• Basic Research •

Rescue of human corneal epithelial cells after alkaline insult using renalase derived peptide, RP-220

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Abstract

• AIM: To study the effect of renalase peptide, RP-220, on cell viability of human corneal epithelial cells after alkali insult.

• METHODS: A dose-response relationship between cell viability and exposure to NaOH solution were characterized using cultured human corneal epithelial cells. Viability of corneal epithelial cells was determined using commercially available MTT and CyQUANT® assays.

• RESULTS: At a concentration of 6 mmol/L, insult with NaOH leads to reduced corneal epithelial cell viability by approximately 30%. This reduced viability was prevented by treating the cells after initial insult with the 20-amino acid renalase derived peptide (RP-220).

• CONCLUSION: RP-220 has a pro-survival role for RP-220 following alkaline insult to corneal epithelial cells.

• KEYWORDS: corneal alkali injury; renalase; RP-220; human corneal epithelial cells

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INTRODUCTION

Chemical exposure is a significant cause for ophthalmic morbidity[1]. Injury can occur by certain household or occupational agents in an accidental or intentional manner[1]. Military personnel are also at risk, and the use of chemical agents by terrorist groups presents a unique challenge in the modern era[2-4]. Of the possible exposures, alkaline agents are known to be particularly deleterious[1,5]. Notably, epidemiologic studies conducted using data from US emergency room visits have shown children from 1-2 years of age are at highest risk for ocular injury from an alkaline agent, even higher than men ages 18-64, which is another population at particularly high risk[1]. In contrast to acidic agents, which cause protein denaturation leading to slowing of further tissue penetration, alkaline agents cause tissue melting, further enhancing tissue penetration[6]. Notably, the most significant injury to the eye has been shown for substances with pH 11-11.5[6].

Corneal injury represents one of the most significant sources for immediate and delayed ocular morbidity from alkaline agent exposure. There is a wide variability in the literature on prognosis after alkaline injury, also revealing a direct correlation between the severity of initial exposure and prognosis[7-9]. Despite current treatment efforts, individuals presenting with more severe injury typically have a protracted treatment course that may or may not result in useful vision[7]. The downstream events triggered by the initial injury may be angiogenic, fibrotic, or inflammatory in nature, each with potentially devastating consequences for vision and quality of life[10-12].

Our aim in the current study was to investigate the potential for corneal epithelial cell rescue after alkaline insult, using the renalase peptide (RP-220)[13]. Renalase is an amine oxidase secreted from the kidney into the blood[14-15]. There are two prominent isoforms of the flavoprotein oxidase renalase expressed in humans[16]. Despite early evidence suggesting a physiological role in catecholamine metabolism, subsequent studies have refuted this claim[17-19]. Rather, renalase has been found capable of acting as a signaling molecule activating mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2), thereby exerting cytoprotective effects[13]. Plasma membrane calcium ATPase isoform 4b (PMCA4b, encoded by ATP2B4 gene), has been identified as a renalase receptor upstream of MAPK activation[17,19].

RP-220, the 20-amino acid peptide derived from full length renalase sequence, is equally as effective as full-length recombinant renalase in its cytoprotective properties. RP-220, specifically, has been demonstrated to rapidly activate ERK1/2 and p38 MAPK in a human kidney epithelial cell line, suggesting that RP-220 is the critical region of the renalase protein for interaction with PMCA4b receptors[17,19]. A study involving human corneal epithelium showed that PMCA4 is the most prominent PMCA isoform and that splice variant PMCA4b is present in the human cornea[20], confirming the existence of an RP-220-specific receptor in the human cornea. The ability of RP-220 to stimulate an increase in ERK1/2 and p38 MAPK activity would likely protect cells against apoptosis[21] and ultimately increase cell survival, therefore improving clinical outcomes. Furthermore, activation of MAPK and ERK1/2 is expected to encourage epithelial cell migration and proliferation[22], aiding in the wound healing process.

In vivo, renalase peptide reduced ischemic or cisplatin-induced kidney injury and *in vitro*, protected human proximal tubular (HK-2) cells from H_2O_2 or cisplatin-induced apoptosis[13]. The HK-2 cell line has also demonstrated its ability to upregulate renalase expression upon hypoxic insult, *via* hypoxia-inducible factor-1α (HIF-1α)[23]. Renalase expression has also been shown to be controlled by HIF-1α in human cardiomyocytes and implicated in that context as protective against cardiac ischemia[24]. In rats, contrast-induced histological damage to the kidney, as well as apoptosis and inflammation, were all reduced by treatment with intraperitoneal recombinant renalase[25]. Renalase has also been shown to reduce tubulointerstitial fibrosis in a rat model, and it has been suggested that this is due to its ability to interfere with the TGF-β-induced epithelial-mesenchymal transition (EMT)[26]. We have previously shown a marked increase in TGF-β1 expression following alkaline injury in the rat[27], and TGF-β expression is closely linked to corneal wound healing[28]. Taken together, the effects of renalase in various injury models suggest that it may be beneficial in the context of alkaline injury to the cornea.

MATERIALS AND METHODS

Cell Culture Normal primary human corneal epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas VA). Cells were thawed and plated on 100 mm Biocoat culture plates coated with Collagen 1 (Corning Incorporated, Kennebunk ME) and grown to 70% confluence in corneal epithelial cell basal media supplemented with Corneal Epithelial Cell Growth Kit (ATCC). They were then lifted using 0.25% Trypsin-EDTA (Life Technologies Corporation, Grand Island NY) and Trypsin neutralizing buffer (Life Technologies Corporation). Viable cells were counted using 0.4% Trypan Blue Stain (Life Technologies Corporation) and a hemocytometer (VWR, Brooklyn NY). Cells were subsequently plated at a density of 5×10^5 cells/mm^2 in a 96-well plate (CellTreat Scientific Products, Pepperell MA) and grown to 70% confluence for use in multi-well plate assays. Microplate well volumes were 100 μL throughout all experiments and incubation times.

pH of NaOH Dilutions An Accumet AE150 pH meter (Thermo Fisher Scientific, Waltham MA) was used to measure the pH of serial dilutions of a 10 mol/L NaOH stock solution (Sigma Aldrich, St. Louis MO) with sterile water (Life Technologies Corporation) or 0.9% NaCl solution (Saline solution; Braun, Irvine CA, USA).

Alkaline Insult to Corneal Epithelial Cells A stock 50 mmol/L solution of NaOH was prepared from 10 mol/L NaOH stock (Sigma) solution by dilution with saline solution (Braun), then filtered through a 0.22 μm filter (Merck Millipore, Cork Ireland). To each well of a 96-well plate (CellTreat Scientific) containing cultured cells (passage 3) and 100 μL of corneal epithelial cell media (ATCC), 100 μL of the 50 mmol/L NaOH solution were added and mixed gently using a multichannel pipette. Then, 100 μL were withdrawn and placed in the next column of wells containing cells and media. This process was continued across the plate such that there was treatment of cells with NaOH concentrations of 25.0, 12.5, 6.2, 3.1, 1.6, 0.78, 0.39, and 0.20 mmol/L. Incubation of cells with each concentration of NaOH was for 1min, with 4 replicates of each and after insult, cells were rinsed twice with 100 μL each of saline solution (Braun). Cells were then incubated with 100 μL of corneal epithelial cell media (ATCC) for 24h at 37°C in a CO_2 incubator (Thermo Fisher Scientific). MTT assay was performed after 24h using the Vybrant™ MTT Cell Proliferation Assay (Thermo Fisher Scientific), measured using a Varioskan LUX spectrophotometer (Thermo Fisher Scientific) with absorbance at 540 nm.

Effects of RP-220 and Scrambled Peptide on Corneal Epithelial Cells The RP-220 and scrambled peptide (RP-Scr220) was custom-made according to Wang *et al*[17] by Selleck Chemicals (Houston TX) and dissolved in dimethyl sulfoxide (DMSO; Sigma) to yield 10 mg/mL stock solutions. The 100 μg/mL stock solutions of RP-220 and RP-Scr220 were then prepared by diluting the stock with corneal epithelial media (ATCC). These stock solutions were then used to treat cultured corneal epithelial cells on a black-walled 96-well plate (Falcon, Big Flats NY) with serial dilutions of RP-220 and RP-Scr220 at concentrations of 50, 25, 12.5 and 6.25 μg/mL. Cells treated with corneal epithelial media only served as control. All treatments were performed with 6 replicates. Cells were incubated with these peptides for 72h, then the plate was stored at -80°C. CyQUANT® Cell Proliferation Assay Kit (Invitrogen

Molecular Probes, Eugene OR) was then used according to the manufacturer's instructions and fluorescence at the excitation/emission wavelengths of 480/520 nm measured using a Varioskan LUX spectrophotometer (Thermo Fisher Scientific). The fluorescence measurements obtained for cells treated with each concentration of peptide were normalized to those of the control cells treated with corneal epithelial media only.

Rescue of Corneal Epithelial Cells after Alkaline Insult For rescue assays involving RP-220, a 6 mmol/L solution of NaOH was prepared from a stock 10 mol/L NaOH (Sigma) solution, filtered through 0.22 μm filter (Merck Millipore, Cork Ireland). Cells on a 96-well plate (CellTreat Scientific Products) were treated with the 6 mmol/L NaOH solution for 1min to provide alkaline insult, followed by two rinses with 100 μL each of saline solution (Braun). Cells were then treated with 10 or 20 μg/mL RP-220 or RP-Scr220 solution, or with corneal epithelial media (ATCC) alone. A set of cells treated only with saline solution (Braun) rather than NaOH, served as control. The cells were incubated with these solutions for 24h, then MTT assay was performed using the Vybrant™ MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific), measured using a Varioskan LUX spectrophotometer (Thermo Fisher Scientific) with absorbance at 540 nm. Each treatment was with 4 replicates.

Statistical Analysis The data analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using a one-way ANOVA with post-hoc Tukey testing. Differences were considered significant if $P<0.05$. Data are presented as mean±standard error of the mean.

RESULTS

Human corneal epithelial cells were treated with NaOH solution to assess the impact of alkaline insult. The pH of the treatment solution was assessed prior to alkali insult. NaOH solution was diluted with either sterile water or sterile saline solution in various concentrations, and their pH were measured. As shown in Figure 1, the initial pH of saline and sterile water was 4.64 and 9.12, respectfully. As the graph shows in Figure 1, though there was difference in initial pH, no significant pH difference between NaOH dilutions with either sterile water or sterile saline solution was observed. Therefore, NaOH solution was diluted with saline to minimize osmotic pressure. The concentration of NaOH solution used for corneal epithelial cell insult in these experiments was 6 mmol/L, with pH of around 11.5, as 6 mmol/L was the minimum concentration of NaOH that reach the maximum pH. There is no significant difference of pH obtained with 6.2, 12.5 and 25 mmol/L. The pH of the treatment solution was suitable to assess alkali injury as studies show, the most significant eye injury was observed with pH range 11 to 11.5[6].

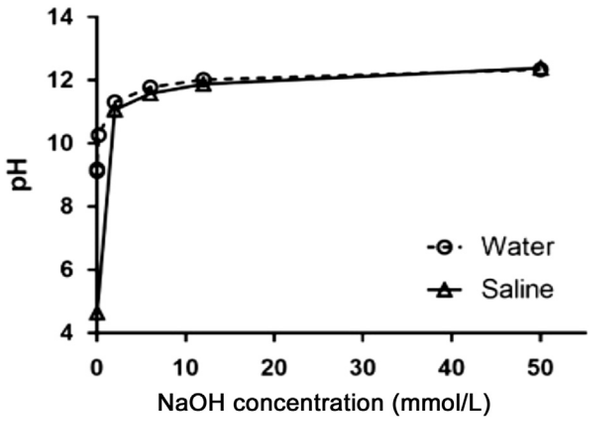

Figure 1 pH change of NaOH solutions with either sterile water or sterile saline solution (0.9% NaCl) Dashed line shows pH of NaOH dilutions with sterile water, and solid line shows pH of NaOH dilutions with sterile saline.

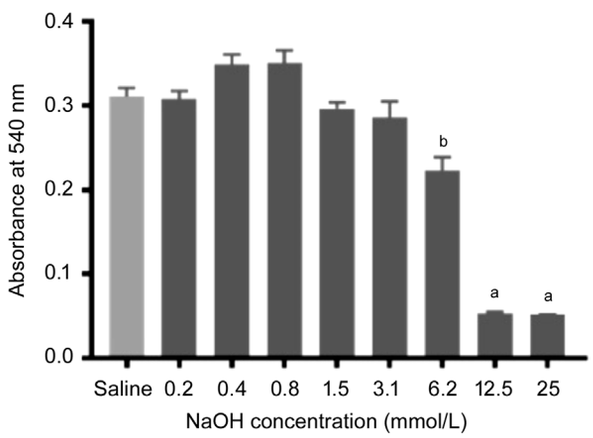

Figure 2 Human corneal epithelial cell viability after NaOH insult Plot shows the MTT assay results denoted with average absorbance values at 540 nm after treatment with various NaOH concentrations. Error bars indicate standard error of the mean of each group (n=4). [a]$P<0.0001$, [b]$P<0.0005$ on Tukey's multiple comparisons test compared with saline control.

To assess the alkali insult, human corneal epithelial cells were treated with various NaOH concentrations (0.2 through 25 mmol/L) for 1min. Cell viability after NaOH insult was observed using MTT assay at 24h after the initial NaOH exposure. As shown in Figure 2, the average absorbance values at 540 nm after treatment was decreased in a dose-dependent manner. While no significant difference in MTT reading from cells treated with 0.2 to 3.1 mmol/L NaOH was observed, compared to saline control, cell viability was reduced significantly with 6.2, 12.5, and 25 mmol/L NaOH insult, approximately 85%, 84%, and 33% of saline control, respectively. This result indicates that NaOH solution can serve as an alkali insult simulation and 6 mmol/L NaOH is appropriate concentration to examine the effect of renalase peptide.

CyQUANT® assay was used to compare the numbers of cells present after 72h incubation with varying concentrations

of either RP-220 or RP-Scr220. These data are presented in Figure 3. Controls are incubated with corneal epithelial cell media only. Cell proliferation induced by RP-220 or scrambled peptide was not observed. The proliferation of cells was significantly reduced in the presence of 50 μg/mL scrambled peptide. Though it is not significant, the cell viability also reduced on treating cells with 50 μg/mL RP-220. This indicates that higher concentration of RP-220 or RP-Scr220 can bring adverse effect to cells. Therefore, based on the CyQUANT® assay, the peptide concentration for further experiments was limited to 10 μg/mL and 20 μg/mL.

To examine whether the renalse peptide rescue the corneal epithelial cells after the alkali insult as simulation of eye injury, the cell viability was observed using MTT assay. Cells were treated with either scrambled or RP-220 following the NaOH insult for 1min, and cell viability was measured after 24h of treatment. Control cells treated with saline solution. As shown in Figure 4, NaOH insult reduced cell viability and following treatment with 10 μg/mL RP-220 significantly rescued cell viability compared to NaOH insult only cells or scrambled peptide treated cells. The scrambled peptides showed no effects on the cell viability after the insult. Higher concentration RP-220 (20 μg/mL) also rescued the cells but it was not significant. This assay indicates that 10 μg/mL RP-220 is suitable to rescue the cells against alkaline insult.

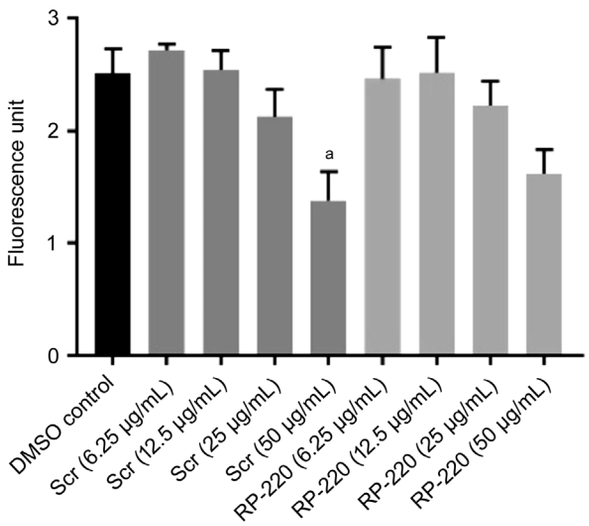

Figure 3 Human corneal epithelial cell proliferation after treatment with various concentrations of RP-220 or RP-Scr220 Plot shows CyQuant® assay results denoted with the average fluorescence values after treatment with peptide or control, corneal epithelial media. Error bars indicate standard error of the mean of each group (n=6). [a]P<0.0005 on Tukey's multiple comparisons test compared with control.

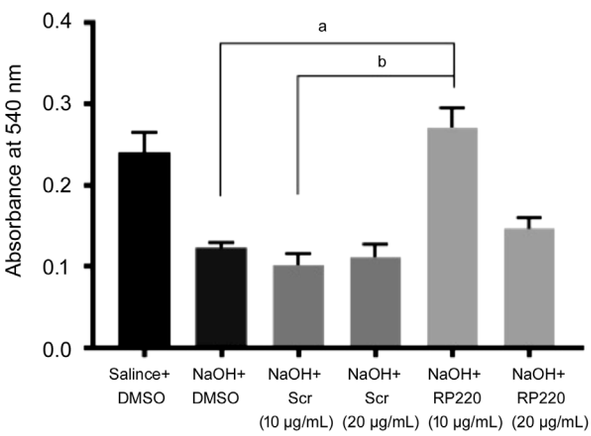

Figure 4 Human corneal epithelial cell viability after insult with 6 mmol/L NaOH, or 6 mmol/L NaOH followed by RP-220 or scrambled peptide for rescue Plot shows the MTT assay results denoted with average absorbance values at 540 nm after treatment. Error bars indicate standard error of the mean of each group (n=4). [a]P<0.0001, [b]P<0.0005 on Tukey's multiple comparisons test compared between groups.

DISCUSSION

In this study, we have demonstrated a pro-survival role for the renalase derived peptide (RP-220) following insult of cultured human corneal epithelial cells with NaOH solution. The alkaline nature of the insult was demonstrated by testing pH of various dilutions of NaOH with sterile water or saline solution, and a reduction in corneal epithelial cell viability shown. We have previously characterized an alkaline injury model in the rat using 1 mol/L NaOH which demonstrated that corneal epithelial injury is a key early pathologic event after alkaline injury and finding ways to alleviate this insult is thus important for limiting the overall damage that results with time after alkaline injury[27].

In the studies outlined herein, we used commercially available human corneal epithelial cells at passage 3. The cells were grown to 70% confluence prior to use, which is slightly lower than that used in some other studies of corneal epithelial cell cultures[29-30]. It has been shown that corneal epithelial cells express more proliferation-type genes at this lower confluence compared to higher confluence[31]. This is relevant in the context of studying alkali injury since cells are in a more proliferative phase during wound healing than they may be at other times. Of note, at later passages we did observe significant phenotypic changes in the cells, taking on a more fibroblast-like appearance. Hence, care was taken to examine culture plates prior to use for the experiments in this study to ensure that the predominant morphology of the cultured cells was epithelial. In future studies, it will be important to further characterize the genotypic expression profile of these cells at different passages and confluences.

Using the commercially available MTT assay, we identified concentrations of NaOH that resulted in reduced numbers of viable corneal epithelial cells after 1min of insult. MTT assay

reading showed slight increase, but not significantly different, in 0.2 and 0.4 mmol/L than in saline control (Figure 2). This can be partially explained as MTT reading increases in higher pH according to Plumb *et al*[32] 1989. The higher pH can decrease the cell number, but the part of the effects can be counteracted by pH dependent MTT absorption increase. There were no statistically significant differences among 0 to 3.1 mmol/L NaOH. Although there was a significant decrease in viability with use of 12.5 and 25 mmol/L NaOH, versus 6.2 mmol/L NaOH, the pH values do not differ much between these solutions (Figure 1). The damage to the cells using 12.5 mmol/L and above was too severe to the cells. During the cornea chemical damage, the cells with high degree of damage cannot be recovered as many cells are subject to immediate cell death. This study was designed to evaluate an alkaline insult that led to reduced viability of cells, yet feasible to potentially prevent or reverse. Accordingly, we choose 6 mmol/L NaOH as that is the minimum concentration showed significant difference with no insult control. It will be informative to repeat the rescue studies using a higher concentration of NaOH to see whether a rescue effect is still observed. In our *in vivo* model of alkaline injury, we have observed severe loss of epithelial cells after 30s treatment with 1 mol/L NaOH[27]. At this much higher concentration, cell-cell contact is disrupted and mechanical sloughing of cells from the surface occurs. This likely occurs in our *in vitro* studies as well, during rinses and various solution changes. The use of control wells where cells were exposed to identical numbers of solution changes was important to account for the loss of cells simply from loss of adhesion and subsequent removal from pipette activity.

Notably, the MTT tetrazolium dye-based assay is dependent upon metabolic activity of cells[33]. Although we have attempted to control for this in our experiments, it is possible that the assay is affected in some way independent of the viability of the cells. One example of the way in which results could be impacted is the variation of metabolic activity (and thus potentially assay results) with cell density[34]. To fully understand the data, it will be important to look at viability with the MTT assay with varying corneal epithelial cell densities. This is an important limitation of the results and it will be useful to further validate the NaOH insult dose-response results using an assay that is independent of cellular metabolism. These limitations also apply to the data presented in Figure 4, which were also obtained using the MTT assay.

Cell proliferation was not affected by incubation with varying concentrations of RP-220 or control scrambled peptide, as assayed by CyQUANT®, except at the highest concentration (50 μg/mL) of the scrambled peptide. Insignificant trends toward reduced viability were seen with 50 μg/mL RP-220 and with 25 μg/mL RP-220 and scrambled peptide, as illustrated in Figure 3. These findings may be related to the concentration of DMSO present as a solvent for these peptides, which would be 0.5% (remainder corneal epithelial media) at the highest peptide concentration. The difference between the 50 μg/mL viabilities for RP-220 versus scrambled peptide may be related to potential proliferation-promoting effect of the RP-220 on corneal epithelial cells. It seems that at the concentration of peptide used for the experiments, there are no adverse effects on the cells from either solvent or peptide. It will be important in future studies to ascertain what effect lower concentrations of peptide, as well as solvent controls, have on corneal epithelial cell proliferation with time. Notably, renalase has been shown to increase proliferation of melanoma cells[35], but whether this also occurs in corneal epithelial cells is unknown.

In this work, we have shown that RP-220 at a concentration of 10 μg/mL has a pro-survival effect on corneal epithelial cells after alkaline insult. Interestingly, this effect is absent at the next highest concentration of 20 μg/mL. This may be due to DMSO solvent effect, although the data presented in Figure 3 would suggest that this effect is negligible. These data would also suggest minimal to no adverse effect from the peptide itself. Studies in HK-2 cells have previously shown anti-apoptotic effects of renalase at concentrations as low as 10 μg/mL[13]. Although signaling mechanism was not analyzed in this work, renalase has been proposed to promote cell survival through activation of STAT3, ERK, p38 and AKT downstream activation[19]. Epithelial wound healing has been shown to be dependent upon p38 and ERK1/2 activation in rabbit and human corneal epithelial cells[22]. This was due to a combination of increased proliferation and migration[22].

As noted previously, we have shown that there is significant upregulation of TGF-β1 in a rat model of NaOH corneal injury[27]. Additionally, collagen 1a1 (Col1a1) and smooth muscle actin (SMA) expression were found to increase at various time points following the initial injury in our model[27]. Work in the HK-2 renal tubular epithelial cell line has demonstrated a dose-dependent reduction in TGF-β1-induced Col1 and SMA expression with renalase *via* decreased ERK1/2 signaling[26]. GF-β1 is known to be expressed in epithelial cells, and this is likely an upstream initiator of corneal fibrosis and thus scarring and opacification[28]. Hence it is possible that *in vivo*, renalase could protect against some of the downstream fibrotic response to alkali injury, thus preventing some degree of visual morbidity. Future studies will further elucidate what effect RP-220 has on corneal epithelial cell gene expression *in vitro*, as well as the effects of the peptide in the context of *in vivo* alkali injury.

RP-220 signaling is initiated *via* PMCA4b as a receptor[17]. Importantly, this isoform is expressed in human corneal epithelial cells[20], and ostensibly this is an early event in pro-survival signaling in these cells leading to the findings in this study. Further work is needed to confirm the expression of this isoform under the culture conditions used in these experiments, and to confirm its role in the protective effect. We will also be examining the expression of PMCA isoforms in the rat given the use of this animal model for *in vivo* studies of renalase. The findings in this study provide a foundation for several new lines of experimental questions relating to the renalase peptide and its potential as a therapeutic in the context of alkali injury.

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