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

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Received: 2008-12-18 Accepted: 2009-01-08

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 hydroperoxide (t-BHP), H₂O₂, sodium azide (NaN₃), 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, H₂O₂ and hypoxia induced cell damage but not NaN₃. 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 hydroperoxide (t-BHP)

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 calcium 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), uveitis and AMD [17]. 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\(_2\)O\(_2\), including tert-butyl hydroperoxide (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.

**Methods**

**Culture of human retinal pigment epithelial cells** The human RPE 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\(^{\circ}\)C 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\(_2\)O\(_2\) (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\(^{\circ}\)C 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\(^{\circ}\)C. 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 a modified of method described by Swanson and Varming \textit{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\(^{\circ}\)C 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\(^{\circ}\)C. 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 \textit{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 N₂ and CO₂ 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°C. 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 nitrite/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 1×10⁶ 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% (P<0.001) and 100.84%±13.00% (P<0.001) as compared to controls of 89.90%±2.80% and 55.80%±4.08%, respectively.

**Effect of Hydralazine on H₂O₂ Induced Toxicity in ARPE-19 Cells** As shown in Figure 3, ARPE-19 cells were exposed to H₂O₂ (0.01-10mmol/L) combined with hydralazine (1-100mg/L) for 24 hours. Hydralazine concentration-dependently (10-100mg/L) inhibited H₂O₂ (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 H₂O₂ treated were at 30mg/L (74.30%±0.80%; P<0.001) and 100mg/L (68.04%±0.67%; P<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 NaN₃ (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.
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% ; *P*<0.001) and 72 hours (103.55% ± 1.75%; *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$_2$·*), the hydroxyl radical (OH·), and their intermediary, hydrogen peroxide (H$_2$O$_2$), 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 sulphydryl groups, and polypeptide chain scission [32,33]. Three oxidative stress, H$_2$O$_2$, 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$_2$O$_2$ induced oxidative stress damage, but not by NaN$_3$.

Hydralazine, an organic hydroperoxide [30], 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, leading to loss of cell viability [30]. Alternatively, t-BHP can be converted into its peroxy 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 obvisous that, ROS [38,41], t-BHP radical [42,36] 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$_2$O$_2$ 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$_2$O$_2$ 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 $^{[30,44,45]}$ 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 $^{[45]}$. 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 $^{[46]}$, 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 10mM 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 hyponxia-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.

Acknowledgement: We thank the support in part from the Institute of Ocular Pharmacology, Texas A&M University Health Science Center, USA and Stroke Center of Taipei Medical University, Taipei, Taiwan, China.

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