AVA (%) or $AVA_{\%}$.

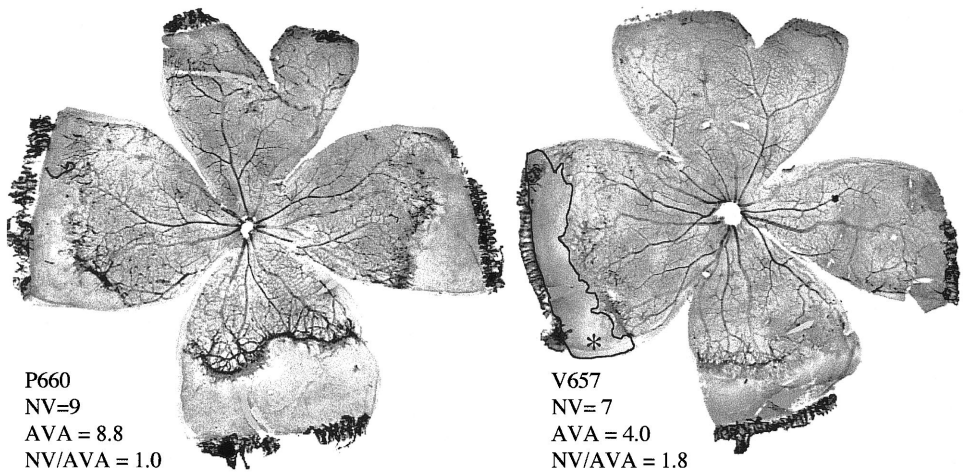

FIGURE 1. Digital images of retinas from animals given daily intraperitoneal injections of PTX (*left*) or vehicle (*right*) from experimental days 6 through 16. There were no PTX-induced abnormalities in vessel architecture. The AVA in one quadrant of the vehicle retina is outlined; the inner retina is rolled back at *. Neovascular score (NV) and AVA in square millimeters are given for each retina. Magnification, ×9.5.

Statistical Analysis

ANOVA and multiple regression routines in commercial software (Statview ver. 4.5; Abacus Concepts, Berkeley, CA) were used to assess the influence of PTX on AVA and on neovascularization. Comparisons between groups were performed with Fishers' protected least-significant difference procedure for multiple comparisons. The relative contribution of AVA and PTX dose to the observed neovascular response was analyzed by multiple regression.

Results

Treatment with PTX had no effect on vascular pattern, but caused striking increases in the area of avascular retina (Fig. 1). The mean $AVA_{\%}$ increased 65% in animals given 25 mg PTX/kg ($P < 0.01$; ANOVA) and 33% in those treated with 75 mg PTX/kg ($P < 0.15$). There was no significant difference between the two treatments ($P = 0.2$; Fig. 2). When all PTX-treated animals (i.e., regardless of dose) were compared with all controls, there was still a significant PTX-induced increase in $AVA_{\%}$ ($P < 0.03$; ANOVA).

Neovascular scores were significantly increased in animals treated with 25 mg PTX/kg, the group that also had the largest AVAs (Fig. 2B). As seen in Figure 3, neovascular scores increased with increase in AVA in all treatment groups, and the regression lines overlapped. Although $r^2 > 0.5$, and $P < 0.0001$ (Table 2) indicated that each of the regression lines in Figure 3 were reasonable estimates of the data, overlaps in the 95% confidence intervals for the slopes and intercepts indicated they were not significantly different from each other. Therefore, multiple regression analysis (Table 3) was used to assess the relationship between neovascular scores and AVA, drug dose, and/or the interaction of the two. The resultant R^2 indicated that 65% of the observed variation in neovascular scores could be attributed to the combined positive influences of AVA ($P < 0.0001$) and drug dose ($P < 0.05$). Moreover, the influence of AVA was almost six times greater than the effect of PTX (standard coefficients 0.81 and 0.14, respectively). The interaction term was not significant ($P > 0.6$) and was omitted from the model.

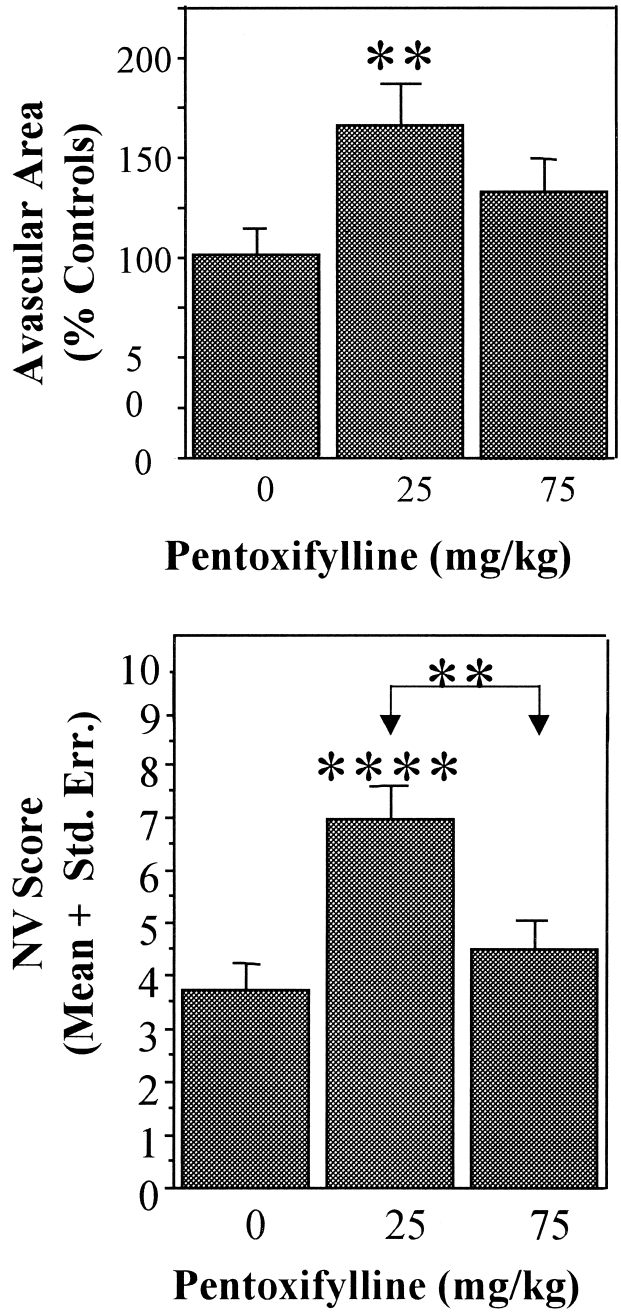

Figure 2. **(A)** Area (in square millimeters) of avascular retina in animals given intraperitoneal injections of saline containing 0, 25, or 75 mg/kg PTX. Animals given 25 mg/kg had significantly larger AVAs than animals injected with saline. Those given the higher dose tended to have larger AVAs than the control animals but were not significantly different from those given 25 mg/kg ($P = 0.2$). Data are means ± SE, expressed as a percentage of the vehicle controls in each experiment (**$P < 0.01$; ANOVA). **(B)** Neovascular scores in animals given intraperitoneal injections of saline containing 0, 25, or 75 mg/kg PTX. Animals treated with 25 mg/kg had higher neovascular scores than controls or those given the higher dose. Data are means ± SE (**$P < 0.01$; ****$P < 0.0001$; ANOVA).

Discussion

The strong inhibition of vasculogenesis in the oxygen-exposed rat retina is consistent with reports that PTX inhibits hypoxia-induced expression of VEGF,[16] and evidence that vasculogenesis fails if VEGF signaling is disrupted.[26–28] Recent literature suggests that PTX inhibits hypoxia-induced transcription and production of VEGF[12,16] and VEGF-induced proliferation of endothelial cells[13] or angioblasts. Both activities would slow vasculogenesis and reduce survival of newly formed vessels.[25] However, because VEGF was not measured in these retinas, the possibility that PTX acted through a different mechanism cannot be excluded.

Failure to Inhibit Neovascularization

The observed stimulation of neovascularization is inconsistent with previous reports that PTX inhibits neovascularization in a rabbit corneal model[22] and in a small number of patients with vitreous hemorrhage,[23] and with a recent report that pyrrolidine dithiocarbamate, another inhibitor of NFκB activation, suppresses retinal neovascularization in mice.[29] Multivariate analysis indicated that the increases in neovascularization were primarily due to the hypoxic consequences of PTX inhibition

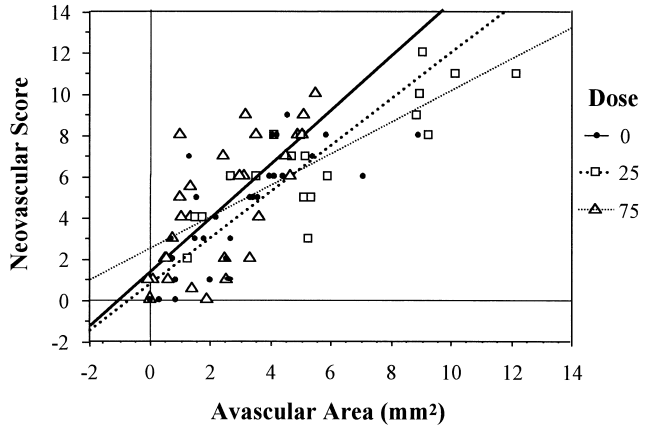

FIGURE 3. Relationship between the AVA and neovascular score in 81 animals treated with 25 or 75 mg/kg PTX or saline. The three regression lines are not significantly different, in that they have overlapping coefficients for slope and intercept (Table 2). Multiple regression analysis resulted in the model in Table 3.

TABLE 3. Multiple Regression Model

	Coefficient	SE	Standard Coefficient	*P*
AVA	0.97	0.08	0.81	<0.0001
Dose	0.01	0.01	0.14	0.05
Intercept	1.15	0.42	1.15	0.007

$NV = 1.15 + 0.97$ (AVA) $+ 0.14$ (Dose); overall $R^2 = 0.65$; $P < 0.0001$. Results from multiple regression analysis of variables possibly influencing neovascularization indicate that 65% of the variability in neovascular score could be attributed to the avascular area and the dose of PTX given. The standard coefficients (the relative contribution of each variable) indicate that AVA was approximately five times more important than dose in predicting the neovascular score. Dose: 0, 25, or 75 mg/kg PTX. NV, neovascular score.

of vasculogenesis (i.e., the PTX-induced increase in AVA). The PTX-induced increase was small and barely significant. Possible explanations for this observation include (among others) participation of angiogenic factors not sensitive to PTX, stimulation of neovascularization through inhibition of phosphodiesterase by PTX, and/or pharmacokinetic considerations.

Because VEGF expression is upregulated in the AVAs, and because PTX resulted in even larger AVAs, it is clear that the proangiogenic stimulus would be greater in the PTX-treated animals. The most parsimonious explanation for the observed absence of effect on neovascularization is that the PTX was sufficient to tip the balance of pro- and antiangiogenic factors regulating intraretinal vessel growth during periods of hyperoxia, but was not sufficiently antiangiogenic to suppress neovascular responses to higher levels of VEGF[7,8] and/or other angiogenic factors produced by the enlarged AVAs in the PTX-treated animals. The ability of PTX to have a deciding influence on the balance of pro- and antiangiogenic factors may be exacerbated by reduced clearance of PTX during periods of hyperoxia. Blood flow in the retina and/or optic nerve head was reduced 25% to 30% in human subjects breathing 100% oxygen.[30,31] If blood flow in neonatal mice were similarly reduced during periods of hyperoxia, it is feasible that intraretinal vessel growth would be exposed to higher average levels of PTX than those present during the final days in room air when the neovascular growth was most active.

The differential effects observed in this model may also derive from the opposing effects of PTX on two pathways stimulating VEGF expression in hypoxic tissues. Vasculogenesis is primarily dependent on the hypoxia-induced expression of VEGF.[1,25–28,32] Recent evidence indicates that this pathway is PKC_ζ-dependent[12,13] and inhibited by PTX.[15,16] Another pathway may also be important in neovascularization. Adenosine accumulating in hypoxic retina[33] can activate A-2 receptors on endothelial cells, stimulating a rise in cyclic adenosine monophosphate (cAMP) and increased transcription of VEGF.[34,35] Although PTX is an inefficient phosphodiesterase inhibitor, the doses used in these experiments may have achieved concentrations sufficient ($\sim 10^{-4}$ M)[36] to elevate cAMP and thus increase VEGF in hypoxic endothelial cells. This explanation would be consistent with the stronger inhibition of vasculogenesis at the lower dose of PTX, and further suggests that adenosine may play a significant role in the neovascular response to large areas of ischemic retina.

Clinical Implications

Small clinical studies have suggested beneficial effects of PTX on retinal blood flow and appearance of microvascular abnormalities.[19–21,37] The present data and prior evidence that PTX inhibited corneal neovascularization in rabbits[22] and extraretinal neovascularization in patients[23] suggest that PTX at appropriate doses may inhibit vasculogenesis and/or neovascularization. Both retinal vasculogenesis (data shown here) and corneal neovascularization were sensitive to PTX, and both involve the recruitment of endothelial precursors.[1,5] Although far from perfect, PTX may have some application for neovascularization associated with inflammation, until newer more effective drugs are available. However, the use of PTX in proliferative retinopathies is limited by the possibility of exacerbating neovascular responses to retinal ischemia. In conclusion, systemic treatment with PTX inhibited vasculogenesis, but not neovascularization, in the rat model of retinopathy of prematurity.

TABLE 2. Regression Analysis of the Relation between Neovascular Score and AVA in Animals Given Saline or PTX

Dose (mg/kg · day)	*n*	Intercept (a)	95% Confidence Limits	Slope (b)	95% Confidence Limits	r^2	*P*
0	33	0.86	−0.10 to 1.81	1.1	0.83 to 1.39	0.68	<0.0001
25	25	2.6	0.96 to 4.26	0.8	0.50 to 1.00	0.70	<0.0001
75	75	1.4	−0.03 to 2.89	1.3	0.79 to 1.79	0.51	<0.0001

Regression coefficients for the model: $NV = a + b\,(AVA)$. Regression analysis for lines in Figure 3 confirms that neovascular scores were strongly related to the AVA. However, overlap in the 95% confidence limits indicates that neovascular responses in PTX-treated animals were not significantly different from the vehicle controls. *n* = Number of animals/retinas in group.

References

1. Stone J, Itin A, Alon T, et al. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosci.* 1995;15:4738–4747.
2. McLeod DS, Lutty GA, Wajer SD, Flower RW. Visualization of a developing vasculature. *Microvasc Res.* 1987;33:257–269.
3. Chan-Ling T, Gock B, Stone J. The effect of oxygen on vasoformative cell division: evidence that "physiological hypoxia" is the stimulus for normal retinal vasculogenesis. *Invest Ophthalmol Vis Sci.* 1995;36:1201–1214.
4. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci.* 1996;37:1625–1632.
5. Asahara T, Takahasi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* 1999;18:3964–3972.
6. Miller JW, Adamis AP, Shima DT, et al. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. *Am J Pathol.* 1994;145:574–584.
7. Dorey CK, Aouididi S, Reynaud X, Dvorak HF, Brown LF. Correlation of vascular permeability factor/vascular endothelial growth factor with extraretinal neovascularization in the rat. *Arch Ophthalmol.* 1996;114:1210–1217.
8. Donahue ML, Phelps DL, Watkins RH, LoMonaco MB, Horowitz S. Retinal vascular endothelial growth factor (VEGF) mRNA expression is altered in relation to neovascularization in oxygen induced retinopathy. *Curr Eye Res.* 1996;15:175–184.
9. Goebeler M, Roth J, Brocker EB, Sorg C, Schulze–Osthoff K. Activation of nuclear factor-kappa B and gene expression in human endothelial cells by the common haptens nickel and cobalt. *J Immunol.* 1995;155:2459–2467.
10. Diaz–Meco MT, Berra E, Municio MM, et al. A dominant negative protein kinase C zeta subspecies blocks NF-kappa B activation. *Mol Cell Biol.* 1993;13:4770–4775.
11. Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. *J Biol Chem.* 1999;274:13594–13603.
12. Pal S, Claffey K, Cohen H, Mukhopadhyay D. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. *J Biol Chem.* 1998;273:26277–26280.
13. Wellner M, Maasch C, Kupprion C, Lindschau C, Luft FC, Haller H. The proliferative effect of vascular endothelial growth factor requires protein kinase C-alpha and protein kinase C-zeta. *Arterioscler Thromb Vasc Biol.* 1999;19:178–185.
14. Mhashilkar AM, Biswas DK, LaVecchio J, Pardee AB, Marasco WA. Inhibition of human immunodeficiency virus type 1 replication in vitro by a novel combination of anti-Tat single-chain antibodies and NF-kappa B antagonists. *J Virol.* 1997;71:6486–6494.
15. Biswas DK, Ahlers CM, Dezube BJ, Pardee AB. Pentoxifylline and other protein kinase C inhibitors down-regulate HIV-LTR NF-kappa B induced gene expression. *Mol Med.* 1994;1:31–43.
16. Amirkhosravi A, Meyer T, Warnes G, et al. Pentoxifylline inhibits hypoxia-induced upregulation of tumor cell tissue factor and vascular endothelial growth factor. *Thromb Haemost.* 1998;80:598–602.
17. Hu DE, Fan TP. Protein kinase C inhibitor calphostin C prevents cytokine-induced angiogenesis in the rat. *Inflammation.* 1995;19:39–54.
18. Stoltz RA, Abraham NG, Laniado–Schwartzman M. The role of NF-kappaB in the angiogenic response of coronary microvessel endothelial cells. *Proc Natl Acad Sci USA.* 1996;93:2832–2837.
19. Sonkin PL, Kelly LW, Sinclair SH, Hatchell DL. Pentoxifylline increases retinal capillary blood flow velocity in patients with diabetes. *Arch Ophthalmol.* 1993;111:1647–1652.
20. Sebag J, Tang M, Brown S, Sadun AA, Charles MA. Effects of pentoxifylline on choroidal blood flow in nonproliferative diabetic retinopathy. *Angiology.* 1994;45:429–433.
21. Kruger A, Martulla B, Wolzt M, et al. Short-term oral pentoxifylline use increases choroidal blood flow in patients with age related macular degeneration. *Arch Ophthalmol.* 1998;116:27–30.
22. Ueda T, Ueda T, Fukuda S, et al. Lipid hydroperoxide induced TNFα, VEGF, and neovascularization in the rabbit cornea: effect of TNFα inhibition. *Angiogenesis.* 1997;1:174–184.
23. Iwafune Y, Yoshimoto H. Clinical use of pentoxifylline in haemorrhagic disorders of the retina. *Pharmatherapeutica.* 1980;2:429–438.
24. Reynaud X, Dorey CK. Extraretinal neovascularization induced by hypoxic episodes in the neonatal rat. *Invest Ophthalmol Vis Sci.* 1994;35:3169–3177.
25. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med.* 1995;1:1024–1028.
26. Shalaby F, Rossant J, Yamaguchi T, et al. Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature.* 1995;376:62–66.
27. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996;380:435–439.
28. Ferrara N, Carver–Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* 1996;380:439–442.
29. Yoshida A, Yoshida S, Ishibashi T, Kuwano M, Inomata H. Suppression of retinal neovascularization by the NF-kappaB inhibitor pyrrolidine dithiocarbamate in mice. *Invest Ophthalmol Vis Sci.* 1999;40:1624–1629.
30. Langhans M, Michelson G, Groh MJ. Effect of breathing 100% oxygen on retinal and optic nerve head capillary blood flow in smokers and non-smokers. *Br J Ophthalmol.* 1997;81:365–369.
31. Harris A, Anderson DR, Pillunat L, et al. Laser Doppler flowmetry measurement of changes in human optic nerve head blood flow in response to blood gas perturbations. *J Glaucoma.* 1996 5:258–265.
32. Aiello LP, Northrup JM, Keyt BA, Takagi H, Iwamoto MA. Hypoxic regulation of vascular endothelial growth factor in retinal cells. *Arch Ophthalmol.* 1995;113:1538–1544.
33. Ghiardi GJ, Gidday JM, Roth S. The purine nucleoside adenosine in retinal ischemia-reperfusion injury. *Vision Res.* 1999;39:2519–2535.
34. Takagi H, King GL, Robinson GS, Ferrara N, Aiello LP. Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. *Invest Ophthalmol Vis Sci.* 1996;37:2165–2176.
35. Cheng T, Cao W, Wen R, Steinberg RH, LaVail MM. Prostaglandin E_2 induces vascular endothelial growth factor and basic fibroblast growth factor mRNA expression in cultured rat Muller cells. *Invest Ophthalmol Vis Sci.* 1998;39:581–591.
36. Meskini N, Nemoz G, Okyayuz–Baklouti I, Lagarde M, Prigent AF. Phosphodiesterase inhibitory profile of some related xanthine derivatives pharmacologically active on the peripheral microcirculation. *Biochem. Pharmacol.* 1994;47:781–788.
37. Ferrari E, Fioravanti M, Patti AL, Viola C, Solerte SB. Effects of long-term treatment (4 years) with pentoxifylline on haemorheological changes and vascular complications in diabetic patients. *Pharmatherapeutica.* 1987;5:26–39.