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A Possible Role of Acrolein in Diabetic Retinopathy: Involvement of a VEGF/TGF β Signaling Pathway of the Retinal Pigment Epithelium in Hyperglycemia

Jeffery Grigsby¹, Brandi Betts¹, Eileen Vidro-Kotchian¹, Richard Culbert², and Andrew Tsin¹

¹University of Texas at San Antonio, One UTSA Circle, San Antonio, TX, USA

²Department of Surgery, Texas Tech School of Medicine, Midland, TX, USA

Abstract

Purpose—Acrolein has been implicated in retinal pigment epithelium (RPE) cell death, and has been associated with diabetic retinopathy. Our purpose was to investigate the potential effect of high glucose in influencing acrolein-mediated RPE cytokine production and cell death. We investigated the influence of the acrolein effect on ARPE-19 cells in high glucose conditions and quantified the release of transforming growth factor β (TGF β 1 and 2) and vascular endothelial growth factor (VEGF). We assessed the ability of N-benzylhydroxylamine (NBHA) as well as TGF β pathway inhibitors SIS3 and SB431542 to prevent this effect of acrolein on ARPE-19 cells.

Materials and methods—Confluent ARPE-19 cells were treated with acrolein and/or NBHA in both 5.5 and 18.8 mM glucose conditions. Cells were also pretreated with SIS3, a specific inhibitor of the SMAD3 pathway, and SB431542, a specific inhibitor of TGF β signaling pathway, before treating them with acrolein. Viable cells were counted and ELISAs were performed to measure the cytokines TGF β 1 and 2, and VEGF released into the conditioned media.

Results—In ARPE-19 cells exposed to acrolein and hyperglycemia there was reduced cell viability and an increase in the cell media of VEGF, TGF β 1, and TGF β 2, which was reversed by NBHA. Acrolein/hyperglycemia-induced cell viability reduction and cytokine overproduction was also reduced by TGF β pathway blockade.

Conclusions—We conclude that the effect of acrolein on the reduction of viability and VEGF increase by ARPE-19 cells in hyperglycemic media is conducted through the TGF β signaling pathway. Our results suggest that benefits of sequestering acrolein by NBHA and the blockage of the TGF β pathway by SB431542 and SIS3 offer suggestions as to potential useful pharmacological drug candidates for the prevention of diabetes-induced complications in the eye.

Keywords

Acrolein; NBHA; Age-related macular degeneration; TGF β ; VEGF and RPE

Correspondence: Dr. Andrew Tsin, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249, USA. Andrew.Tsin@utsa.edu.

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INTRODUCTION

The reactive aldehyde, acrolein (2-propenal), is formed environmentally and physiologically by oxidation. Environmentally, this oxidation is primarily from incomplete combustion of fossil fuels, wood, and plastic and during the heating of carbohydrates, vegetable oils, animal fats, and amino acids which occurs during cooking.¹ Tobacco smoke, however, equals or exceeds the total human exposure to acrolein from all other environmental sources.² Endogenous acrolein is formed from peroxidation of fats,³ including docosahexaenoic acid (DHA),⁴ the predominant unsaturated fatty acid in the retina,⁵ and from glucose auto-oxidative peroxidation.⁶ It is also formed from the oxidation of spermine⁷ and low-density lipoprotein (LDL).⁸ Acrolein interacts with biomolecules to form DNA adducts, amino acid adducts⁹ and protein cross links.¹⁰

In the retina, acrolein has been found adjacent to the retinal pigment epithelium (RPE) in the cones of *rdl* mice undergoing oxidative damage,¹¹ and acrolein-derived products have been found in Muller cells.¹² Acrolein, or acrolein-derived products, have also been found in blood serum, hemoglobin¹³ and vitreous (Vidro, 2010, Ph.D. dissertation).¹⁴ Spermine, a precursor of acrolein, has been found in the vitreous of patients with proliferative retinopathy at a level that is 15 times that of those without diabetes.¹⁵ Zhang et al.¹³ found that the acrolein-derived hemoglobin product N^ε(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine) measurement could be a marker for risk evaluation of proliferative diabetic retinopathy (PDR). The importance of the RPE in the pathogenesis of diabetic retinopathy relates not only to its role in maintaining the external blood-retinal barrier but also in its importance in the secretion into the retina of various factors known to play a role in angiogenesis such as vascular endothelial growth factor (VEGF),¹⁶ pigment epithelial-derived factor (PEDF)¹⁷ and transforming growth factor β (TGF β).^{18,19} Exogenous exposure to acrolein by humans is well documented,²⁰ as well as the fact that hyperglycemia leads to production of reactive molecules such as acrolein.^{6,21,22} Acrolein has been associated with diabetic nephropathy.²³

The generation of oxidative stress in the eyes of patients with diabetes is known to result from an increase in the production of reactive oxygen species (ROS), presumably emanating from the mitochondrial respiratory chain.²⁴ Recently, the oxidative stress marker heme oxygenase-1 has been demonstrated to increase in the retinas of diabetic rats compared to normal controls.²⁵ We hypothesized that this increased oxidative environment, compared to that of the normoglycemic, might be more permissive to acrolein-induced damage to RPE cells, and that N-benzylhydroxylamine (NBHA), through its ability to sequester acrolein, would be effective in preventing these changes in viable cell number, and pro-angiogenic cytokine regulation. In the present study, experiments were also performed to assess the roles of the TGF β receptor I (TGF β RI) and Smad3 in these acrolein-induced effects.

Acrolein-lysine adducts were found to be significantly higher in the urine of young type 1 diabetes patients,²⁶ type 2 diabetes patients,²⁷ and in the serum of patients with type 2 diabetes with end-stage renal disease as compared with normal controls.²⁸ Our previous studies in normal glucose conditions concluded that NBHA and SIS3 are capable of reducing acrolein-mediated RPE cell death.²⁹ We also reported that the action of acrolein in

the reduction of cell viability and the increase of VEGF are partially mediated by TGF β 2.²⁹ Acrolein, itself, though associated with diabetes complications and retinal degeneration in age-related macular degeneration has not been studied in the eyes of subjects with diabetes. We studied *in vitro*, using ARPE-19 cells cultured in low and high glucose media, the effect of acrolein on the production of TGF β and VEGF, the ability of NBHA to prevent these effects of acrolein on cytokine release, its effect on cell viability, and explored the pathways by which acrolein causes an increase in VEGF synthesis.

MATERIALS AND METHODS

ARPE-19 Cell Culture

ARPE-19 cells (ATCC #CRL 2302) were received at passage 22. They were grown to confluence in T-75 flasks in DMEM (Mediatech No. 10-014-CV) (containing phenolred), with 18 mM glucose. This was supplemented with 10% FBS (Hyclone No. SH30088.03), 0.4% sodium bicarbonate (Sigma No. 55761), 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. All media and treatments were warmed to 37°C before use. 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 for testing after they had been confluent for 1 week. These cells were ovoid at confluence, and non-melanized, as described by Heimsath et al.³⁰

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

Experiments were performed with a postconfluent monolayer in 24-well plates. Acrolein (Sigma No. 01680) and NBHA (Sigma No. 13454) were dissolved in serum-free 5.5 or 18 mM glucose media immediately before use. Cells were serum-starved for 24 hours in either 5.5 or 18 mM glucose media before being treated in serum-free 5.5 or 18 mM glucose 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 72 hours. At this time, the conditioned media (CM) 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 because of the potential for the estrogen-like effects of phenol red.³¹ Estrogen has been shown to increase cell proliferation in retinal vascular endothelial cells.³² No significant cell viability differences were found between ARPE-19 grown with, or without phenol red (data not shown).

Acrolein and Cell Transduction Pathway Inhibitors

Postconfluent 24-well plates of ARPE-19 were serum-starved in 5.5 or 18 mM glucose for 24 hours. They were then pre-treated³³ for 24 hours with serum-free 5.5 or 18 mM glucose media containing SIS3 (Calbiochem No. 566405), a specific inhibitor of SMAD3^{33,34} at a concentration of 2 μ M (concentration per C. Nagineni personal communication). Cells were also pretreated^{35,36} for 24 hours with 10 μ M SB431542 (Sigma No. S4317), a TGF β R1 receptor blocker,^{35,36} at an amount that would completely inhibit the pathway without being toxic.³⁶ The inhibitors SIS3 and SB431542 were dissolved in DMSO at a concentration of 2

parts per 1000 which has been determined by control experiments to not affect either cell viability or VEGF concentration in the CM in this experimental protocol (data not shown). After this, the media containing the inhibitors was removed and the cells were then treated for 72 hours with serum-free 5.5 or 18 mM glucose media containing 50 μ M acrolein. At this time, the CM was collected and stored at -20°C until use. ARPE-19 cells in the wells were counted immediately.

Viability of ARPE-19 Cells

Viable cells were counted using a trypan blue dye exclusion method with a Neubauer Hemocytometer.

Cytokine Determination

VEGF 165 and 121 and TGF β 1 and 2 were quantified in the CM using Quantikine ELISA kits from R&D Systems (DVE00, DB100 and DB250, respectively) according to manufacturer's instructions.

Statistical Analysis

Shapiro-Wilks test was done on the data to determine normality, Bartlett's test was used to determine homogeneity of variances and data were analyzed using either one or two-way ANOVA. A p -value < 0.05 was considered to be statistically significant. In all cases, each experiment was performed at least in triplicate ($n = 3$).

RESULTS

Acrolein Decreases ARPE-19 Cell Viability in 18 mM Glucose

ARPE-19 cells were treated for 72 hours with increasing concentrations of acrolein (0, 12.5, 25 and 20 μ M) in both normal (5 mM) and hyperglycemic (18 mM) treatment media (Figure 1A). With no acrolein in the treatment medium, there was no significant difference in viable cell number between the two glucose concentrations. However, with increasing acrolein concentration, differences in cell viability arose between the two glucose concentrations and two-way ANOVA was significant for the interaction of glucose and acrolein concentration, $p < 0.05$. There were 15% fewer viable cells in the 25 μ M acrolein/18 mM glucose treatment condition than the equivalent 5.5 mM condition, $p < 0.05$. In addition, there were 34% fewer viable cells in the 18 mM glucose/50 μ M acrolein than in the 5.5 mM glucose/50 μ M acrolein group, $p < 0.001$ (Figure 1A).

Acrolein Increases VEGF per Viable ARPE-19 Cells in 18 mM Glucose

Confluent ARPE-19 cells were treated with increasing concentrations of acrolein in serum-free normal (5.5 mM) and high glucose (18 mM) media for 72 hours (Figure 1B). A two-way ANOVA was significant for this interaction between acrolein and glucose, $p < 0.0001$, and VEGF increase. This demonstrated a significant increase in VEGF secreted into the CM in the 18 mM glucose with the 25 or 50 μ M acrolein group compared to the 5.5 mM glucose with the equivalent acrolein group. 25 μ M acrolein/18 mM glucose had a 34% increase in VEGF per 10,000 cells compared to the 25 μ M acrolein/5.5 mM glucose group, $p < 0.01$, and

the 50 μ M acrolein/18 mM glucose group had 50% more VEGF per 10,000 cells compared to the 50 μ M acrolein/5.5 mM glucose group, $p < 0.001$ (Figure 1B).

NBHA Reduces the Acrolein Effect on Cell Numbers

ARPE-19 cells were treated with either acrolein, NBHA, or a combination of the two in the media (Figure 2A). In this experiment, we found that when 200 μ M NBHA was used alone in the 18 mM glucose group, without acrolein, viable cell numbers decreased by 19% compared to cells grown in treatment media without NBHA, $p < 0.05$. However, when used in combination with 50 μ M acrolein, 50 μ M NBHA increased the number of viable cells 74%, 100 μ M NBHA increased the number 103% and 200 μ M NBHA increased the number 186%, $p < 0.001$, over the 50 μ M acrolein-alone treatment group. There was a significant treatment effect for NBHA on acrolein-induced reduction of viable cell numbers, $p < 0.0001$, using two-way ANOVA. When 200 μ M NBHA was used with acrolein there were no viable cell count differences between the high and low glucose treatment groups.

The CM corresponding to these cell counts were collected, and VEGF (Figure 2B), TGF β 1 (Figure 2C), and TGF β 2 (Figure 2D), protein levels were determined per 10,000 cells. In combination with 50 μ M acrolein, a nonsignificant reduction in VEGF was seen with 50 μ M NBHA, but when 100 μ M NBHA was used, the VEGF levels decreased significantly 44%, $p < 0.001$, and decreased further with 200 μ M NBHA, reducing it 56% below the level of acrolein treatment alone $p < 0.001$, restoring the VEGF level to within 4% of the non-acrolein, non-NBHA control level. Two-way ANOVA showed that there was a significant interaction between NBHA and glucose levels, $p < 0.0001$, that accounted for 34% of the total variance of VEGF levels.

Acrolein and NBHA Affect 18 mM Glucose-induced TGF β 2 Regulation

Having established an optimal acrolein concentration, 50 μ M, and an optimal NBHA concentration, 200 μ M, for ARPE-19 cells, experiments were performed to determine the effect of acrolein on TGF β 2 levels (Figure 3D). Cells were treated with no acrolein or NBHA, 200 μ M NBHA alone, 50 μ M acrolein alone, or a combination of 50 μ M acrolein and 200 μ M NBHA in both high (18 mM) and low glucose (5.5 mM). In the 18 mM glucose groups, two-way ANOVA performed on results from ARPE-19 cells treated with 50 μ M acrolein and 50 and 200 μ M NBHA did not demonstrate a treatment effect of the NBHA (data not shown). However, the combination of 18 mM glucose and 50 μ M acrolein did cause a 75% increase, $p < 0.05$, in TGF β 2 per 10,000 cells levels, which was eliminated with 200 μ M NBHA co-treatment (Figure 2D). In the nonacrolein, non-NBHA groups, the 18 mM glucose group had 23% more TGF β 2 per 10,000 cells than the 5.5 mM group indicating that glucose levels alone play a significant role in the upregulation of TGF β 2 and enhance the acrolein effect.

A similar experiment was performed with ARPE-19 to examine the roles of acrolein and glucose in the regulation of TGF β 1 (Figure 2C). There was no difference in the low and high nonacrolein glucose treatment groups both with and without NBHA addition. However, in the presence of 50 μ M acrolein, the 18 mM glucose cells had 30% more TGF β 1 in the CM, compared to the 5.5 mM group. The 18 mM glucose treatment with 50 μ M acrolein

increased TGF β 1 levels by 230%, $p < 0.001$, compared to 18 mM glucose levels in the nonacrolein group. This treatment effect was eliminated by the addition of 200 μ M NBHA to the 18 mM glucose/50 μ M acrolein treatment media. Results of two-way ANOVA show a significant difference between the acrolein/NBHA group and glucose levels, $p < 0.0001$.

The Effect of TGF β Pathway Inhibition on Acrolein Effects in 18 mM Glucose

ARPE-19 cells in 5 and 18 mM glucose were pretreated with 2 μ M SIS3 or 10 μ M SB431542 for 24 hours before being treated with 50 μ M acrolein for 72 hours. Figure 3A shows that these two blockers of T β R1 signaling were equally effective in preventing acrolein-induced loss of viable cells, both of them resulting in viable cell numbers not significantly different from controls or from each other. Pretreatment with these two inhibitors also prevented the acrolein-induced increase of VEGF (Figure 3B). Acrolein alone in 18 mM glucose increased the VEGF level 88%, $p < 0.001$, but this was prevented by both SIS3 and SB431542, restoring VEGF levels to the control level.

DISCUSSION

Acrolein has been associated with disorders including exacerbation of asthma, increased pulmonary edema, increased bronchial responsiveness,³⁷ hemorrhagic cystitis,³⁸ atherosclerosis,³⁹ stroke,^{40,41} impaired immune function,⁴² renal failure,⁴³ and Alzheimer's disease.^{44–46} Acrolein has also been associated with the RPE cell disruptions seen in age-related macular degeneration.^{47–51} Acrolein adducts have been found in the serum and urine of both type 1 and type 2 diabetes patients,^{27,28,52} and recently Zhang et al.¹³ have found an association between the serum level of acrolein-derived FDP-lysine and the degree of diabetic retinopathy.

There have been several studies investigating substances that could mitigate the effects of acrolein-induced damage of cultured RPE cells. These substances include (R)- α lipoic acid,⁴⁷ hydroxytyrosol,⁴⁸ resveratrol,⁴⁹ and vitamin E.⁵⁰ All of these function as antioxidants and aid in mitochondrial protection. Hydroxylamines have been used in the amelioration of reactive aldehyde effects in neurodegeneration,⁵² damage to rat retinal precursor cells,⁵³ and damage to mouse mammary cells.⁵⁴ NBHA was successfully used in this study to prevent the toxic effects of acrolein on cultured ARPE-19 cells, and at 200 μ M, NBHA was able to prevent loss of ARPE-19 cell viability caused by acrolein. NBHA also blocked increased production of TGF β 1, TGF β 2, and VEGF.

Previously, we have shown that acrolein at normal glucose levels (5.5 mM) is associated with decreased cell viability in ARPE-19 cells, which is reversed by NBHA, or the TGF β pathway blocker SIS3. Furthermore, we found that acrolein increased levels of VEGF and TGF β 2 in the cell media of ARPE-19 cells, that the increase of TGF β 2 could be partially blocked by NBHA, and the increase in VEGF could be partially blocked by NBHA and the TGF β pathway blocker SIS3 supporting the hypothesis that acrolein exerts its effects through modulation of the TGF β pathway.²⁹ We now demonstrate that acrolein has a greater effect on reducing ARPE-19 cells in higher than in normal glucose levels and that this reduction in cell number can be modulated by co-administration of NBHA (sequestering the acrolein) which reduced the effects of the glucose concentration difference in cell viability.

This is most likely due to the enhanced oxidizing effect of the higher glucose levels and is not unexpected considering the increased mitochondrial potential associated with hyperglycemia as evidenced by the work of Du et al.⁵⁵ and Brownlee et al.⁵⁶ Acrolein disrupted TGF β and VEGF regulation by the remaining cells, causing each cell to contribute more of each cytokine to the CM, and this, too, could be mitigated by co-treatment with NBHA. In addition, we are the first to show that this enhanced high glucose/acrolein damage proceeds through TGF β pathways, and could be blocked equally by the SIS3 transduction inhibitor, or the TGF β 1 receptor I (ALK 5) blocker SB431542. Further investigation is needed to evaluate the potential effects of NBHA and TGF β pathway blockers on cell viability, TGF β and VEGF regulation. Nevertheless, the results of the present study suggest several potentially useful pharmacological interventions useful not only in the neutralization of reactive aldehydes associated with retinopathy, but also in the systemic neutralization of reactive aldehydes accompanying many degenerative disorders.

Particularly interesting were the results from the SIS3 transduction inhibitor and TGF β RI (ALK 5) blocker SB431542 studies. Both of these substances either directly or indirectly prevent phosphorylation of Smad3, part of the pathway stimulated by TGF β RI.^{33,35} Signaling by TGF β isoforms is initiated by an oligomeric receptor complex consisting of two types of transmembrane subunits that each possesses serine/threonine kinase activity. Binding of ligand to the activated type II receptor (T β RII) promotes complex formation with the type I receptor (T β RI/ALK 5). TGF β RII then trans-phosphorylates TGF β RI/ALK 5 in a juxtamembrane region rich in glycine and serine residues (G-S region), leading to activation of TGF β RI/ALK 5 and propagation of TGF β signaling by several signaling cascades. Signaling by TGF β through the Smad pathway has been extensively characterized and is considered the canonical pathway.^{33,35}

SB431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide) is a potent and selective inhibitor of ALK 4, 5, and 7, with selectivity for the kinase activity of ALK 5, and lesser selectivity for ALKs 4 and 7. It acts as a competitive ATP-binding site kinase inhibitor and has been shown to inhibit the *in vitro* phosphorylation of Smad3 and Smad2, but has no effect on Smad-independent pathways such as ERK, JNK, or p38 MAPK.⁵⁷ SIS3 (specific inhibitor of Smad3) is a cell-permeable pyrrolopyridine compound that selectively inhibits TGF β 1-dependent Smad3 phosphorylation and Smad3-mediated cellular signaling with no effect on Smad2, p38 MAPK, ERK, or PI3K signaling.³³ Smad3 is a receptor-activated molecule that, once phosphorylated by a member of the TGF β receptor I family, i.e., ALK 4, 5, or 7, combines with Smad4 and the R-Smad/Smad4 complex then undergoes nuclear translocation and stimulates gene expression.

Smad3 can be activated through ALK 4, 5, and 7, TGF β receptor I family members that can likewise be activated by activin, TGF β s and nodal. Thus, the use of these inhibitors, by giving virtually identical results has demonstrated not only that acrolein-induced cell loss and VEGF regulation proceeds not only through a TGF β RI-mediated pathway, but specifically through a TGF β RI receptor-mediated Smad3 pathway, negating the possibility of non-TGF β RI activated Smad3 activation which has been shown to occur in osteoblastic cells in response to parathyroid hormone.⁵⁸ ALK 7 has been shown to be specific for

adipose tissue,⁵⁹ so the mechanism for acrolein reduction of cell number is narrowed down to activation of ALK 5 and/or ALK 4, both activated by nodal, activin, and TGF β .

In RPE cells, TGF β 1 has been shown to inhibit proliferation and has been linked to apoptosis,⁶⁰ but most evidence points to the ability of TGF β to reduce proliferation of RPE cells.^{61–63} A recent study linked the induction of senescence in RPE cells to hydrogen-peroxide-induced subtoxic oxidative stress mediated by TGF β release, but, again, these were subtoxic amounts. The acrolein used in the study, the action apparently modulated by Smad3 was toxic to RPE.

Acrolein toxicity *in vivo* is a complex issue. Normally, acrolein is detoxified by its conjugation with glutathione (GSH) via glutathione S-transferase (GST),⁶⁴ and acrolein reacts 110–150 times faster with GSH than does the lipid peroxidation-derived reactive aldehyde 4-hydroxynonanal (HNE).¹ It is suggested that some individuals may be more susceptible to acrolein toxicity due to impaired glutathione activity, especially in smokers.⁶⁵ Reactive aldehydes like acrolein are also detoxified by aldehyde dehydrogenase (ALDH), a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids.⁶⁶ Both of these mechanisms have been observed in cultured RPE cells in response to lipid-derived aldehydes.⁶⁷ There is evidence, however, that reactive aldehydes can deactivate ALDH and lead to dopaminergic cell death as seen in Parkinson's disease.⁶⁸ Diabetes has also been shown to impair the enzymatic disposal of HNE by both GST and ALDH in rat liver,⁶⁹ and ALDH in rat retina has been shown to co-localize with lipid peroxidation products. Thus, it seems that acrolein defenses are present in the eye, but they can be disrupted by either the toxin they are meant to detoxify, or by genetic polymorphisms that render antioxidant defenses less than optimum.

Betts et al.⁷⁰ demonstrated an increase in cell viability with increasing levels of exogenous VEGF in the CM. However, Betts et al. also show a decrease in ARPE-19 viability with increasing levels of VEGF, when the ginsenoside-Rb-1 is added to the CM. In this current work, we also show a decrease in ARPE-19 viability, this time in response to acrolein in the treating media, even though VEGF levels increased.

The results of this study show that acrolein in 18 mM glucose exerts toxic effects on cultured APRE-19 cells that were even more pronounced than the effects in 5.5 mM glucose in terms of loss of viable cell number and increases in VEGF and TGFB's, suggesting that the increased glucose concentration exacerbated the effects of the acrolein. It was also shown that, as in 5.5 mM glucose, these effects could be prevented by the co-treatment of these cells with NBHA. Investigation into the signaling pathways involved in acrolein-mediated toxicity again implicated members of the TGFB signaling pathway-TBRI and Smad3, because by specifically blocking the actions of these proteins with SB431542 and SIS3, virtually all of the reduction in cell number and increase in cytokine level could be prevented. While NBHA is known to be an irritant to some human tissues, there is no available toxicological information on SB431542 or SIS3⁷¹. Their use in this work, does suggest a possible approach in the prevention, or treatment of eye pathologies, which are accompanied by high glucose.

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FIGURE 1.

High glucose-enhances acrolein effects on ARPE-19 cell viability and VEGF levels. Confluent ARPE-19 cells were treated with either 5.5 or 18 mM glucose in combination with either 0, 12.5, 25, or 50 μM acrolein for 72 hours. Viable cell numbers (A) or VEGF (B) was determined for each treatment group. (A) 18 mM glucose increased cell loss at the 25 and 50 μM level of acrolein compared to cells treated with 5.5 mM glucose and an equivalent acrolein level, $p < 0.05$, $p < 0.01$. Two-way ANOVA was significant for the synergistic effect of glucose and acrolein on the loss of cell viability, $p < 0.05$. (B) 18 mM

glucose increased the level of VEGF secreted into the conditioned media at each level of acrolein compared to cells treated with 5.5 mM glucose and an equivalent acrolein level. This reached significance at the 25 and 50 μ M acrolein levels, $p < 0.01$, $p < 0.001$. Two-way ANOVA was significant for the synergistic effect of glucose and acrolein on the increase of VEGF, $p < 0.0001$. Single asterisk indicates a significant difference between 5.5 and 18 mM glucose at the same acrolein concentration ($n = 3$).

FIGURE 2.

NBHA inhibits the effects of acrolein. Confluent ARPE-19 cells were treated with either 5.5 or 18 mM glucose in combination with either no acrolein, 50 μM acrolein, 200 μM NBHA, or a combination of 50 μM acrolein with either 50, 100, or 200 μM NBHA for 72 hours. Viable cell numbers were counted (A) and from the cell media the proteins VEGF (B), TGFβ2 (C), and TGFβ1 (D) were determined per 10,000 cells. Protein levels were divided by their corresponding cell count. (A) 200 μM of NBHA when used alone in 18 mM glucose decreased viable cell numbers by 25% compared to cell numbers from cells with no NBHA or acrolein treatment, $p < 0.05$. 50 μM acrolein used with 50 μM NBHA increased viable cell numbers by 73%, 100 μM NBHA increased by 103% and 200 μM NBHA by 186%, $p < 0.001$ compared to acrolein alone. A treatment effect was found by two-way ANOVA for the NBHA on acrolein-induced reduction in cell number, $p < 0.0001$. Results are average viable cell number $\pm$ SE, $n = 3$. (B) In combination with 50 μM acrolein in 18 mM glucose, 100 μM NBHA reduced VEGF by 44%, $p < 0.001$ and 200 μM NBHA reduced VEGF by 56%, $p < 0.001$, bringing it to within 4% of the nonacrolein, non-NBHA group. Results are the mean pg VEGF per 10,000 viable cells $\pm$ SE, $n = 3$. (C) Treatment with 50 μM acrolein and 18 mM glucose resulted in 230%, $p < 0.001$, more TGFβ1 in the condition media compared to the corresponding 18 mM glucose group without acrolein. Addition of 200 μM

NBHA to the treating media eliminated this effect. Two-way ANOVA found a significant correlation between the acrolein/NBHA group and glucose levels, $p < 0.0001$. Single asterisk indicates a significant difference within the same glucose level; “#” indicates a significant difference between glucose levels with the same treatment. Results are the mean pg TGF β 1 per 10,000 viable cells $\pm$ SE ($n = 3$). (D) In the 18 mM glucose groups, 50 μ M of acrolein resulted in a 75% increase, $p < 0.05$, of TGF β 2 levels per 10,000 cells which was reduced to control with a combination of 200 μ M NBHA to the treatment media. Asterisk refers to a significant difference between acrolein effect within its own glucose level. Results are the mean pg TGF β 2 per 10,000 viable cells $\pm$ SE ($n = 3$).

FIGURE 3.

SIS3 and SB431542 inhibits the effects of acrolein effects in high glucose. Treatment with the Smad3 inhibitor SIS3 or the TGF β 1 receptor blocker SB431542 was performed on confluent ARPE-19 cells before they were treated with acrolein in both low (5.5 mM glucose), or high (18 mM glucose) for 72 hours. (A) Pretreatment with SB431542 and SIS3 prevented acrolein-induced loss of cell viability in both 5.5 and 18 mM glucose. These blockers were equally effective in preventing 50 μ M acrolein-induced viable cell loss and resulted in treated cell numbers equivalent to those without acrolein treatment in both

glucose groups. Results are average viable cell number $\pm$ SE ($n=3$). (B) Pretreatment with SB431542 and SIS3 prevent acrolein-induced VEGF increases per 10,000 cells in the 5.5 and 18 mM glucose/50 μ M acrolein treatment groups. These blockers were equally effective in preventing VEGF increase and resulted in VEGF per 10,000 cells found in the condition media equivalent to that without acrolein treatment in both glucose groups. ELISA for VEGF was performed on the conditioned media from each well, and the results were divided by the corresponding viable cell counts. Results are the mean pg VEGF per 10,000 viable cells $\pm$ SE ($n=3$).