Inhibition of APE1/Ref-1 redox activity rescues human retinal pigment epithelial cells from oxidative stress and reduces choroidal neovascularization

Y. Li a, b, X. Liu a, T. Zhou a, M.R. Kelley c, P. Edwards a, H. Gao a, X. Qiao a, * 

a Department of Ophthalmology, Henry Ford Health System, 1 Ford Place 5D, Detroit, MI, United States 
b Department of Ophthalmology, Xijing Hospital, Fourth Military Medical University, Xi’an, Shanxi, People’s Republic of China 
c Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, United States

Abstract

The effectiveness of current treatment for age related macular degeneration (AMD) by targeting one molecule is limited due to its multifactorial nature and heterogeneous pathologies. Treatment strategy to target multiple signaling pathways or pathological components in AMD pathogenesis is under investigation for better clinical outcome. Inhibition of the redox function of apurinic endonuclease 1 (APE1) was found to suppress endothelial angiogenesis and promote neuronal cell recovery, thereby may serve as a potential treatment for AMD. In the current study, we for the first time have found that a specific inhibitor of APE1 redox function by a small molecule compound E3330 regulates retinal pigment epithelium (RPEs) cell response to oxidative stress. E3330 significantly blocked sub-lethal doses of oxidized low density lipoprotein (oxLDL) induced proliferation decline and senescence advancement of RPEs. At the same time, E3330 remarkably decreased the accumulation of intracellular reactive oxygen species (ROS) and down-regulated the productions of monocyte chemotactrant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF), as well as attenuated the level of nuclear factor-kB (NF-kB) p65 in RPEs. A panel of stress and toxicity responsive transcription factors that were significantly upregulated by oxLDL was restored by E3330, including Nrf2/Nrf1, p53, NF-kB, HIF1, CBF/NF-Y/Y1, and MTF-1. Further, a single intravitreal injection of E3330 effectively reduced the progression of laser-induced choroidal neovascularization (CNV) in mouse eyes. These data revealed that E3330 effectively rescued RPEs from oxidative stress induced senescence and dysfunctions in multiple aspects in vitro, and attenuated laser-induced damages to RPE–Bruch’s membrane complex in vivo. Together with its previously established anti-angiogenic and neuroprotective properties, E3330 is implicated for potential use for AMD treatment.

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1. Introduction

Age-related macular degeneration (AMD) is the leading cause of central vision loss in the elderly in developed world, and an increasing public health problem due to population aging [1, 2]. There are two forms of AMD, the dry form (or non-exudative; atrophic) and the wet form (or exudative; neovascular). Currently the most widely used therapies for AMD are limited to laser photocoagulation and intravitreal anti-vascular endothelial growth factor (VEGF) agents for wet AMD [3]. However, these treatments have various limitations such as insufficient effectiveness or requirement of repetitive intravitreal injections [4–6]. In addition, no treatment is approved so far for dry AMD which could develop into irreversible vision loss as severe as neovascular AMD. Although the mechanism of AMD is not fully understood, it is well known that AMD affects different cell types in the retina with a broad
spectrum of corresponding pathologies involving photoreceptor and RPE degeneration caused by cumulative oxidative stress and local inflammation, as well as endothelial cell activation and angiogenesis (for in-depth review, see Refs. [7,8]). Therefore, strategies targeting a single molecule are not sufficient to control the disease process. Agents that regulate multiple signaling pathways or multiple pathological components are more likely to produce a greater therapeutic effect for AMD.

In the past decade, the reduction/oxidation (redox) regulation function of apurinic endonuclease 1/redox factor-1 (APE1/Ref-1, here referred to as APE1) has been studied for its extended repertoire in controlling cellular response to oxidative stress. APE1 has two major functions in mammalian cells: DNA repair and redox regulation of gene transcription [11–13]. The DNA repair function of APE1 dominates the base excision repair pathway that responsible for repairing DNA damaged by oxidative stress, alkylating agents, and ionizing radiation [14]. Equally important, the redox function of APE1 enhances the DNA binding of numerous redox-sensitive transcription factors via reduction of disulfide bonds [13,15,16]. APE1 thereby represents an interesting therapeutic target in different mechanistic contexts. While the DNA repair function was a underlying mechanism of resistance to chemotherapy in various cancers [9], the redox function may serve as a target for modulating cell stress response, proliferation, angiogenesis, and processes such as inflammation.

Many stress responsive transcription factors including nuclear factor-κB (NF-κB) [17], hypoxia inducible factor-1α (HIF-1α) [10,18], cAMP response element binding protein (CREB) [19], activator protein-1 (AP-1) [17,20], p53 [21], and others have been reported to be activated by APE1 dependent redox activation. Theoretically, specific inhibition of the APE1 redox function may influence multiple signaling pathways and downstream molecules that could lead to various pathologies, without affecting the genomic integrity as long as the DNA repair function is left intact. In fact, it is possible to precisely eliminate the redox function of APE1 without interfering with its DNA repair function, because the two functions of APE1 have distinctively independent domains and mechanisms [10,22]. Previous studies have identified a small molecule inhibitor, E3330 (also called APX3330) which is specific to APE1 redox activity [10,23–26]. E3330 significantly reduced the transcriptional activities of NF-κB [25] and HIF-1α [10,18] in various cancer cells and endothelial cells without affecting the DNA repair function [10,25,27]. These factors and their downstream effectors have been closely associated with the etiology and pathogenesis of AMD [5,6,28–32]. We have recently reported that E3330 effectively suppressed retinochoroidal angiogenesis [33,34] and reduced neuronal cell loss [35]. These evidences indicate that E3330 may affect multiple signaling pathways and rectify multiple pathologies relevant to AMD pathogenesis through inhibition of APE1 redox function. However, the role of APE1 redox activity in retinal pigment epithelium cells (RPEs) is not known.

RPEs are highly specialized pigmented epithelium lining between the neural retina and Bruch's membrane, that plays critical roles in maintaining the homeostasis of retinal neurons and choroid [36,37]. RPEs are subject to lifelong chronic oxidative injury due to the constant exposure to light, a high level of metabolic activity, and accumulation of oxidized lipoprotein component in the cell [38,39]. Oxidative stress induced senescence and dysfunction of the macular RPEs is believed to herald AMD [8,40]. This study focused on the role of E3330 in regulating RPE responses to the oxidative stimulus elicited by oxidized low-density lipoprotein (oxLDL) in vitro and laser-induced RPE–Bruch’s membrane damage in vivo. We found that specific inhibition of APE1 redox activity with E3330 significantly rescued human RPEs from proliferation decline and senescence advancement induced by oxLDL. These effects were associated with reduced intracellular ROS, down-regulated secretion of monocyte chemoattractant protein–1 (MCP-1) and VEGF, and suppressed nuclear accumulation of NF-κB p65. The DNA binding activities of multiple redox-sensitive transcription factors including Nrf2/Nrf1, p53, NF-κB, HIF1, CBF/NF-Y/YY1, and MTF-1 were directly repressed by E3330. A single intravitreal injection of E3330 markedly reduced the laser-induced lesion size in RPE–Bruch’s membrane complex in mouse eyes. These findings suggest that E3330 holds great therapeutic promise for AMD by rescuing RPEs from oxidative stress in multiple aspects and reducing in vivo RPE–Bruch’s membrane complex damage. APE1 redox function could serve as a potential therapeutic target in the management of AMD.

2. Results

2.1. E3330 blocks sub-lethal doses of oxLDL-induced proliferation decline of RPEs

OxLDL has been widely used as an experimental stimulant to induce pathological stresses in RPE cells and mimic oxidative damages seen in AMD [41,42]. In a TUNEL assay to exclude the potential cell toxicity induced by oxLDL, no significant RPE apoptosis was observed at the doses of less than 450 μg/mL oxLDL (p > 0.05 when compared with the negative control group;

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**Fig. 1.** Effects of oxLDL and E3330 on RPE apoptosis (A) and proliferation (B) in vitro. A, TUNEL assay of RPEs exposed to various doses of oxLDL. A negative control without terminal transferase and a positive control with DNase I was included. All data points were normalized to the total number of the cells seeded in each well. OxLDL at or under 300 μg/mL did not induced significant cell apoptosis when compared with the negative control group. Significant apoptosis was only seen at 450 μg/mL and 600 μg/mL oxLDL treated groups. Bars represent mean ± SEM of three independent experiments. *p < 0.05 versus the negative control. B, MTS assay of RPE proliferation in response to 0, 50, 100, or 150 μg/mL oxLDL alone or combined with 30 μM E3330. All treatment groups were normalized to be a percentage of the control group. OxLDL inhibited the RPE proliferation dose dependently. 30 μM E3330 remarkably reversed the suppression effect of 100 μg/mL and 150 μg/mL oxLDL on RPE proliferation, but had no effect on the cell growth by itself. Bars represent mean ± SEM of three independent experiments. *p < 0.05 versus the no treatment control.
E3330 effectively protected RPEs from oxLDL-induced stress without affecting the basal senescence-related function.

According to these above mentioned apoptosis, proliferation and senescence phenotype assays, 150 μg/mL oxLDL was adopted as a sub-lethal dose to challenge RPEs for all the subsequent experiments.

2.3. E3330 reduces the accumulation of intracellular ROS, secretion of MCP-1 and VEGF, and nuclear level of NF-κB p65 in oxLDL-challenged RPEs

Accumulation of intracellular ROS is a major source of oxidative stress in AMD [40,45]. 150 μg/mL oxLDL significantly increased the intracellular ROS in RPEs by 20.23 ± 8.03% (p < 0.05 when compared with the control group; Fig. 3A). This elevated intracellular ROS was eliminated by 30 μM E3330 (p < 0.05 when compared with 150 μg/mL oxLDL treated group; Fig. 3A). This is an interesting finding that may at least partly explain the protective effect of E3330 on oxLDL stressed RPEs.

RPEs derived MCP-1 [46], VEGF [47] and ApoE [48,49] are critical contributors to local inflammation and angiogenesis in the development of AMD. Co-treatment of E3330 remarkably eliminated the striking upsurge secretion of both MCP-1 and VEGF stimulated by oxLDL (p < 0.01 and p < 0.05 respectively when compared with 150 μg/mL oxLDL challenged group; Fig. 3B and C). In addition, 30 μM E3330 significantly decreased MCP-1 production by 40% in the unchallenged RPEs (p < 0.01; Fig. 3B), but did not affect much of the basal VEGF secretion from RPEs (Fig. 3C). However, the increase of ApoE production by oxLDL was not altered by the addition of 30 μM E3330 (Fig. 3D).

Several studies have reported that E3330 inhibits NF-κB mediated transcription via different mechanisms [25,50–53]. In this study, 150 μg/mL oxLDL significantly increased the NF-κB p65 level in RPE nucleus, which was prominently reduced by concomitant 30 μM E3330 (Fig. 3E). E3330 alone did not affect the level of p65 in un-challenged RPE nucleus.

2.4. E3330 blocks the activation of multiple transcription factors induced by oxLDL in RPEs

To identify the transcription factor(s) that the APE1 redox inhibitor E3330 regulates, a reporter gene assay array containing a set of ten known stress and toxicity responding genes in ten different pathways was applied. ARPE-19 cells were exposed to 150 μg/mL oxLDL alone or with 30 μM E3330. OxLDL remarkably upregulated all the ten transcriptional activities in RPEs (p-value varies when compared with the control group; Fig. 4 and Table 1). The presence of E3330 significantly repressed seven of the ten transcription factors that stimulated by oxLDL. When compared with the oxLDL challenged groups, E3330 decreased the transcriptional activities of Nrf2/Nrf1 (59.91 ± 7.03%, p < 0.01), NF-κB (57.66 ± 8.06%, p < 0.05), HIF1 (62.05 ± 11.06%, p < 0.05), CBP/NF-Y/Y1 (44.24 ± 9.03%, p < 0.01), MTF1 (80.12 ± 16.06%, p < 0.05), and HSF-1 (63.18 ± 2.95%, p < 0.01) in RPEs. When compared with the un-treated control groups, six of the seven transcription factors had their DNA binding activities almost completely restored to the basal levels by E3330 (p < 0.05 when compared with the control group; Fig. 4 and Table 1), with the exception of HSF1. Though HSF1 of heat shock pathway and AhR in xenobiotic drug metabolism were also markedly repressed by E3330, the end point levels of these two transcription factors were still significantly higher than that in the control groups (both p < 0.05 when compared with the control group). The rest two transcription factors, glucocorticoid receptor and AP1, demonstrated a trend towards reduction by E3330 but did not reach statistic significant points (both p > 0.05 when...
compared with 150 μg/mL oxLDL challenged group). The fold change and corresponding p-value are summarized in Table 1. These data clearly revealed that E3330 was able to restore multiple signaling pathways that stimulated by oxLDL which in turn may prevent a range of pathogenic responses in RPEs.

2.5. E3330 reduces laser-induced CNV lesion in mouse eyes

A laser-induced CNV model is a classic animal model of wet AMD which is an ocular wound-healing response to damage of the RPE–Bruch’s membrane–choroidal complex [7]. We adopted this model to determine the in vivo effect of E3330. A single intravitreal injection of either E3330 or vehicle was given right after the laser photocoagulation. The treatment dosage of 1 μL 200 μM E3330 was equivalent to a final concentration of approximately 20 μM in the retinochoroidal tissue. After 2 weeks survival, a remarkable 40% reduction of the laser-induced CNV size was found in E3330 treated eyes when compared with the control eye (Fig. 5 A, B). Mean value of the CNV size treated by E3330 was 10,568 μm² while that of vehicle control treated was 16,531 μm². The difference was statistically significant (n=6; p=0.0200). These results are consistent with our previous finding that a single intravitreal treatment of E3330 inhibited the retinal angiomatous proliferation (RAP)-like neovascularization in Vldlr−/− mice [33].

3. Discussion

Age-related macular degeneration (AMD) is a progressive disease encompassing multifactorial pathogenesis involving metabolic, functional, genetic and environmental factors, and affecting multiple components in the retina including photoreceptors, retinal pigment epithelium (RPE), Bruch’s membrane and choriocapillaries. Current therapies still have limitations in effectively controlling this complex disease. The oxidative stress induced degeneration and dysfunction of macular RPEs are early and crucial changes contributing to local drusogenesis, inflammation and neovascularization [8,40]. Protecting RPEs from oxidative damage is thereby an
essential consideration for AMD treatment. In this study, we demonstrated for the first time that specific inhibition of APE1 redox function with E3330 effectively protected RPEs from oxLDL-induced impairment and reduced the area of laser-induced damage in RPE–Bruch’s–choroidal complex. Taken together with our previous findings that E3330 significantly suppressed in vitro angiogenesis of retinal and choroidal endothelial cells [33,34], and reduced retinal neovascularization in vivo [33], these data strongly suggest E3330 is a promising therapeutic agent for AMD by rescuing RPE degeneration and dysfunction from oxidative stress and reducing endothelial angiogenesis at the same time.

3.1. APE1 redox function and its role in the retina and choroid

The presence of APE1 in the retina and choroid has been established due to its critical roles in mammalian cells. We have

Table 1

| Transcription factor | Reporter gene activity fold change | Pathway               |
|----------------------|-----------------------------------|-----------------------|
|                      | oxLDL/Control | p Value | oxLDL+ E3330/oxLDL | p Value | oxLDL+ E3330/Control | p Value |
| Nrf2/Nrf1            | 1.71          | 0.008   | 0.60               | 0.003   | 0.84               | 0.409   | Antioxidant response  |
| p53                  | 1.70          | 0.001   | 0.59               | 0.000   | 0.82               | 0.130   | DNA damage            |
| NF-κB                | 1.76          | 0.008   | 0.58               | 0.011   | 1.04               | 0.850   | NF-κB                 |
| HIF-1                | 1.65          | 0.012   | 0.63               | 0.021   | 1.08               | 0.658   | Hypoxia               |
| CBF/NF-Y/YY1         | 2.33          | 0.002   | 0.44               | 0.004   | 1.16               | 0.549   | ER stress             |
| MTF1                 | 1.26          | 0.043   | 0.80               | 0.042   | 1.00               | 0.980   | Heavy metal stress    |
| HSF1                 | 1.59          | 0.000   | 0.63               | 0.002   | 1.22               | 0.024   | Heat shock            |
| Glucocorticoid Receptor (GR) | 1.49      | 0.015   | 0.68               | 0.312   | 1.33               | 0.065   | Glucocorticoid        |
| AP-1                 | 1.43          | 0.039   | 0.71               | 0.113   | 1.13               | 0.465   | MAPK/JNK              |
| AhR                  | 1.65          | 0.004   | 0.61               | 0.094   | 1.36               | 0.047   | Xenobiotic            |

OxLDL versus Con, 150 μg/mL oxLDL challenge versus vehicle control.
OxLDL+E3330 versus oxLDL, concomitant treatment of 150 μg/mL oxLDL, plus 30 μM E3330 versus 150 μg/mL oxLDL challenge.
OxLDL+E3330 versus Con, concomitant treatment of 150 μg/mL oxLDL plus 30 μM E3330 versus vehicle control.
reported high levels of APE1 in the neural retina, purified retinal vascular endothelial cells (RVECs) and retinal pericytes in mice [33]. We also found abundant expression of APE1 in human ARPE-19, RVECs and choroidal endothelial cells (CECs) [34]. Chiariini et al. [34,55] demonstrated that APE1 was mainly located in the nucleus of all retinal cells and positively associated with the retina cell differentiation during postnatal development in rats. APE1 was postulated to accomplish both functions of DNA repair and reductively activation of transcription factors in the nucleus [56].

In previous studies, the function of APE1 was evaluated by approaches such as gene knockout [57,58], RNA antisense [59,60] and siRNA [61]. While valid, these approaches are not suitable for the current study as they affect the entire APE1 molecule regardless of its distinctive dual functions. The subsequent changes are the mixture of multiple effects in which the specific role of APE1 redox activity cannot be depicted. In addition, such approaches could lead to cell cycle arrest [58], cell death [61] and early embryonic lethality [57]. Recent identification of the novel small molecule inhibitor that specifically suppresses APE1 redox function, namely E3330, makes it possible to investigate the redox activity individually [11,16,23]. The specificity of E3330 has been well documented by several studies. E3330-fixed latex beads specifically bind to APE1 protein [24], and E3330 effectively blocks multiple redox sensitive transcription factors without interfering with the DNA repair function of APE1 [10,23,25,26,51]. By using E3330, we have previously reported that APE1 redox function was required for retinal and choroidal angiogenesis [33,34], and involved in neural tissue recovery after stroke in diabetic rats [35]. These and our current findings in RPEs clearly indicate that inhibition of APE1 redox function have multiple beneficial effects in the retinorchoroidal region including anti-angiogenesis, anti-inflammation and neural protection.

3.2. Inhibition of APE1 redox function rescued degeneration and dysfunction of RPEs induced by oxidative stress

OxLDL is known as a major contributor of oxidative stress and inflammation in AMD pathology. Previous studies showed that oxLDL inhibits RPE viability, promotes RPE senescence and apoptosis in a dose dependent manner [42], and disrupts multiple functions of RPE cells at sub-lethal dosages [42,62]. OxLDL-stimulated intracellular accumulation of ROS plays an important role in linking oxidative stimulus with early dysfunctions of RPEs [40,45]. The biological effects of oxLDL are mainly mediated through signaling pathways, especially via activation of transcription factors such as NF-κB [41,63]. In this study, E3330 effectively attenuated sub-lethal oxLDL induced viability decline and senescence advancement of RPEs, at the same time blocked intracellular accumulation of ROS in the cell. It is quite possible that these events occurred through the suppression of multiple transcription factors via inhibition of APE1 redox function, since the ROS generation mediated by NADPH oxidase (Nox) was actually controlled by transcription factors including NF-κB [64], HIF-1 [65] and Nrf2 [66,67]. Intracellular ROS reduction may subsequently relieve the cellular macromolecules such as DNA, proteins, and cell membrane lipids from oxidative stress [40,68], and suppress the ROS triggered “activation signals” and the cross talk between ROS and redox-sensitive transcription factors such as NF-κB [69] and HIF-1 [70]. These changes presented as the regain of RPE proliferation and reduction of senescence-like phenotype in RPEs in our study. The fact that we saw such a broad spectrum of effects by E3330 on RPE functions strongly indicated that all of these aspects were affected in various degrees.

By examining the production of MCP-1 and VEGF by RPEs, we found that the inhibitory effect of E3330 was translated from redox sensitive transcription factors to downstream effector molecules capable of mediating chronic inflammation and angiogenesis in the retina and choroid. These findings supported our previous reports that intravitreal application of E3330 significantly reduced retinal and choroidal neovascularization in mice [33,34], as RPEs is a considerable source of VEGF and MCP-1 in the retinochoroidal tissue. ApoE [49], another AMD related molecule that synthesized by RPEs, was minimally regulated by E3330. The transcriptional regulation of ApoE gene expression is known to be cell-type specific [71]. Ishida et al. reported that ApoE secretion by human RPEs was transcriptionally regulated by ligands for nuclear hormone receptors such as thyroid hormone receptor, liver X receptor and retinoid X receptor [48]. While little is known so far about APE1 redox regulation on these transcription factors, our data indicated that oxLDL-induced ApoE production in RPEs was not regulated by APE1 redox signals.

NF-κB is arguably the most studied transcription factor in the pathogenesis of AMD. It is known NF-κB can be activated by oxidative stress and regulates expressions of various pro-inflammatory and pro-angiogenesis factors in RPEs, including MCP-1, ICAM-1, IIs, and VEGF [28,41,72]. E3330 is able to suppress the NF-κB activity through different mechanisms. For example, Handa's team reported that E3330 inhibited the NF-κB DNA binding through reversing the APE1-dependent reduction of Cys-62 residue of p50, without affecting the IkB-α degradation and p65 translocation or phosphorylation in Jurkat cells [25,50]. Goto et al. showed that E3330 impaired the nuclear translocation of NF-κB and degradation of IkB-α in human peripheral monocytes [51]. The differences noted in these reports are probably due to the usage of different methodology and cell-specific differences. In the current study, we examined the effect of E3330 on the nuclear level of NF-κB p65 as nuclear localization is required for both APE1 and NF-κB to accomplish their functions. We found E3330 markedly reduced the nuclear accumulation of p65 protein, which was compiled with the inhibitory effect of E3330 on NF-κB DNA binding as is shown in luciferase reporter gene assay. As other NF-κB proteins such as p50, p52, RelB and cRel may also contribute to the transcriptional activity of NF-κB in RPEs, additional experiments investigating the underlying mechanism are necessary.
3.3. Inhibition of APE1 redox function represses multiple stress-
responding transcription factors in RPEs

We have provided data in this study that E3330 significantly
repressed the oxlDL-induced stimulation of at least six (Nrf2/Nrf1,
P53, NfkB, HIF1, CBF/NF-YY1, and MTF1) transcription factors in
RPEs. Some of these transcription factors are directly implicated in
dry and/or wet AMD pathogenesis. Nrf2/Nrf1 is critical in retinal
antioxidant and detoxification responses [73,74]. p53 stimulates
RPE apoptosis and cell cycle arrest [75,76]. NfkB regulates the
production of pro-inflammatory factors and growth factors in
several stress scenarios including immune reactions, inflammation
and hypoxia in RPEs [28,29,77]. HIF1 mainly controls the VEGF
production induced by hypoxia [30]. It is noteworthy that although
the effect of E3330 is quit potent affecting multiple transcription
factors, at the given dose it only blocked the aberrantly up-
regulated transcriptional activities and brought them back down
to the levels comparable to their basal activities. These data
demonstrated that targeting APE1 redox activity could modulate
multiple signaling pathways closely related to AMD pathogenesis
and may achieve a greater therapeutic effect than any mono-target
therapies, without causing side effects from complete inhibition
of any given transcription factor.

3.4. APE1 redox function in inhibiting choroidal neovascularization

Lasering-CNV is a wound-healing response to the damage of
RPE–Bruch’s–choroidal complex [7]. The growth of
CVN is largely promoted and directed by RPE derived signals,
typically increased expression of VEGF via HIF-1 [78] and NF-kB
[72] signaling pathways, and MCP-1 via NF-kB pathway [28,79,80].
Therefore, the finding that intravitreal E3330 significantly reduced
the laser-induced CVN in mouse eyes can be at least partly
attributed to the attenuated transcription activities of NF-kB and
HIF-1, as well as decreased production of MCP-1 and VEGF in RPEs
by E3330. However, E3330 also directly inhibits the angiogenic
activities of endothelial cells [33,34]. Due to the nature of laser-
induced CVN animal model, it is difficult for us to distinguish
whether the actual effect of APE1 redox inhibition is from CEC or
RPE or both at this point. Further studies are underway for a more
comprehensive understanding of this issue.

In summary, we demonstrated that specific inhibition of APE1
redox activity with E3330 significantly protected RPEs from
oxLDL-induced stresses and reduced laser-induced CVN in mouse
eyes. This effect was mediated by inhibiting ROS generation,
repressing multiple redox sensitive transcription factors, and
preventing upsurge of downstream pro-inflammatory and pro-
angiogenic molecules. These findings in RPEs along with
our previous reports of the anti-angiogenic effect on retinochoroidal
endothelial cells [33,34,79] suggested that specific inhibition of
APE1 redox activity with E3330 could be a promising therapeutic
strategy for both dry and wet AMD by targeting multiple signaling
pathways in the context of hypoxia, chronic inflammation, and
angiogenesis. Further studies are necessary for a better under-
standing of the underlying mechanisms for this novel AMD
treatment.

4. Materials and methods

4.1. Materials

A RPE cell line from adult human, ARPE-19 (American Type
Culture Collection, Manassas, VA), was cultured in DMEM (Gibco®
Life Technologies, Grand Island, NY) supplemented with 10% FBS in
a 37 °C incubator with 5% CO2. ARPE-19 cells near confluence were
resuspended by 0.25% Trypsin–EDTA (Gibco® Life Technologies,
Grand Island, NY) and used for sub-culture and various assays.

OxLDL, a widely used experimental paradigm for AMD [41,42],
was commercially purchased (Kalen Biomedical, Montgomery
Village, MD) and used to induce pathological stress in ARPE-
19 cells. E3330 [2E]-3-[5-2,3-dimethoxy-6-methyl-1,4-benzoqui-
nonyl]-2-nonyl-2-propenoic acid] was first dissolved in dimethyl-
sulphoxide (DMSO, Sigma-Aldrich Co. Saint Louis, MO) at
a concentration of 20 mM, then diluted in culture medium to reach
various end point doses. The final concentration of DMSO in the
medium did not exceed 0.01% (vol/vol). A dose of 30 μM of E3330
was applied throughout this study according to our previous
observation that 20–50 μM E3330 significantly reduced retino-
choroidal angiogenesis in vitro and in vivo in the absence of dose-
related adverse effects [33,34], as well as our pilot dose-response
experiments in ARPE-19 cells. Cells were treated with vehicle
control, 30 μM E3330 alone or in combination with various doses
of oxLDL. A vehicle control with matched concentration of DMSO
without E3330 was routinely included in each experiment.

4.2. Cell apoptosis and proliferation assay

The effects of oxLDL and/or E3330 on RPE apoptosis and
proliferation was measured by a TdT mediated dUTP-fluorescein
nick end-labeling (TUNEL) Reaction kit (In Situ Cell Death Detec-
tion Kit, Roche, Indianapolis, IN) and a CellTiter 96® AQueous One
Solution Reagent (Promega Corporation, Madison, WI) respec-
ively. ARPE-19 cells were seeded in 96-well plates at 2000 cell/
well and exposed to various doses of oxLDL alone or combined
with 30 μM E3330 for 48 h in a 37 °C incubator. These assays
were performed following the manufacturer’s protocol.

4.3. Cell senescence associated β-gal (SA-β-gal) assay

A cellular senescence assay kit (Cell Biolabs, San Diego, CA) was
used to determine the effect of E3330 on senescence-like pheno-
type of RPEs. ARPE-19 cells seeded at 2000 cells/well in 96-well
plates were exposed to oxLDL alone or combined with 30 μM
E3330 for 48 h in a 37 °C incubator. After centrifugation, 50 μL of
the supernatant of each sample was collected and immediately
 incubated with 50 μL of freshly prepared fluorometric substrate
for 2 h at 37 °C in darkness. The fluorescent intensity of each
reaction mixture was determined by a SpectraMax M2e microplate
reader ( Molecular Devices LLC, Sunnyvale, CA) at an excitation
wavelength of 360 nm and emission wavelength of 465 nm.

4.4. Intracellular ROS measurement

The intracellular ROS accumulation in RPEs was measured by
2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA) using an
intracellular ROS assay kit (Cell Biolabs, San Diego, CA). ARPE-19
cells were pre-loaded with DCFH-DA by exposure to freshly
preparing oxLDL alone or combined with 30 μM E3330 for
48 h. DCFH fluorescence of the cell lysate was measured with
excitation and emission settings of 485 and 530 nm by a micro-
plate reader.

4.5. ELISA assay

The protein concentrations of MCP-1, VEGF and ApoE in the
conditioned medium of ARPE-19 cells were determined using
commercially available ELISA kits (R&D Systems, Minneapolis,
MN) following the manufacturer’s instructions.
4.6. Western blot

ARPE-19 cells were treated with 150 μg/mL oxLDL or 30 μM E3330 alone or in combination for 48 h. Nuclear proteins were extracted from ARPE-19 cells with a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s instruction. The nuclear proteins were separated by SDS-PAGE, and western blot was performed with an anti-NF-κB p65 antibody (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. β-actin (Abcam, Cambridge, MA) was used as a loading control. Quantitative densitometry analysis was performed using an Image Lab™ Software (Bio-Rad, Hercules, CA). The ratio of NF-κB p65 and β-actin was presented.

4.7. Luciferase reporter gene assay

The reporter gene assay was performed using a Cignal Finder Stress & Toxicity 10-Pathway Reporter Array (Qiagen-SABiosciences, Valencia, CA). Briefly, ARPE-19 cells were exposed to 150 μg/mL oxLDL alone or combined with 30 μM E3330 for 48 h. The treated cells at 40–60% confluence were transfected with equal amounts of the mixed firefly luciferase (Fluc) and Renilla luciferase (Rluc) reporter vector for 24 h using Attractene transfection reagent (Qiagen, Valencia, CA). Then the fresh medium was added and incubated for another 6 h. Fluc and Rluc expressions were detected using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) with a Luminometer SpectraMax M2e according to the manufacturer’s instruction. The transcription activities, as indicated by the Fluc reading, were normalized to Rluc. The ratio of Fluc and Rluc was divided by the ratio of the promoterless positive control luciferase to determine the fold change.

4.8. Animals

Male wild-type C57BL/6J mice of 6–8 weeks were used in the in vivo studies. Animals were housed on a 12-h light-dark cycle with free access to food and water. All procedures were performed with strict adherence to the guidelines for animal care and experimentation prepared by the Association for Research in Vision and Ophthalmology and approved by the Henry Ford Health System Institutional Animal Care and Use Committee.

4.9. Laser photocoagulation, E3330 intravitreal injection, and quantification of the CNV lesions

Mice were anesthetized by 50 mg/kg ketamine HCl and 10 mg/kg xylazine. Topical 1% tropicamide, and 2.5% phenylephrine were administered for pupil dilation. Animals were positioned before a slit-lamp with a laser-delivery system (Coherent Novus 2000 Argon Laser System, Santa Clara, CA, USA). The fundus was visualized using a cover slip with Cornea Coat® (Hydroxypropylmethylcellulose, Insight Instruments Inc., Stuart, FL, USA). Laser photocoagulation (532 nm wavelength, 100 mW power, 50 μm methylcellulose, Insight Instruments Inc., Stuart, FL, USA) was used as a loading control. Quantitative densitometry analysis was performed using an Image Lab™ Software (Bio-Rad, Hercules, CA). The ratio of NF-κB p65 and β-actin was presented.

Fluorescent labeled isocitron was used to stain CNV spots according to previous reports [33,80]. Briefly, 4% PFA fixed eye cups were incubated with blocking solution containing 3% normal donkey serum, 0.2% Triton x-100 in PBS and then stained with a 1:100 diluted isocitron in blocking buffer at 4 °C overnight. With 4–6 relaxing radial cuts, the RPE-choroid-sclera complex was flattened and mounted with the RPE side upward. The area of CNV lesion in each sample was measured using SPOT Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

4.10. Statistical analysis

Results of quantitative studies were expressed as mean ± SEM of at least three independent experiments. Differences were assessed using one-way analysis of variance (ANOVA) for repeated measures followed by Fisher’s least significant difference test when appropriate. The statistical analyses were performed using IBM SPSS Statistics 19 (Armonk, NY). A value of p < 0.05 was considered statistically significant.

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References

[1] W.J. Tomany SC, R. Van Leeuwen, R. Klein, P. Mitchell, J.R. Vingerling, B.E. Klein, W. Smith, P.T. De Jong, Risk factors for incident age-related macular degeneration: pooled findings from 3 continents, Ophthalmology 111 (2004) 1280–1287.
[2] T.K. Ryskulova A., D.M. Makuc, M.F. Cotch, R.J. Klein, R. Janiszewski, Self-reported age-related eye diseases and visual impairment in the United States: results of the 2002 national health interview survey, Am. J. Public Health 98 (2008) 454–461.
[3] F.S. Ferris FL, L. Hyman, Age-related macular degeneration and blindness due to neovascular maculopathy, Arch. Ophthalmol. 102 (1984) 1640–1642.
[4] M.M. Brown DM, P.K. Kaiser, J.S. Heier, J.P. Sy, T. Ianchulev, ANCHOR Study Group, Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: two-year results of the ANCHOR study, Ophthalmology 116 (2009) 57–65.e55.
[5] CATT Research Group, M. D., M.G. Maguire, G.S. Ying, J.E. Grunwald, S.L. Fine, G. Jaffe, Ranibizumab and bevacizumab for neovascular age-related macular degeneration, N. Engl. J. Med. 364 (2011) 1907–1908.
[6] A.P.P. Tufail, C. Egan, P. Hykin, L. da Cruz, Z. Gregor, J. Dowler, M.A. Majid, C. Bailey, Q. Mohamed, R. Johnston, C. Bunce, W. Xing, ABC Trial Investigators, Bevacizumab for neovascular age-related macular degeneration (ABC Trial): multicentre randomised double masked study, Br. Med. J. 340 (2010) 101136/ bmj.c2439.
[7] C. WR, Histopathology of age-related macular degeneration, Mol. Vis. 27 (1999).
[8] H. JC, Age-related macular degeneration: the molecular link between oxidative damage, tissue-specific inflammation and outer retinal disease: the Proctor lecture, Invest. Ophthalmol. Vis. Sci. 51 (2010) 1275–1281.
[9] K.M. Fishel ML, The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target, Mol. Asp. Med. 28 (2007) 375–395.
[10] D.S. Luo M, A. Jiang, A. Reed, Y. He, M. Fishel, R.L. 2nd Nyland, R.F. Borch, X. Qiao, M.M. Georgiadis, M.R. Kelley, Role of the multifunctional DNA repair protein APE-endonuclease (APE1/Ref-1), an essential multifunctional DNA repair enzyme, Antioxid. Redox Signal 11 (2009) 601–620.
[11] Q.F. Tell G, C. Tiriell, M.R. Kelley, The many functions of APE1/Ref-1: not only a DNA repair enzyme, Antioxid. Redox Signal 11 (2009) 601–620.
[12] M.A. Bhakat KK, S. Mitra, Transcriptional regulatory functions of mammalian AP-endonuclease (APE1/Ref-1), an essential multifunctional protein, Antioxid. Redox Signal 11 (2009) 621–638.
[13] L.-F.M. Evans AR, M.R. Kelley, Going APE over ref-1, Mutat. Res. 461 (2000) 83–108.
Y. Li et al. / Redox Biology 2 (2014) 485–494

[14] B.D. Wilson DM, The major human abasic endonuclease: formation, con-sequences and repair of abasic lesions in DNA, Mutat. Res. 485 (2001) 283–307.
[15] D.G. Tell G, D. Caldwell, M.R. Kelley, The intracellular localization of APE1/Ref-1 is more than a passive phenomenon? Antioxid. Redox Signal 7 (2005) 367–384.
[16] G.M. Kelley M.R., M.L. Fishel, APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/reduct protein APE1/Ref-1, Curr. Med. Pharmacol. 5 (2012) 36–53.
[17] H.S. Ando K, Y. Kabek, O. Ogura, I. Sato, Y. Yamaguchi, T. Wada, H. Handa, A new APE1/Ref-1dependent pathway leading to reduction of NF-kappaB and AP-1, and activation of their DNA-binding activity, Nucleic Acids Res. 36 (2008) 4327–4336.
[18] K.C. Zou GM, Y. Kabe, H. Handa, R.A. Anders, A. Maitra, The Ape-1/Ref-1 redox function in age-related macular degeneration, Prog. Retin. Eye Res. 20 (2001) 385–414.
[19] B.N. Witmer, V.G. Van Noorden, C.J. Schlingemann R, Vascular endothelial growth factors and angiogenesis in eye disease, Prog. Retin. Eye Res. 22 (2003) 127–150.
[20] J.H. Kim, L.S. Kim, K.W. Yu, Y.S. Kim JH, Oxidized low density lipoprotein-induced senescence of retinal pigment epithelial cells is followed by outer blood-retinal barrier dysfunction, Int. J. Biochem. Cell Biol. 44 (2012) 808–814.
[21] R. M.R. RPE cell senescence, a key contributor to age-related macular degeneration, Med. Sci. Monit. 18 (2012) 505–510.
[22] S. Beatty, K.H. Phil, M. Henson, D. Boulton M, The role of oxidative stress in the pathogenesis of age-related macular degeneration, Surv. Ophthalmol. 45 (2001) 115–134.
[23] M.K. Jayaraman L, C. Zhu, T. Curran, S. Xanthoudakis, C. Prives, Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity, J. Biol. Chem. 11 (1992) 23–315.
[24] N. Shimizu, S.K. Tang, J. Nishi, T. Sato, N. Yamauchi, I. Hiramoto, M. Aizawa, S. Hatakeyama, A. Jiang, G.H. Kelley, M.R. Qiao X., Inhibition of APE1/Ref-1 redox activity with antagonists E3330 inhibits the growth of tumor endothelium and endothelial progenitor cells, J. Lipid Res. 51 (2010) 263–274.
[25] M.K. Jayaraman L, C. Zhu, T. Curran, S. Xanthoudakis, C. Prives, Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity, J. Biol. Chem. 11 (1992) 558–570.
[26] M.G. Xanthoudakis S, F. Wang, Y.C. Pan, T. Curran, Redox activation of Fos-Jun dimerization and activation of their DNA-binding activity, Nucleic Acids Res. 36 (2008) 4327–4336.
[69] X.S. Rossello, I.J. Weisman, G.A. Sun, G.Y. Wood WG., AP-2β regulates amyloid beta-protein stimulation of apolipoprotein E transcription in astrocytes, Brain Res. (2012) 87–95.

[70] W. Ma, L.S. Guo, J. Qu, W. Hudson, B.I. Schmidt, A.M. Barile GR., RAGE ligand upregulation of VEGF secretion in ARPE-19 cells, Invest. Ophthalmol. Vis. Sci. 48 (2007) 1355–1361.

[71] Z. Zhao, C.Y. Wang, J. Sternberg, P. Freeman, M.L. Grossniklaus, H.E. Cai J., Age-related retinopathy in NRF2-deficient mice, PLoS One 6 (2011).

[72] K.A. Rezaei, C.Y. Cai, J. Sternberg P., Modulation of NRF2-dependent antioxidant functions in the RPE by Zip2, a zinc transporter protein, Invest. Ophthalmol. Vis. Sci. 49 (2008) 1665–1670.

[73] S. Bhattacharya, C.E. Johnson, D.A. Johnson LR., Age-related susceptibility to apoptosis in human retinal pigment epithelial cells is triggered by disruption of p53–Mdm2 association, Invest. Ophthalmol. Vis. Sci. 53 (2012) 8350–8366.

[74] A.K. Kwok, Y.C. Lai, T.Y. Chan, K.P. Pang CP., Effects of trypan blue on cell viability and gene expression in human retinal pigment epithelial cells, Br. J. Ophthalmol. 88 (2004) 1590–1594.

[75] C.N. Nagineni, K.V. William, A. Detrick, B. Hooks Jl., Regulation of VEGF expression in human retinal cells by cytokines: implications for the role of inflammation in age-related macular degeneration, J. Cell. Physiol. 227 (2012) 116–126.

[76] G.W. Smith, D.C. Prentice, H. Blanks J., The importance of hypoxia-regulated, RPE-targeted gene therapy for choroidal neovascularization, Adv. Exp. Med. Biol. (2012) 269–277.

[77] Y. Chen, K.A. Chen, Y. Yang P., IL-17A stimulates the production of inflammatory mediators via Erk1/2, p38 MAPK, PI3K/Akt, and NF-κB pathways in ARPE-19 cells, Mol. Vis. (2011) 3072–3077.

[78] D. Ping, B.G. Rogers, E.M. Boss J.M., Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene, J. Immunol. 162 (1999) 727–734.

[79] X. Qiao, L.Y. Liu, X. Zhou, T. Kelley, M.R. Edwards, P. Gao H., APE1/Ref-1 redox inhibitor APX3330 modulates choroidal endothelial cells by transcriptional regulation of NF-κB and STAT3 activity, Abstr. Assoc. Res. Vis. Ophthalmol. 274 (2013).

[80] R.F. Mullins, G.M. Skeie J.M., Glycoconjugates of choroidal neovascular membranes in age-related macular degeneration, Mol. Vis. (2005) 509–517.