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NBHA Reduces Acrolein-Induced Changes in ARPE-19 Cells: Possible Involvement of TGF β

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Abstract

Purpose—Acrolein, a toxic, reactive aldehyde formed metabolically and environmentally, has been implicated in the damage to and dysfunction of the retinal pigment epithelium (RPE) that accompanies age-related macular degeneration (AMD). Our purpose was to investigate the potential of acrolein to influence the release of transforming growth factor beta-2 (TGF β 2) and vascular endothelial growth factor (VEGF), to assess the ability of N-benzylhydroxylamine (NBHA) to prevent the effect of acrolein on cytokine release and reduction of viable cells, and to explore the pathway by which acrolein might be causing the increase of VEGF.

Materials and Methods—Confluent ARPE-19 cells were treated with acrolein and/or NBHA. They were also pretreated with SIS3, a specific inhibitor of SMAD 3, and ZM39923, a JAK3 inhibitor, before being treated with acrolein. Viable cells were counted; ELISA was used to measure the TGF β 2 and/or VEGF in the conditioned media.

Results—Acrolein was shown to reduce the number of viable ARPE-19 cells and to upregulate the release of the proangiogenic cytokines TGF β 2 and VEGF. Co-treatment with 200 μ M NBHA significantly reduced the effects of acrolein on viable cell number and TGF β 2 release. Pretreatment of the cells with SIS3 partially blocked the action of acrolein on decreased viable cell number and VEGF upregulation, suggesting that part of the effects of acrolein are mediated by the increased levels of TGF β and its signaling.

Conclusions—Our results suggest that the action of acrolein on the reduction of viability and VEGF increase by ARPE-19 cells is partially mediated by TGF β 2. By reducing the effects of acrolein, NBHA and SIS3 could be potential pharmacological agents in the prevention and progression of acrolein-induced damage to the RPE that relates to AMD.

Keywords

Acrolein; Age-related macular degeneration; Retinal pigment epithelium; TGF β ; VEGF

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INTRODUCTION

In the industrialized world, age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in people over the age of 50, and of legal blindness in people over 65. At present, there is no effective treatment for most of those afflicted and because life expectancy in the Western world is increasing, AMD is becoming an even more serious health and socioeconomic problem. The pathogenesis of AMD is multifactorial and includes many clinical changes, commonly including degeneration of the retinal pigment epithelium (RPE), the formation of drusen subadjacent to the basement membrane of the RPE, and degeneration of the macula, a cone-rich area of the retina essential for central, high-resolution color vision.¹

The RPE, the outermost layer of the retina, is a pigmented monolayer posterior to the retina that plays a crucial role in retinal physiology. It is responsible not only for the regeneration of visual chromophore² and the blood-retinal barrier,³ but also for the phagocytosis of continuously shed photoreceptor outer segments.⁴ These phagocytosed outer segments form undegradable lipofuscin granules due to oxidative stress within the RPE, impairing its function.^{5,6} A2E, a major component of lipofuscin, has been associated not only with complement system dysregulation, but with increased levels of reactive aldehydes.^{7,8} The RPE also produces vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF β),⁹ both of which are found in increased amounts in eyes affected with proliferative (wet) AMD.

VEGF is necessary for cell survival, but has been implicated as a key protein involved in proliferative AMD, and is the major target for its pharmacologic intervention.¹⁰ TGF β is not only associated with induction of cellular senescence,^{11,12} but recent evidence has shown that subtoxic oxidative stress via TGF β release induces senescence in RPE cells, as measured by senescence-associated biomarkers.¹³ TGF β and some of its superfamily members are known to upregulate VEGF from ARPE-19^{14,15} and RPE cells.^{9,16}

Epidemiologic studies have associated the development of AMD with several environmental factors which contribute to oxidative stress. These include sunlight exposure, low antioxidant intake, and cigarette smoking. Smoking is a primary risk factor associated with the prevalence and incidence of neovascular AMD and geographic atrophy; indeed, there is a strong association between AMD and pack-years of cigarette smoking.^{17,18} Smoking causes oxidative stress due to high concentrations of aldehydes and NO_x in the cigarette smoke which deplete ascorbate levels and cause oxidation of lipids and proteins.¹⁹⁻²² Among the reactive aldehydes formed, including malondialdehyde and 4-hydroxynonenal, acrolein is the most toxic formed during this combustion process.²³ Acrolein is also formed by the peroxidation of polyunsaturated fatty acids like docosahexaenoic acid (DHA),²⁴ photoreceptor outer segments having the highest DHA content of any cell type.²⁵ In vitro, acrolein has been shown to be toxic to RPE cells by increasing cell death²⁶ and apoptosis²⁷ and by decreasing mitochondrial potential, glutathione, antioxidant capacity, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression, and mitochondrial enzyme activity.²⁸ In postmortem donor eyes, acrolein markers have been associated with oxidative damage in AMD.²⁹ Reactive aldehydes like 4-hydroxynonenal and acrolein have been shown to

upregulate transcripts³⁰ and protein for VEGF in RPE cells,³¹ and TGF β in human promonocytes.³² However, the effect of acrolein on the production of proangiogenic cytokines by RPE cells has not been studied and, therefore, we are the first to demonstrate this effect.

N-benzylhydroxylamine (NBHA) is a hydroxylamine that sequesters aldehydes by forming stable oximes.³³ It has been used successfully in vivo and in vitro to sequester acrolein and prevent its toxic effects in models of murine neurodegeneration³⁴ and in cultured mouse mammary carcinoma cells.³⁵ Based on these prior studies, we hypothesized that in RPE cells, NBHA reduces cell death, VEGF, and TGF β triggered by acrolein. Results from the present study indicate NBHA has a significant protective effect on these acrolein actions.

Based on investigations describing the role of TGF β in macular degeneration,^{13,36} the association of the JAK3 pathway with retinal degeneration³⁷ and JAK3 upregulation of VEGF in lymphoma cells,³⁸ we used inhibitors that would specifically block these TGF β and JAK3 signaling pathways to investigate their role in acrolein-induced cell loss and VEGF levels. SMAD 3, a component of the TGF β signaling pathway, was blocked as it was shown to be involved in signaling by TGF β 1^{36,39} as well as TGF β 2,⁴⁰ the predominant isoform in the primate eye,⁴¹ and the predominant isoform secreted by RPE cells.⁴²

Based on results from these studies, we found that we were able to directly prevent the effects of acrolein on loss of ARPE-19 cells and increased TGF β 2 by sequestering acrolein with NBHA, and indirectly to prevent ARPE-19 cell loss and VEGF increase by blocking the signaling pathway of TGF β 2.

MATERIALS AND METHODS

ARPE-19 Cell Culture

ARPE-19 cells were obtained from ATCC (Manassas, Virginia, USA) at passage 22. They were grown to confluence in T-75 flasks in DMEM (containing phenol red) with 5.5 mM glucose (Mediatech #10-014-CV, Manassas, VA), supplemented with 10% FBS, 0.348% sodium bicarbonate, 100U/ml penicillin, and 100 μ g/ml streptomycin (Mediatech 30-001-CI). The media was adjusted to pH 7.2, sterile-filtered and stored at 4°C until use. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Growth media was changed every 3–4 days. At confluence, after 5–7 days of culture, they were passaged. At passage 24, they were subcultured into 24-well culture plates and maintained in the medium described above. Cells were used after they had been confluent for a week. These cells were ovoid at confluence, and non-melanized, as described by Heimsath et al., 2006.⁴³ All media and treatments were warmed to 37°C before use.

Co-Treatment of ARPE-19 Cells with Acrolein and NBHA

Experiments were performed with a post-confluent monolayer in 24-well plates. Acrolein (Sigma #01680, Sigma/Fluka BioChemika, Buchs, Switzerland) and NBHA (Sigma #13454, Sigma/Fluka BioChemika, Buchs, Switzerland) were dissolved in serum-free media immediately before use. Cells were serum-starved for 24hr before being treated in serum-free media with acrolein (12.5, 25, or 50 μ M), NBHA (200 μ M), or co-treated with 50 μ M

acrolein plus 50,100, or 200 μ M NBHA for 72hr. At this time, the conditioned media was collected and stored at -20°C until use. ARPE-19 cells in the wells were counted immediately. (Additional acrolein studies were also done in DMEM without phenol red and no significant differences were found—unpublished results.)

Acrolein and Cell Transduction Pathway Inhibitors

Post-confluent 24-well plates of ARPE-19 were serum-starved for 24hr. They were then pretreated with serum-free media containing SIS3 (Calbiochem #566405, EMD Biosciences, La Jolla, CA), a specific inhibitor of SMAD3, and ZM39923 (Tocris #1367, Tocris Bioscience, Ellisville, MO), a JAK3 pathway inhibitor, each at a concentration of 2 μ M, for 24hr. After this, the media containing the inhibitors was removed and the cells were then treated with serum-free media containing 50 μ M acrolein for 72hr. At this time, the conditioned media was collected and stored at -20°C until use. ARPE-19 cells in the wells were counted immediately.

Viability of ARPE-19 Cells

Cells in each well were rinsed with HBSS before being trypsinized using 200 μ L trypsin EDTA 1X (Mediatech 25-053-CI, Mediatech, Manassas VA) warmed to 37°C , for 5–7 min, and the trypsin/cell suspension was neutralized with 800 μ L of warmed serum-containing growth media. Equal volumes of cell suspension and Trypan Blue were added together and allowed to incubate at room temperature for 3 min. Viable cells were counted using a Neubauer hemacytometer (Hausser Scientific, Horsham, PA); cells stained with Trypan Blue were considered non-viable and were not counted. Only one well at a time was trypsinized and counted, the 24-well plate being returned to the incubator in between trypsinizations.

Cytokine Determination

VEGF₁₆₅ and 121 and TGF β 2 were quantified in the conditioned media (CM) using Quantikine ELISA kits from R & D Systems (Minneapolis, MN) (DVE00 and DB250, respectively) according to manufacturer's instructions. Total levels of TGF β 2 were assayed following the acidification and neutralization step which freed the TGF β 2 from its latency-associated protein.

Statistical Analysis

Data was analyzed using one-way ANOVA. A p -value less than or equal to 0.05 was considered to be statistically significant. In all cases, each experiment was performed in triplicate ($N = 3$).

RESULTS

Acrolein Affects Cell Viability, VEGF, and TGF β 2

Treatment of ARPE-19 with increasing concentrations of acrolein for 72hr (Figure 1A) led to a significant reduction in the numbers of viable cells at every acrolein concentration tested: 12.5 μ M acrolein reduced viable cell number by 14% ($p = 0.025$), 25 μ M acrolein

reduced viable cell number by 23% ($p = 0.024$), and acrolein at 50 μM reduced viable cell number by 49% ($p = 0.014$) as compared with control.

Treatment of ARPE-19 with increasing concentrations of acrolein for 72hr led to increased levels of VEGF in the CM per 10,000 viable cells (Figure 1B), with a significant ($p = 0.02$) 53% increase at 50 μM acrolein. Cells treated with 50 μM acrolein for 24, 48, and 72hr (Figure 1C) showed rapid increases of VEGF in the CM, as compared with controls from comparable time points, there being a 113% increase ($p = 0.006$) after 24hr and 176% increase ($p = 0.005$) after 48hr. This leveled off to a 58% increase ($p = 0.05$) after 72hr.

Increasing levels of acrolein (Figure 1D) led to significant increases in TGF β 2 per 10,000 viable cells at every concentration of acrolein tested. Acrolein at 12.5, 25, and 50 μM increased TGF β 2 in the CM by 16% ($p = 0.03$), 54% ($p = 0.001$), and 98% ($p = 0.0003$), respectively, per 10,000 cells.

NBHA Reduces Acrolein Effects

ARPE-19 cells were co-treated with 50 μM acrolein plus NBHA (Figure 2A). NBHA at 50 μM combined with acrolein had no effect on preventing acrolein-induced toxicity ($p = 0.98$) and viable cell number remained at 51% of control. NBHA at 100 μM plus acrolein (50 μM) significantly improved viable cell number over acrolein alone ($p = 0.018$), restoring it to 67% of control. NBHA at 200 μM added to 50 μM acrolein led to further protection against acrolein-induced cell death ($p < 0.001$), completely preventing the toxic effect so that the viable cell number was 97% of control. NBHA at 200 μM , itself, had no effect on viable cell number.

When cells were treated with 50 μM acrolein plus increasing amounts of NBHA (Figure 2B) for 72hr, a trend in the reduction of the amount of VEGF per 10,000 viable cells was noted, though a significant level was not observed.

Addition of increasing concentrations of NBHA to 50 μM acrolein (Figure 2C) significantly reduced TGF β 2 by 15% at 100 μM NBHA ($p = 0.015$) and by 34% at 200 μM NBHA ($p = 0.05$).

SIS3 Reduces Acrolein Effects

As shown in Figure 3A, 50 μM acrolein for 72hr reduced the number of viable cells to 45% of the control number ($p = 0.005$). Pretreatment of cells with the inhibitor SIS3 before the addition of acrolein led to a 32% ($p = 0.03$) increase in viable cell number compared with acrolein alone, suggesting that a TGF β -mediated mechanism was at least partially responsible for decreased cell viability due to acrolein. Pretreatment with ZM39923 before the addition of acrolein had no significant effect on acrolein-mediated loss of viable cell number ($p = 0.86$), indicating that the JAK3 pathway was not affected by acrolein and played no role in acrolein-mediated cell loss. All cells, including those pretreated with inhibitors showed an increase in VEGF levels in the CM in response to acrolein treatment (Figure 3B). ARPE-19 cells treated with 50 μM acrolein alone had 142% more VEGF in the CM ($p < 0.001$) after 72hr. Pretreatment of cells with SIS3 before the addition of acrolein led to a 30% decrease in the amount of VEGF secreted by the viable cells ($p = 0.003$).

compared with acrolein alone. This suggests that the increase in VEGF due to acrolein treatment was partially mediated by TGF β 2. In contrast, cells pretreated with ZM39923 showed no change in acrolein-mediated increase of VEGF ($p = 0.38$), suggesting that the JAK3 pathway also played no role in acrolein-mediated upregulation of VEGF.

DISCUSSION

Acrolein is a ubiquitous environmental pollutant; roughly three fourths of ambient acrolein in the U.S. is from mobile sources, with the remainder from agriculture, industrial processes, tobacco smoke, and forest fires. It and other reactive aldehydes have been implicated in many medical disorders,⁴⁴⁻⁵³ as well as in ocular pathogenesis³⁰⁻⁵⁴ and pathology of the RPE present in AMD.^{31,55,56} Potential agents for ameliorating aldehydes' toxic effects on RPE and ARPE-19 cells have been explored, including glutathione precursors,³¹ hydroxytyrosol,⁵⁷ resveratrol,²⁶ and (R)- α -lipoic acid.^{27,28} Jia et al.²⁸ showed that in response to acrolein, the ARPE-19 cell line gave results comparable to those obtained using primary human fetal RPE cells, although the level of differentiation of ARPE-19 used in their study was not described. The lack of complete differentiation of ARPE-19 cells could be a limitation to the interpretation of the data obtained in the present study, but the results obtained by Jia et al. in their study suggest that ARPE-19 as used in the present study could be a suitable model for RPE in acrolein studies.

We are the first to demonstrate that acrolein leads to increased TGF β 2 and VEGF by RPE cells. TGF β 2 and VEGF have long been known to be associated with AMD,^{9,58-60} but their ocular upregulation due to reactive aldehydes has not been previously explored. We have shown that a 72-hr treatment of ARPE-19 cells with 50 μ M acrolein, in addition to reducing cell number in a concentration-dependent fashion, as has been previously demonstrated,²⁸ significantly increased VEGF/10,000 viable cells in a concentration- and time-dependent fashion, and significantly increased the secretion of TGF β 2/10,000 viable cells. Increased VEGF is usually associated with proliferation of ARPE-19 cells,⁴³ but in this case the cells may be producing more VEGF in an autocrine attempt to increase cell number. Increased TGF β is frequently associated with decreased cell number, but this can vary with the concentration of TGF β .⁶¹ These results fit into a pattern suggested by the results of others. Glutathione depletion is associated with oxidative stress and toxicity from hydrogen peroxide (H₂O₂)⁶² as well as from acrolein,⁶³ but acrolein toxicity has been shown to be more severe than that of H₂O₂.³⁵ Subtoxic levels of oxidative stress due to H₂O₂ induce senescence in RPE cells via TGF β 2 release¹³ which can directly upregulate VEGF by ARPE-19 cells¹⁴ and RPE primary cultures via mitogen-associated protein kinases,⁹ as well as JNK, PI3K, NF- κ B, and others.¹⁶ We have shown that acrolein leads not only to increased cell death and increased TGF β 2 release by the surviving cells, but appears to increase VEGF release by the stressed cells. Thus, a sequence is suggested leading from acrolein to increased VEGF and TGF β 3 secretion by RPE cells to cell death. To explore this, we performed experiments with cell transduction pathway inhibitors and showed that by inhibiting SMAD3, a member of the transduction pathway stimulated by TGF β , we could prevent some loss of viable cell number, a 48% increase in the number of viable cells compared to cells treated with acrolein alone, and also partially block the upregulation of VEGF by acrolein. This suggests that the upregulation of VEGF and loss of cell number due

to acrolein is at least partly due to the acrolein-induced upregulation of TGF β . Therefore, it is likely that SMAD3, which mediates cytostatic and apoptotic cellular responses induced by TGF β , also mediates some of the toxic effects of acrolein, although it is possible that SIS3 directly protects the cells. We have shown that acrolein also increases TGF β secretion by ARPE-19 cells. This relationship between acrolein and TGF β with respect to cell viability is consistent with a previous study showing decreased ARPE-19 cell viability in response to TGF β .⁶⁴

We are also the first to show the cytoprotective effects of N-benzylhydroxylamine (NBHA) on acrolein-induced toxicity of ARPE-19 cells. By sequestering acrolein before it could exert its toxic downstream effects, NBHA prevented the upregulation of TGF β 2 and the loss of cells caused by acrolein, but did not significantly reduce the upregulation of VEGF. Hydroxylamines have been used to delay lung fibroblast senescence,⁶⁵ evaluated for their “anti-aging” activities,⁶⁶ and used for radioprotection.⁶⁷ In particular, NBHA has been utilized in the following: in vivo to prevent hippocampal neurodegeneration due to reactive aldehydes, in vitro to prevent aldehyde-induced damage to the retinal precursor cell line E1A-NR.3,³⁴ and to mitigate acrolein-induced toxicity in FM3A mouse mammary carcinoma cells.³⁵ In the micromolar concentrations used in these studies, as in ours, NBHA was found not to have an effect on animal or cell viability. Additionally, in our study, NBHA alone was shown to have no effect on VEGF or TGF β 2 levels.

We are exposed to environmental and metabolic reactive aldehydes on a daily basis, and cumulative acrolein damage to the RPE is a likely mediator of some of the pathology seen in the chronic eye disease AMD; acrolein exposure through cigarette smoke is generally considered to make up a large proportion of total human exposure.⁶⁸⁻⁶⁹ AMD is also associated with several genetic factors that can predispose one to the disease and it has also been shown that smoking, in combination with these genetic variants, confers additional susceptibility.⁷⁰ Although there are no studies that have quantified exposure history and the retinal concentration of acrolein, increased oxidative damage due to acrolein in the eyes of post-mortem patients with AMD has been demonstrated.²⁹ Acrolein has previously been shown to cause dysfunction of the RPE and in this study we demonstrated that it also increases proangiogenic cytokines that are associated with AMD. We prevented acrolein-induced upregulation of TGF β 2 by ARPE-19 cells using the hydroxylamine NBHA and simultaneously protected the cells from acrolein toxicity. Thus, therapeutic agents that could sequester and buffer the effect of reactive aldehydes, as well as compounds like SIS3 that interfere with TGF β signaling, are promising drug candidates to test for the prevention and progression of acrolein-induced damage associated with age-related macular degeneration.

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FIGURE 1.

Acrolein affects ARPE-19 cell viability, VEGF, and TGFβ2 release. Confluent ARPE-19 cells were treated with 12.5, 25, or 50 μM acrolein for 72hr. (A) Viable cells were counted using a hemacytometer with Trypan Blue. Results are average viable cell number ± SE ($N = 3$). (B) ELISA for VEGF was performed on the conditioned media from each well, and the results were divided by the viable cell counts in Figure 1A. Results are the mean pg VEGF per 10,000 viable cells ± SE ($N = 3$). (C) Confluent ARPE-19 were treated with 50 μM acrolein; the conditioned media was collected after 24, 48, and 72hr. Results were obtained by comparing treatment groups to serum-free time-matched controls. Results are the mean pg VEGF per 10,000 viable cells ± SE ($N = 3$). (d) ELISA for TGFβ2 was performed on the conditioned media from each well, and the results were divided by the viable cell counts in Figure 1A. Results are the mean pg TGFβ2 per 10,000 viable cells ± SE ($N = 3$). Asterisks indicate statistical significance of $p < 0.05$.

FIGURE 2.

NBHA reduces acrolein effects. Confluent ARPE-19 cells were treated with 50 μ M acrolein, 200 μ M NBHA, or a combination of 50 μ M acrolein with 50, 100, or 200 μ M NBHA for 72hr. (A) Viable cells were counted using a hemacytometer with Trypan Blue. Results are average viable cell number $\pm$ SE ($N = 3$). (B) ELISA for VEGF was performed on the conditioned media from each well, and the results were divided by the viable cell counts in Figure 2A. Results are the mean pg VEGF per 10,000 viable cells $\pm$ SE ($N = 3$). (C) ELISA for TGF β 2 was performed on the conditioned media from each well, and the results were

divided by the viable cell counts in Figure 2A. Results are mean pg TGF β 2 per 10,000 viable cells $\pm$ SE ($N = 3$). Asterisks indicate statistical significance of $p < 0.05$.

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FIGURE 3.

SIS3 reduces acrolein effects. Confluent ARPE-19 cells were treated 2 μ M SIS3 or ZM39923 (ZM) for 24hr before being treated with 50 μ M acrolein for 72hr. (A) Viable cells were counted using a hemacytometer with Trypan Blue. Results are average viable cell number $\pm$ SE ($N = 3$). (B) ELISA for VEGF was performed on the conditioned media from each well, and the results were divided by the viable cell counts in Figure 3A. Results are the mean pg VEGF per 10,000 viable cells $\pm$ SE ($N = 3$). Asterisks indicate statistical significance of $p < 0.05$ between the acrolein-treated group and those treated with acrolein plus SIS3 or ZM.