·Basic Research·

Antioxidant effect of hydralazine on retinal pigment epithelial cells and its potential use in the therapy of age-related macular degeneration

Yu-Wen Cheng, George C Y Chiou

Institute of Ocular Pharmacology, College of Medicine, Texas A&M System Health Science Center College Station, TX 77843, USA

Correspondence to: George C Y Chiou. Institute of Ocular Pharmacology, College of Medicine, Texas A&M System Health Science Center College Station, TX 77843, USA. Chiou@medicine.tamhsc.edu

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Abstract

• AIM: To investigate the antioxidant effect of hydralazine under hypoxia-induced damage on retinal pigment epithelial (ARPE-19) cells and the role of reactive oxygen species (ROS) in this effect.

• METHODS: Human retinal pigment epithelial (hRPE) cells were used to investigate the effect of hydralazine on oxidative stress, including tert-butyl hydroxyperoxide (t-BHP), H_2O_2, sodium azide (NaN_3), and hypoxia induced cell damage. Cell viability was determined by MTT assay.

• RESULTS: When ARPE-19 cells were treated with oxidative stress induced by ROS, hydralazine showed concentration-dependent protection against t-BHP, H_2O_2 and hypoxia induced cell damage but not NaN_3. Nitric oxide (NO) was not involved in this effect.

• CONCLUSION: Hydralazine showed antioxidant potential against oxidative stress induced damage in ARPE-19 cells. These effects might be caused through scavenger of ROS. Thus, hydralazine could be used for the treatment of age-related macular degeneration (AMD).

• KEYWORDS: age-related macular degeneration (AMD); reactive oxygen species (ROS); tert-butyl hydroxyperoxide (t-BHP)

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INTRODUCTION

Hydralazine, a potent arterial vasodilator that reduces peripheral resistance directly by relaxing the smooth muscle in arterial vessels [1], is one of the first orally active antihypertension drugs developed. It has long been used for the management of hypertensive disorders and heart failure [2,3]. Despite numerous studies with this drug, the mechanism of action of the vasodilation induced by hydralazine is not yet well understood. Recent observations suggest that it inhibits calcium release of the vascular smooth muscle sarcoplasmic reticulum by blocking the inositol triphosphate (IP3) induced calcuim release, therefore reducing calcium turnover inside the cell [4]. The resultant vasodilation reduces cardiac after load, increasing cardiac function in patients with heart failure. However, some evidence exists concerning a direct action in the myocardium by an increase in calcium influx through the sarcolemma. This may be partially due to the stimulation of the beta-adrenoreceptors [5]. In addition, hydralazine exerts the antioxidation which was reported to be via scavenging reactive oxygen species (ROS) and peroxynitrite[6,7].

Age-related macular degeneration (AMD) is a progressive neurodegenerative disease of central retinal area, and is the leading cause of blindness in people over the age of 65 in the United States and Western Europe [8,9]. Risk factors common to both continents include increasing age, cigarette smoking, hypertension, angina and a positive family history [10]. AMD exists in both non-exudative and exudative forms. The non-exudative form involves atrophy of the central retina with a slow and progressive loss of central vision. The exudative form is characterized by the growth of new blood vessels through Bruch's membrane into the subretinal space, and the development of choroidal neovascularization (CNV) through an angiogenic process. Angiogenesis is often triggered by ischemia and hypoxia [11]. As the macula has only a single blood supply, and the retina has the highest uptake of oxygen in the body, it is not surprising to observe that ischemia is strongly associated with AMD development [12].

The retinal pigment epithelium (RPE) is a monolayer cell located between the retinal photoreceptors and the choroidal blood vessels [13,14] which plays a key role in the mechanical and metabolic support of the photoreceptors [15]. In addition, RPE cell[16] is the main element of some ocular diseases, such as proliferative vitreoretinopathy (PVR), uvetitis and AMD [17,18]. AMD and other diseases, such as diabetic retinopathy (DR),

are probably linked to the effects of oxygen radicals derived from light or metabolic reactions. Since the epithelium is very vulnerable to changes in oxygen tensions and oxygen radical-linked stress, ROS produced in the RPE during ischemia-linked diseases may be injurious to RPE cells[15,19]. An important "early" event of AMD is the loss of RPE cells due to oxidative damage[20,21].

Oxidative stress has been recognized to be involved in the etiology of several age-related chronic diseases, such as cancer, diabetes, neurodegenerative and cardiovascular diseases[22,23]. In this study, we plan to investigate the effect of hydralazine on oxidative stress and hypoxia-induced damage on retinal pigment epithelial (ARPE-19) cells and the involvement of ROS in this effect.

MATERIALS AND METHODS

Materials Hydralazine, H_2O_2, including tert-butyl hydroxyperoxide (t-BHP), and NaN_3 (sodium azide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ProOx hypoxia system (Proox Model 110 and $ProCO_2$ Model 120) was purchased from BioSpherix Ltd. (Redfield,NY,USA).The chemical structure of hydralazine is presented in Figure 1.

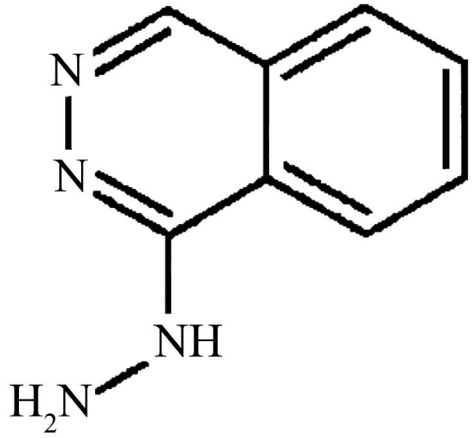

Figure 1 Chemical structure of hydralazine

Methods

Culture of human retinal pigment epithelial cells The human REP cell line ARPE-19 obtained from American Type Culture Collections (ATCC; Manassas, VA, USA) was cultured in Dulbecco's modified essential medium (DMEM) supplement with 100mL/L fetal bovine serum (FBS), 50units/mL penicillin/streptomycin and 2.5mmol/L glutamine at 37℃ incubator with 50mL/L CO_2. The cell line is not transformed and has structure and function properties characteristic of RPE *in vivo*. The cells were seeded into 96-well plates, and subconfluent cell monolayers were studied within three to ten passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose DMEM supplemented with 10mL/L calf serum, 0.6g/L glutamine and 10% penicillin-streptomycin.

Quantitative determination of cell damage induced by oxidative stress with MTT assay Cultured RPE cells were seeded onto 96-well plates with a cell concentration of 1×10^5 cells/mL, and were grown to 80% confluence before treatment to prevent contact inhibition. The cells were exposed to the control or various concentrations of hydralazine (1, 3, 10, 30, 100mg/L) in the presence of oxidative stress inducer, H_2O_2 (0.01-10mmol/L),t-BHP (0.01-10mmol/L) and chemical ischemia, NaN_3 (0.1-100mmol/L). The cultures were then incubated at 37℃ for 24 hours. Cell viability was assessed by using a 3- (4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay to determine the proportion of living cells in each culture (living cells are those with mitochondrial function of dehydrogenase)[24]. The 96- well cultures, after exposure to the control medium or the test compounds for 24 hours, were incubated with 5mg/mL MTT at a dilution of 1∶10 base on the volume of culture medium for 3 hours at 37℃. At the end of incubation, the MTT solution was removed, and the cells were dissolved in 0.1mL/well DMSO. The proportion of viable cells (those with mitochondria capable of cleaving the MTT molecule to produce the dark purple substance, formazan) was determined by measuring the optical density (OD) of each sample at 570nm using a SpectraCount plate reader (Packard BioScience, Meridan, CT, USA). Exact cell number was determined by using Trypan blue exclusion method with a hemacytometer, counting cells, and simul-taneously performing the MTT assay on cells seeded at identical densities to establish a standard curve of A570 *vs* cell number.

Effect of hydralazine on chemical induced hypoxia in ARPE −19 cells Chemical hypoxia was induced using amodified of method described by Swanson and Varming *et al*[25,26]. RPE cells were washed and replaced with fresh DMEM-F12 medium containing various concentrations of NaN_3 (0.1-100mmol/L). Hydralazine was then added for 24 hours incubation at 37℃ in the incubator. Reactions were stopped by washing out the medium and 5g/L MTT at a dilution of 1∶10 base on the volume of culture medium was added for 3 hours at 37℃. At the end of incubation, the MTT solution was removed, and the cells were dissolved in 0.1mL/well DMSO. The proportion of viable cells was determined by measuring the OD of each sample at 570nm using a Spectra- Count plate reader (Packard BioScience,Meridan, CT,USA).

Hypoxia treatment *In vitro* hypoxia treatment was carried out by using a ProOx hypoxia system. ARPE-19 cells were allowed to attach overnight, and then exposed to hydralazine (0.1-100mg/L) under normoxic or hypoxic condition for 24, 48 and 72 hours. In hypoxia, oxygen concentrations of 10mL/L O_2 and 50mL/L CO_2 were maintained using a temperature- and humidity-controlled environmental C-chamber by O_2 and CO_2 controllers (Proox Model 110 and $ProCO_2$

Model 120, BioSpherix Ltd., Redfield, NY, USA) with N_2 and CO_2 gas sources. Reactions were stopped by washing out the medium and 5g/L MTT at a dilution of 1:10 base on the volume of culture medium was added for 3 hours at 37℃. At the end of incubation, the MTT solution was removed, and the cells were dissolved in 0.1mL/well DMSO. The proportion viable cells were determined by measuring the OD of each sample at 570nm using a SpectraCount plate reader (Packard BioScience, Meridan, CT, USA).

Nitric oxide (NO) determination Following incubations with chemicals, samples of phenol red and dexamethasone-free culture media were extracted and levels of nitrite and nitrate, the relatively stable end products of NO, were determined using the nitrite/nitrate.

Gress reagent system Fifty μL aliquots phenol red-free culture medium in 60μL assay buffer were incubated with 10μL each of nitrate reductase preparation and nitrate reductase cofactor preparation (proprietary concentrations), which converts nitrate into nitrite, for 60 minutes at room temperature in 96-well microassay plates. After the required incubation time, 10μL of DAN reagent was added to each well, incubated for 10 minutes, and then the reaction was stopped with 20μL of NaOH added to each well. The total nitrte/nitrate was determined by measuring the OD of each sample at 540nm using a SpectraCount plate reader (Packard BioScience, Meridan, CT, USA). Data were calculated with nitrate standard curve.

Statistical Analysis All data were presented as mean ±SEM. A unpaired Student's *t*-test was performed to analyze the significance between the two means at a certain time point. The differences were considered significant at $P<0.05$.

Results for NO measurements were expressed in μmol/L nitrite and nitrate per 1×10^6 cells.

RESULTS

Effect of Hydralazine on t-BHP Induced Toxicity in ARPE-19 Cells In order to evaluate the potential protective effect of hydralazine against t-BHP-induced cytotoxicity, ARPE-19 cells were exposed to t-BHP (0.01-10mmol/L) combined with hydralazine (1-100mg/L) for 24 hours. As seen in Figure 2, hydralazine showed concentration-dependent rela-tionship against t-BHP induced cell damage at 0.01mmol/L and 0.03mmol/L. The maximum viability protection effect of 100mg/L hydralazine on ARPE-19 cells at 0.01mmol/L and 0.03mmol/L t-BHP treated as compared were 109.99%±2.41% ($^cP<0.001$) and 100.84%±13.00% ($^cP< 0.001$) as compared to controls of 89.90%±2.80% and 55.80%±4.08%, respectively.

Effect of Hydralazine on H_2O_2 Induced Toxicity in ARPE-19 Cells As shown in Figure 3, ARPE-19 cells were exposed to H_2O_2 (0.01-10mmol/L) combined with hydralazine (1-100mg/L) for 24 hours. Hydralazine concentration-dependently (10-100mg/L) inhibited H_2O_2 (0.3mmol/L and 1.0mmol/L) induced damage on ARPE-19 cells. The maximum viability protection effect of hydralazine on ARPE-19 cells at 0.3mmol/L and 1.0mmol/L H_2O_2 treated were at 30mg/L (74.30%±0.80%; $^cP<0.001$) and 100mg/L (68.04% ±0.67% ;$^cP<0.001$) as compared to controls of 48.74%±2.40% and 41.28%±0.80%, respectively.

Effect of Hydralazine on Chemical Induced Hypoxia in ARPE-19 Cells As shown in Figure 4, ARPE-19 cells were exposed to NaN_3 (0.1-100mmol/L) combined with hydralazine (1-100mg/L) for 24 hours. No significant effect was seen in hydralazine treated ARPE-19 cells induced by chemical hypoxia.

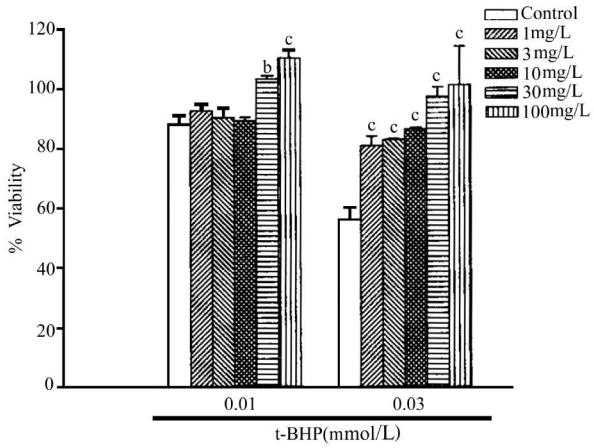

Figure 2 Effects of hydralazine on tert-butyl hydroxyperoxide (t-BHP) induced toxicity in ARPE-19 cells. ARPE-19 cells were incubated with t-BHP 0.01 and 0.03mmol/L for 24 hours and cell viability measured by MTT assay. Data are expressed as the means±SEM for six individual determinations. $^aP<0.05$, $^bP<0.01$, $^cP<0.001$, significantly different from control value by unpaired Student's *t*-test

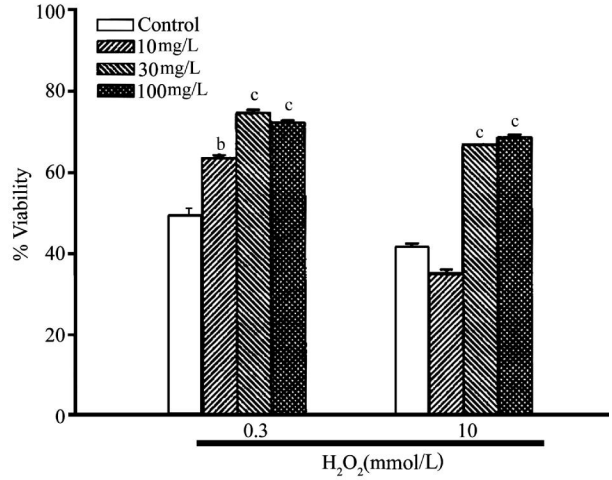

Figure 3 Effects of hydralazine on H_2O_2 induced toxicity in ARPE-19 cells. ARPE-19 cells were incubated with H_2O_2 at 0.3 and 1.0mmol/L for 24 hours and cell viability measured by MTT assay. Data are expressed as the means±SEM for six individual determinations. $^aP<0.05$, $^bP<0.001$, $^cP<0.0001$, significantly different from control value by unpaired Student's *t*-test

Effect of Hydralazine on Hypoxia Induced Cell Damage in ARPE-19 Cells ARPE-19 cells were exposed to hydralazine (0.01-100mg/L) under hypoxic conditions for 24, 48 and 72 hours. In hypoxia, 10mL/L O_2 and 50mL/L CO_2 were maintained using a temperature- and humidity-controlled environmental C-chamber by O_2 and CO_2 controllers with N_2 and CO_2 gas sources. The data showed that hydralazine significantly reversed hypoxia induced cell damage and the maximum reversion effects were 1mg/L at 48 hours (101.210% ±0.54%; $^c P<0.001$) and 72 hours (103.55% ± 1.75%; $^c P<0.001$), as compared to controls of 98.91% ± 0.56% and 97.45%±0.52%, respectively (Figure 5).

Effect of Hydralazine on Nitric Oxide Production in ARPE-19 Cells After incubation of ARPE-19 cells with hydralazine (1-100mg/L) for 24 hours, samples of phenol-red free culture media were extracted and the level of nitrite and nitrate were determined with Gress Reagent assay system. No significant effect of NO production was detected when treated with hydralazine (data not shown).

DISCUSSION

It is proposed that the loss of RPE cells is the main reason during the early phase of AMD [27]. Oxidative stress may play a role in the pathogenesis of AMD [28,29]. An increase in oxidative stress due to a reduction in protective mechanisms or an increase in number and concentration of active photo-oxidative reaction species are believed to contribute in part to the pathogenesis of AMD [30,31].

In order to investigate the antioxidant and vasodilator effects of hydralazine, in the potent ional treatment of AMD, we used in vitro human retinal pigment epithelium cells to evaluate the effect of hydralazine on the involvement of ROS in this study and on the role of hypoxia-induced cell damage in this effect.

A free radical is a molecule with an odd, unpaired electron; this unpaired electron makes the molecule unstable and highly reactive [32-34]. Oxygen free radicals, the superoxide anion ($O_2^{-}\bullet$), the hydroxyl radical (OH^-), and their intermediary, hydrogen peroxide (H_2O_2), are believed to be generated during ischemia and at the time of reperfusion. These ROS can be cytotoxic to cells by attacking fatty acids, which leads to lipid peroxidation of membranes, and reacting with proteins, including destruction and oxidation of amino acids, oxidation of sulfhydryl groups, and polypeptide chain scission[32, 35]. Three oxidative stress, H_2O_2, t-BHP and NaN_3 were used in this study to evaluate the mechanism of hydralazine against ROS damage in ARPE-19 cells. The data showed that hydralazine concentration-dependently inhibited t-BHP and H_2O_2 induced oxidative stress damage, but not by NaN_3.

t-BHP, an organic hydroperoxide [36], can be metabolized in the hepatocyte by glutathione peroxidase, generating oxidized glutathione (GSSG) [37]. Depletion of GSH and NADPH oxidation are associated with altered calcium homeostasis,

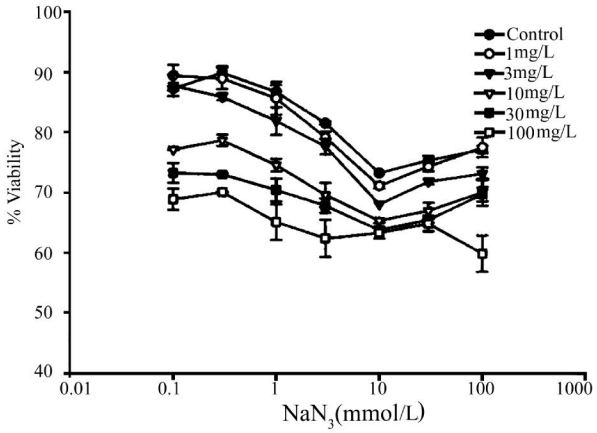

Figure 4 Effects of Hydralazine on NaN3 induced ischemia toxicity in ARPE-19 cells ARPE-19 cells were incubated with NaN_3 for 24 hours and cell viability measured by MTT assay. Concentration scale was logarithmized in order to screen more wide range of NaN_3. Data are expressed as the means± SEM for six individual determinations

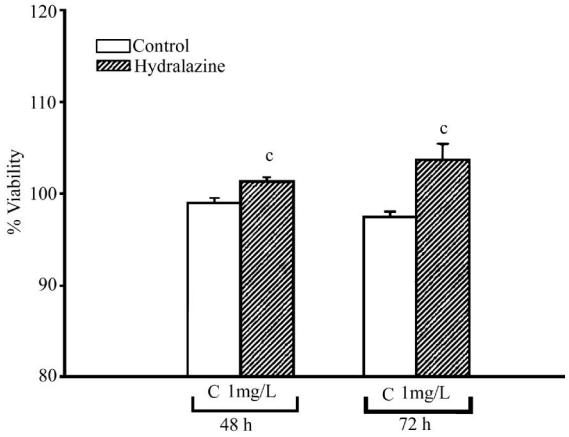

Figure 5 Effect of hydralazine against hypoxia-induced cell damage on ARPE-19 cells Data are expressed as the means± SEM for six individual determinations. $^a P<0.05$, $^b P<0.01$, $^c P< 0.001$, significantly different from control value by unpaired Student's *t*-test

leading to loss of cell viability [38]. Alternatively, t-BHP can be converted into its peroxyl and alkoxyl free radicals by cytochrome P450 enzymes and by free iron-dependent reactions. These free radicals can subsequently initiate lipid peroxidation, forming covalent bonds with cellular molecules (such as DNA and proteins) and further decrease GSH levels. The latter effect, in addition to altering calcium homeostasis, affects mitochondrial membrane potential, eventually causing cell death [39, 40]. It is obsvious that, ROS [38,41], t-BHP radical [42,39] and intracellular iron ion [40] are involved in the toxicity of t-BHP, direct effects on these parameters would tend to reduce the level of damage. H_2O_2 can form highly reactive hydroxyl radicals (•OH) by Fenton's reaction, which are capable of degrading most organic materials [43]. According to our data, hydralazine might be able antagonize t-BHP

and H_2O_2 induced cytotoxicity.

Mitochondrial toxins could offer an alternative to glutamate intoxication to modify the reversible energy failure that occurs during transient ischemia *in vivo*. Sodium azide (NaN_3) has already been used to induce "chemical ischemia" in cell cultures [26,44,45] as well as in *in vivo* experiments [46]. Its precise mechanism of action remains partially obscure. The effects are usually attributed to cytochrome c oxidase-respiratory chain complex IV-inhibition, and superoxide might be the major product after blockage of the electron transfer [47]. Our data show that, hydralazine cannot reverse NaN_3 induced cytotoxicity, indicating that hydralazine might not antagonize mitochondria-derived ROS. In addition, the potent vasodilator, NO, also did not involve in this effect (data not shown).

Reports suggested that hypoxia can cause cell death on RPE cells through an oxidative stress-induced mechanism [27]. There are several effects linked to hypoxia and oxidative injury [48], including uncoupling of mitochondrial oxidative phosphorylation and degradation of ATP to ADP. Moreover, the sudden decrease in O_2 tension allows the release of free radicals from the tightly controlled electron transport chain and their reactions with neighboring membrane lipids, resulting in membrane and cell damage. In order to test the effects of hydralazine in hypoxia-induced damage, we treated various concentrations of hydralazine in ARPE-19 cells for 24-72 hours, within 10mL/L O_2 of hypoxia controller chamber. We found, 1mg/L hydralazine, significantly released the hypoxia-induced ARPE-19 cells damage at 48 hours and 72 hours. Results indicated that hydralazine can prevent hypoxia-induced cell damage, but not chemical (NaN_3)-induced cell damage. This different might be due to the different mechanism of cell injury and the resource of ROS.

It is concluded, that hydralazine significantly antagonizes hypoxia-induced ARPE-19 cells damage, and this effect might be through the free radical scavenger by quenching the ROS. Although, the intracellular signaling involved in oxidative stress-mediated RPE cells death is still poorly understood, searching the chemicals involved in either antioxidant defense or mediation of the oxidative stress in RPE cells should allow the future development of therapeutic strategies against AMD. Hydralazine has the potential on improving the RPE cells damaged by hypoxia and ROS, and could be considered for the treatment of AMD and ischemic retinopathy.

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REFERENCES

1 Alarcon-Segovia D, Wakin KG, Worthington JW, Ward L E. Clinical and experimental studies on the hydralazine syndrome and its relationship to systemic lupus erythematosus. *Medicine* 1967;46(1):1-33

2 Herting RL. Hunter H. The physiologic and pharmacologic basis for the clinical treatment of hypertension. *Med Clin North Am* 1967;51(1):25-37

3 Klein L, O'Connor CM, Gattis WA, Zampino M, de Luca L, Vitarelli A, Fedele F, Gheorghiade M. Pharmacologic theraphy for patients with chronic heart failure and reduced systolic function: review of trials and practical considerations. *Am J Cardiol* 2003;91(9A):18F-40F

4 Gurney AM, Allan M. Inhibition of calcium release from the sarcoplasmic reticulum of rabbit aorta by hydralazine. *Br J Pharmacy* 1995;114(1):238-244

5 Azuma J, Sawamura A, Harada H, Awata N, Kishimoto S, Sperelakis N. Mechanism of direct cardiostimulating actions of hydralazine. *Eur J Pharmac* 1987;135(2):137-144

6 Daiber A, Mülsch A, Hink U, Mollnau H, Warnholtz A, Oelze M, Münzel T. The oxidative stress concept of nitrate tolerance and the antioxidant properties of hydralazine. *Am J Cardiol* 2005;10;96(7B):25i-36i

7 Daiber A, Oelze M, Coldewey M, Kaiser K, Huth C, Schildknecht S, Bachschmid M, Nazirisadeh Y, Ullrich V, Mulsch A, Munzel T, Tsilimingas N. Hydralazine is a powerful inhibitor of peroxynitrite formation as a possible explanation for its beneficial effects on prognosis in patients with congestive heart failure. *Biochem Biophys Res Commun* 2005;338(4):1865-1874

8 Bressler NM, Bressler SB, Fine SL. Age-related macular degeneration. *Surv Ophthalmol* 1988;32(6):375-413

9 Leibowitz HM, Krueger DE, Maunder LR, Milton RC, Kini MM, Kahn HA, Nickerson RJ, Pool J, Colton TL, Ganley JP, Loewenstein JI, Dawber TR. The Framingham Eye Study monograph: an opthalmological and epidermiological study of cataract, glaucoma, diabetic retinopathy, macular degeneration, and visual acuity in a general population of 2631 adults, 1973-1975. *Surv Ophthalmol* 1980;24(Suppl):335-610

10 Shima DT, Adamis AP, Ferrara N, Yeo KT, Yeo TK, Allende R, Folkman J, D'Amore PA. Hypoxic induction of endothelial cell growth factor in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Mol Med* 1995;1(2):182-193

11 Ryan SJ, Schacht AP. Macular disease. In: Ryan SJ, Schacht AP, eds: Retina. 3rd edn, Vol. 4. Medical Retina. Philadelphia: Mosby 2001:1039-1135

12 Strauss O. The retinal pigment epithelium in visual function. *Physiol Res* 2005;85(3):845-881

13 Steinberg RH, Wood I. The relationship of the retinal pigment epithelium. In: Zin KM, Marmor MF, eds: The retinal pigment epithelium. Mass, Cambridge: Harvard University Press 1979:205-225

14 Basinger SF, Hoffman RT. Biochemistry of the pigment epithelium. In: Anderson RE, ed: Biochemistry of the eye. San Francisco: American Academy of Ophthalmology Manuals Program, 1983:pp256-264

15 Augustin AJ, Hunt S, Breipohl W, Böker T, Spitznas M. Influence of oxygen free radicals and free radical scavengers on the growth behaviour and oxidative tissue damage of bovine retinal pigment epithelium cells *in vitro*. *Graefe's Arch Exp Ophthalmol* 1996;234(1):58-63

16 Harper FH, Liversidge J, Thomson AW. Interphotoreceptor retinoid binding protein induced experimental autoimmune uveitis: an immunophenotypic analysis using alkaline phosphatase anti-alkaline phosphatase staining, dual immunofluorescence and confocal microscopy. *Curr Eye Res* 1992;11(Suppl):129-134

17 Charteris DC, Hiscott P, Grierson I, Lightman SL. Proliferation vitreoretinopathy.Lymphocytes in epiretinal membranes. *Ophthalmology* 1992;99(9):1364-1367

18 Lopez PF, Grossniklaus HE, Lambert HM, Aaberg TM, Capone A Jr, Sternberg P Jr, L'Hernault N. Pathologic features of surgically excised subretinal neovascular membranes in age-related macular degeneration. *Am J Ophthalmol* 1991;112(6):647-656

19 Newsome DA, Dobard EP, Liles MR, Oliver PD. Human retinal epithelium contains two distinct species of superoxide dismutase. *Invest Opthalmol Vis Sci* 1990;31(12):2508-2513

20 Dorey CK, Wu G, Ebenstein D, Garsd A, Weiter JJ. Cell loss in the aging retina. Relationship to lipofuscin accumulation and macular degeneration. *Invest Ophthalmol Vis Sci* 1989;30(8):1691-1699

21 Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related muscular degeneration. *Prog Retin Eye Res* 2001;20(6):705-732

22 Cui K, Luo X, Xu K, Ven Murthy MR. Role of oxidative stress in neurodegen-

eration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Prog Neuropsychopharmacol Biol Psychiatry* 2004;28(5):771-799

23 Gibson GE, Huang HM. Oxidative stress in Alzheimer's disease. *Neurobiol Aging* 2005;26(5):575-578

24 Mossman T. Rapidcolorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65 (1-2): 55-63

25 Swanson RA. Astrocyte glutamine uptake during chemical hypoxia *in vitro*. *Neurosci Lett* 1992;147(2):143-146

26 Vaming T, Drejer A, Frandsen A, Schousboe A. Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide: protection by nifidipine and MK-801. *J Neurosci Res* 1996;44(1):40-46

27 Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP. Oxidative damage and protection of the RPE. *Prog Retin Eye Res* 2000;19(2):205-221

28 Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 2000;45 (2):115-134

29 Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of aging. *Nature* 2000;408(6809):239-247

30 Boulton M. Aging of the retinal pigment epithelium. In: Osborne NN, Chader GJ, eds; Progress in retinal research. New York: Pergamon 1991:65-68

31 Boulton M, Moriarty P, Jarvis-Evans J, Marcyniuk B. Regional variation and age-related changes of lysosomal enzymes in the human retinal pigment epithelium. *Br J Ophthalmol* 1994;78(2):125-129

32 Thompson JA, Hess ML. The oxygen free radical system: a fundamental mechanism in the production of myocardial necrosis. *Prog Cardiovasc Dis* 1986;28(6): 449-462

33 Fantone JC, Ward PA. Polymorphonuclear leukocytemediated cell and tissue injury: oxygen metabolites and their relations to human disease. *Human Pathol* 1985;16(10):973-978

34 McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985;312(3):159-163

35 Kloner RA, Przyklenk K, Whittaker P. Deleterious effects of oxygen radicals in ischemia/reperfusion.Resolved and unresolved issue. *Circulation* 1989;80 (5): 1115-1127

36 Alía M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L, Goya L. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicol Appl Pharmacol* 2006;212(2):110-118

37 Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid-peroxidation and cell death in isolated hepatocytes. *Toxicol Appl Pharmacol* 1985;78(3):473-483

38 Martín C, Martínez R, Navarro R, Ruiz-Sanz JI, Lacort M, Ruiz-Larrea MB. Tert-Butyl hydroperoxide-induced lipid signaling in hepato-cytes: involvement of glutathione and free radicals. *Biochem Pharmacol* 2001;62(6):705-712

39 VanderZee J, Barr DP, Mason RP. ESR spin trapping investigation of radical formation from the reaction between hematin and tert-butyl hydroxyperoxide. *Free Radic Biol Med* 1996;20(2):199-206

40 Hix S, Kadiiska MB, Mason RP, Augusto O. In vivometabolism of tert-butyl hydroperoxide to methyl radicals. EPR spin-trapping and DNA methylation studies. *Chem Res Toxicol* 2000;13(10):1056-1064

41 Lima CF, Fernandes-Ferreira M, Pereira-Wilson C. Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels. *Life Sci* 2006;79(21):2056-2068

42 Davies MJ. Detection of peroxyl and alkoxyl radicals produced by reaction of hdroperoxides with rat liver microsomal fractions. *Biochem J* 1989;257 (2): 603-606

43 Pesakhov S, Benisty R, Sikron N, Cohen Z, Gomelsky P, Khozin-Goldberg I, Dagan R, Porat N. Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of streptococcus pneumonia. *Biochip Biophys Acta* 2007;1768(3):590-597

44 Grammatopoulos TN, Johnson V, Moore SA, Andres R, Weyhenm-eyer JA. Angiotension type 2 receptor neuroprotection against chemical hypoxia is dependent on the delayed rectifier K+ channel. Na+/Ca2+ exchanger and Na++ ATPase in primary cortical cultures. *Neurosci Res* 2004;50(3):299-306

45 Grammatopoulos T, Morris K, Ferguson P, Weyhenmeyer J. Angiotension protects cortical neurons from hypoxia-induced apoptosis via the angiotension type 2 receptor. *Mol Brain Res* 2002;99(2):114-124

46 Vécsei L, Tajti J, Klivényi P, Pinter S, Karg E. Sodium azide treatment decreases striatal and cortical concentrations of alpha-tocopherol in rats. *J Neural Transm* 2001;108(3):273-278

47 Duranteau J, Chandel NS, Kulisz A, Shao Z, Schumacker PT. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J Biol Chem* 1998;273(19):11619-11624

48 Emerit J, Chaudiere J. Free radicala and lipid peroxidation in cell pathology. In: Miquel J, Quintanilha A, Weber H, eds; Handbook of free radical and antioxidants in biomedicine. Boca Raton: CRC Press; 1998