Increased Glutathione Synthesis through an ARE-Nrf2–Dependent Pathway by Zinc in the RPE: Implication for Protection against Oxidative Stress

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Purpose. To determine the molecular mechanisms underlying the protective effects of zinc against oxidative stress in cultured retinal pigment epithelial (RPE) cells.

Methods. Cultured ARPE-19 cells were treated with different concentrations of zinc for various times. Cellular glutathione (GSH) and glutathione disulfide (GSSG) levels were measured by high-performance liquid chromatography (HPLC). Glutamate-cysteine ligase (GCL) expression was measured by quantitative reverse transcription-PCR (RT-PCR). Nuclear factor erythroid2-related factor (Nrf2) activity was measured in a dual luciferase assay after transfection of reporter plasmids containing the antioxidant response element (ARE). The small interference (si)RNA approach was used to knock down the expression of Nrf2.

Results. Zinc significantly increased GSH levels in ARPE-19 cells through induction of the de novo synthesis pathway. At 150 μM, zinc increased the GSH level by 70%. At similar concentrations, zinc upregulated the mRNA level of GCL and activated the ARE-Nrf2 pathway. The effects of zinc on ARE activation and GSH synthesis were inhibited by knockdown of Nrf2 expression using the siRNA approach.

Conclusions. Induction of the ARE-Nrf2 pathway by zinc provides powerful and prolonged antioxidation and detoxification that may explain the beneficial effects of zinc observed in the treatment of age-related macular degeneration (AMD). (*Invest Ophthalmol Vis Sci.* 2006;47:2709–2715) DOI:10.1167/iovs.05-1322

The Age-Related Eye Disease Study (AREDS) demonstrated that high-dose oral zinc supplementation, alone or with antioxidants, significantly reduced the risk of progression from intermediate to advanced age-related macular degeneration (AMD).[1] A prior clinical trial enrolling a smaller number of patients over a shorter period showed that oral zinc supplementation resulted in significant reduction in the risk of visual acuity loss in patients with AMD.[2] Of note, zinc and zinc with antioxidant supplementation had much greater efficacy than intake of the three antioxidants (β-carotene, vitamin C, and vitamin E) used in AREDS. This finding is consistent with a subsequent study that showed that vitamin E (α-tocopherol) did not significantly decrease the risk of the development or progression of AMD.[3] These studies provide support for a unique protective function of zinc in AMD pathogenesis that may involve mechanisms other than those related to direct antioxidant defenses.

Oxidative stress is thought to play a key role in AMD pathogenesis.[4–8] It has been proposed that zinc may have many direct and indirect antioxidant functions.[9,10] Acutely, zinc can stabilize sulfhydryls (SH) in proteins by either binding directly to these groups, binding to adjacent sites and creating steric hindrance to the sulfhydryls, or binding to distant sites and causing a conformational change in the protein's tertiary structure that results in the sequestration of the sulfhydryls. Zinc can also antagonize redox-active transition metal catalysts, such as copper and iron, in the Fenton pathway, thereby inhibiting the production of reactive oxygen species (ROS).[11] Chronically, zinc can induce enzymes that act as ultimate antioxidants, such as catalase[12] and the metallothioneins (MTs).[13,14] Studies of zinc deficiency in animals showed various physiological changes consistent with increased oxidative damage.[15–18]

Prestera et al.[19] reported that zinc may also induce the expression of the phase II enzymes by activating the antioxidant response element (ARE) in HepG2 and Hepa 1c1c7 cells. To our knowledge, no study has been undertaken to look into the ability of zinc to induce the phase-II genes in other cell lines, such as the retinal pigment epithelium (RPE), believed to be a critical site of oxidative damage in AMD.[20] The phase-II genes encode for a battery of enzymes that are essential in the antioxidation and detoxification of xenobiotics and endogenous reactive electrophilic compounds.[21] One such enzyme is glutamate-cysteine ligase (GCL), which is the rate-limiting enzyme in the de novo synthesis of GSH,[22] the principal nonprotein thiol responsible for maintaining intracellular redox homeostasis.[23] Transcriptional control of the phase II genes is achieved by the binding of Nrf2 (nuclear factor erythroid2-related factor) to the consensus ARE sequence in the promoter regions.[24] The activation of this pathway may provide powerful and prolonged downstream antioxidant effects.[25]

In the present study, we explored the ability of zinc to activate the ARE-Nrf2 pathway in ARPE-19 cells, an immortalized human RPE cell line. At concentrations previously shown to induce protein expression without toxicity,[12] zinc significantly increased the GSH level in ARPE-19 cells by activating the de novo GSH synthesis pathway. We demonstrated that decreased expression of Nrf2 by siRNA abolishes zinc-induced upregulation of ARE activity and increase in GSH synthesis. These results indicate that dietary and pharmacological intervention of the ARE-Nrf2 pathway provides a potential approach for the treatment and prevention of AMD.

Materials and Methods

γ-Glutamylglutamate (γGG), oltipraz, sulforaphane, and $ZnSO_4$ were purchased from MP Biomedicals (Irvine, CA), Rhône-Poulenc Rörer

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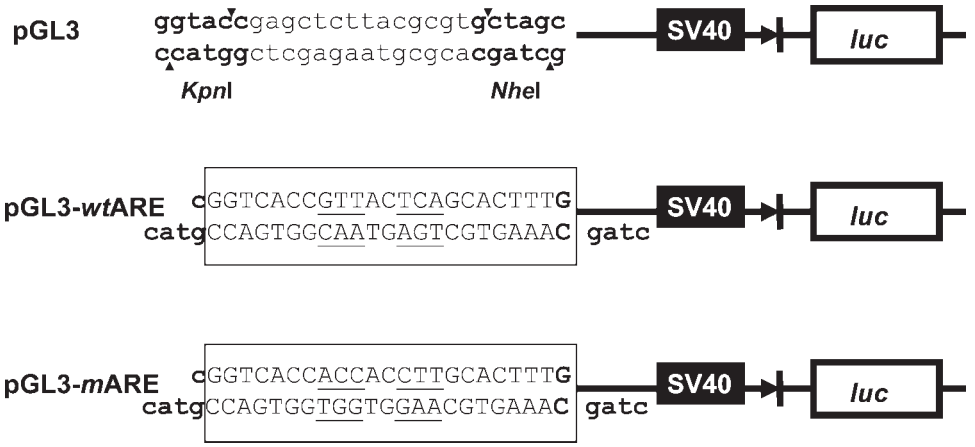

FIGURE 1. Schematic illustration of the ARE reporter constructs. The pGL3-promoter vector consists of the firefly luciferase (*luc*) gene driven by the SV40 minimum promoter downstream from the *Kpn*I and *Nhe*I restriction sites (*bold*), which were used to subclone the ARE transcriptional enhancer sequence. The pGL3-wtARE and the pGL3-mARE constructs contained the wtARE and a mutant ARE (mARE) sequence (*boxed*), respectively. The mARE sequence is a modification of the wtARE sequence (*underscored*).

(Lyons, France), LKT Laboratories, Inc. (St. Paul, MN), and Aldrich (Milwaukee, WI), respectively. Acetone, boric acid, buthionine sulfoximine (BSO), chloroform, dansyl chloride, iodoacetic acid, isopropanol, perchloric acid, KOH, NaOH, potassium tetraborate, $ZnCl_2$, and zinc acetate were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Experimental Conditions

The original stock of ARPE-19 cells from the American Type Culture Collection (ATCC; Manassas, VA) was expanded and stored at −80°C. Each experiment was performed with cells recovered from this stock during a 2-week period. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 50/50 mix; Mediatech; Herndon, VA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Sigma-Aldrich) and grown at 37°C in 95% air and 5% CO_2. Culture medium with or without 10% FBS contained 17.5 or 15.8 mM D-glucose, respectively. Cells were split 1:5 with fresh medium into T75 flasks every third day when they had reached over 90% confluence. For HPLC and quantitative RT-PCR experiments, cells were seeded at 2.5×10^5 cells per 60-mm plate and, when they reached confluence, culture medium was replaced with fresh medium containing different concentrations of zinc. For the dual luciferase reporter assay and Nrf2 siRNA knockdown experiments, cells were seeded at 1.5×10^5 cells per well in six-well plates 24 hours before transfection. For the dual luciferase reporter assay, cells were transfected 6 hours before zinc treatment and for siRNA knockdown experiments they were transfected with Nrf2 siRNA 24 hours before treatment. Each experiment was performed three times in duplicate, with fresh cell preparation.

GSH and GSSG Assay with HPLC

GSH and glutathione disulfide (GSSG) concentrations were measured with HPLC as previously described.[26] After 24 hours of zinc treatment, ARPE-19 cells were washed once with ice-cold phosphate-buffered saline (PBS) and precipitated in 500 μL perchloric acid solution (5% vol/vol) containing 0.2 M boric acid/10 μM γGG (internal standard). After centrifugation at 12,000*g* for 5 minutes, the pellet was lysed in 100 μL of 1N NaOH for protein concentration measurement in a protein assay (Bio-Rad, Hercules, CA), whereas 300 μL of the supernatant (acid soluble extracts) was derivatized first with 60 μL of 40 mM iodoacetic acid and then, after adjustment of the pH to 9.0 ± 0.2 with KOH/potassium tetraborate, with 300 μL dansyl chloride in acetone (20 mg/mL). Samples were then stored at room temperature in the dark for 24 hours. Excess dansyl chloride was extracted by adding 500 μL chloroform. Thiol metabolites in the samples were analyzed with an HPLC system (model 2695 and YMC Pack NH2 Amino column; Waters, Milford, MA). The GSH, GSSG, and γGG peak areas were recorded. The GSH and GSSG concentrations were determined from the γGG measured peak areas and known concentrations. The GSH redox potential, E_h, was calculated using the Nernst equation:

$$E_b = E_o + \frac{RT}{nF} \ln \frac{[GSSG]}{[GSH]^2}$$

where R is the gas constant, T is the absolute temperature, n is the number of electrons transferred (2), and F is Faraday's constant. The standard potential, E_o, for the GSH/GSSG couple at pH 7.4 is −264 mV.

GCL Expression with Quantitative RT-PCR

To determine the time point at which GCL expression peaked in ARPE-19 cells after zinc treatment, we first performed a time course experiment with 100 μM zinc. We then used this time point to study the dose-dependent response of these cells to zinc. Total RNA was isolated (TRIzol Reagent; Invitrogen, Carlsbad, CA), and any trace contaminants of genomic DNA were removed (Turbo DNA Free kit; Ambion, Austin, TX). cDNA was synthesized from total RNA, by using random hexamer primers. One microgram of total RNA was denatured at 72°C for 2 minutes and annealed with 0.5 μg (20 pmol) of random hexamer primer (Applied Biosystems, Inc. [ABI], Foster City, CA). For each reaction, an aliquot from a master mix containing 200 units MMLV reverse transcriptase (RTase), reaction buffer (50 mM Tris-HCl [pH 8.3]; 75 mM KCl; 3 mM $MgCl_2$), 0.5 units recombinant RNase inhibitor, and 0.5 mM of each dNTP (Promega, Madison, WI) was added to the RNA sample already annealed with the random hexamer primer. Complimentary DNA was synthesized at 42°C for 60 minutes and MMLV reverse transcriptase was inactivated at 94°C for 4 minutes. For quantitative RT-PCR, cDNA was added to the master mix (*Taq*Man Universal PCR Mastermix; ABI), and primers for the GCL catalytic subunit (Hs00155249_m1), GCL modulatory subunit (Hs00157694_m1), or 18S rRNA (4308329) provided with the gene expression assay (ABI). Quantitative RT-PCR was performed (model 7300; ABI) with initial denaturation at 50°C for 2 minutes and at 95°C for 10 minutes followed by 40 extension cycles at 95°C for 15 minutes and 60°C for 1 minute. Average threshold cycle (C_t) values were used to determine the relative difference between control and treated groups and were normalized to 18S rRNA.[27] Expression of the catalytic and modulatory subunits were calculated as a ratio of experimental (with zinc) to control (without zinc) and presented as *x*-fold multiples of the control.

Plasmid Constructs

The pGL3-promoter plasmid (Promega) contains a firefly luciferase (*luc*) gene driven by a simian virus 40 (SV40) minimum promoter with upstream *Kpn*I and *Nhe*I restriction sites (Fig. 1). This vector was inserted with either the wild-type (wt)ARE or a mutant (m)ARE sequence as previously described.[28] Oligonucleotides of wtARE (top strand: 5′-cGG TCA CCG TTA CTC AGC ACT TTG-3′; bottom strand: 5′-cta gCA AAG TGC TGA GTA ACG GTG ACC ggt ac-3′) and mARE (top strand: 5′-cGG TCA CCA CCA CCT TGC ACT TTG-3′; bottom

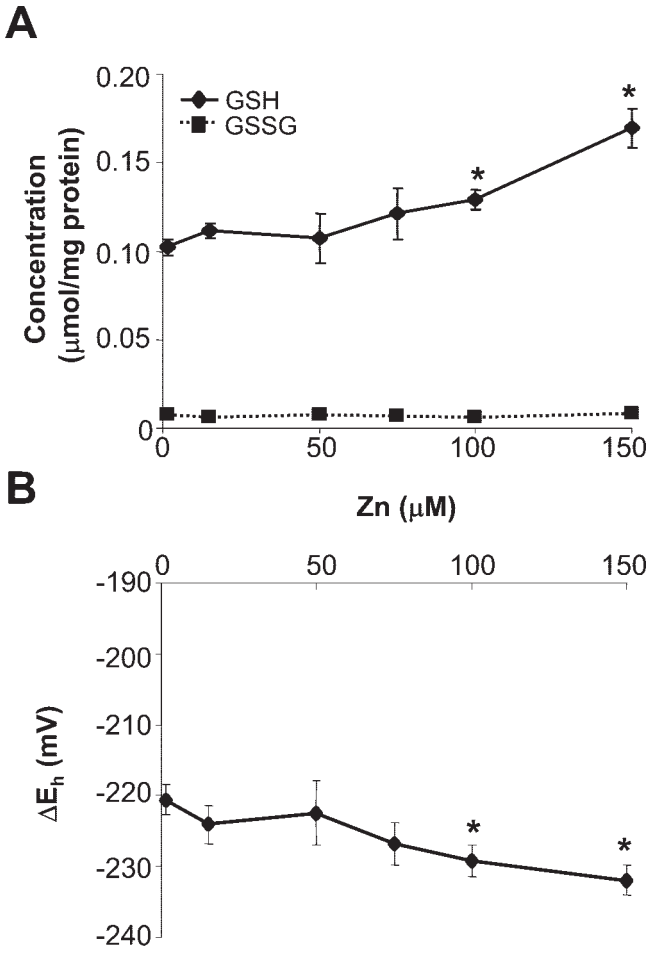

FIGURE 2. Zinc treatment increased the intracellular GSH level in ARPE-19 cells. ARPE-19 cells were treated with $ZnSO_4$ at the indicated concentrations for 24 hours. The intracellular concentrations of glutathione (GSH) and glutathione disulfide (GSSG) were measured by HPLC after derivatization with iodoacetic acid and dansyl chloride. Data presented are dose-dependent changes in the intracellular GSH and GSSG levels (**A**) and the GSH redox potential (ΔE_h) (**B**). Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^*P < 0.05$; one-way ANOVA and the Dunnett post hoc test.

strand: 5′-cta gCA AAG TGC AAG GTG GTG ACC ggt ac-3′) were synthesized by Integrated DNA Technologies (Coralville, IA). These oligonucleotides, containing *Kpn*I and *Nhe*I sticky ends, were phosphorylated, annealed, and subcloned into the pGL3-promoter vector between the *Kpn*I and *Nhe*I sites. The sequences of the constructs were verified by the Vanderbilt DNA Sequencing facility.

Transfection and Dual Luciferase Reporter Assay

The ARPE-19 cells were seeded into six-well plates at 1.5×10^5 cells per well. After 24 hours, cells were transfected for 6 hours with 1.0 μg of the pGL3 vector (control), the pGL3-wtARE vector, or the pGL3-mARE vector (Fugene 6 Transfection Reagent; Roche, Branchburg, NJ) in serum-free DMEM/F12. To control for transfection efficiency, cells were cotransfected with 10 ng of the pRL-CMV vector (Promega) containing the *Renilla* luciferase (*rluc*) gene driven by the cytomegalovirus promoter. After 6 hours, medium was replaced with fresh medium containing zinc, sulforaphane, or oltipraz. In the first set of experiments, cells were treated with 100 μM zinc, 5 μM sulforaphane, or 50 μM oltipraz. In the second set, cells were treated with different concentrations of zinc to test for a dose-dependent response. After 24 hours' treatment, the cells were lysed with 500 μL passive lysis buffer (Promega). With a dual luciferase assay kit (Promega), firefly luciferase activity (measured with a FB12 luminometer; Zylux, Pforzheim, Germany) in relative light units per second (RLU/s) was normalized to *Renilla* luciferase activity and expressed as *x*-fold multiples of the control, a ratio of the experimental (with zinc) to control (without zinc).

Knockdown Nrf2 Expression in RPE by siRNA

Transfection of Nrf2 siRNA was performed as previously described[29] using the target sequence 5′-AAG AGT ATG AGC TGG AAA AAC-3′ for human Nrf2 siRNA (Integrated DNA Technologies, Coralville, IA). The ARPE-19 cells were seeded at 1.5×10^5 cells per well into two six-well plates: one for Western blot analysis and the other for double transfection and luciferase assay. After 24 hours, the cells were transfected with Nrf2 siRNA. Briefly, appropriate amounts of Nrf2 siRNA in 250 μL serum-free DMEM/F12 medium and 5 μL of transfection reagent (Lipofectamine 2000; Invitrogen) in 245 μL serum-free DMEM/F12 medium were prepared in separate centrifuge tubes. After incubation for 5 minutes, the siRNA and Lipofectamine were mixed, incubated for an additional 20 minutes, and added to each well. The degree of Nrf2 knockdown was determined 24 hours after transfection by Western blot analysis,[27] using an anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bands from the Western blot were scanned and their intensities were quantified (Quantity One 1D Analysis Software;

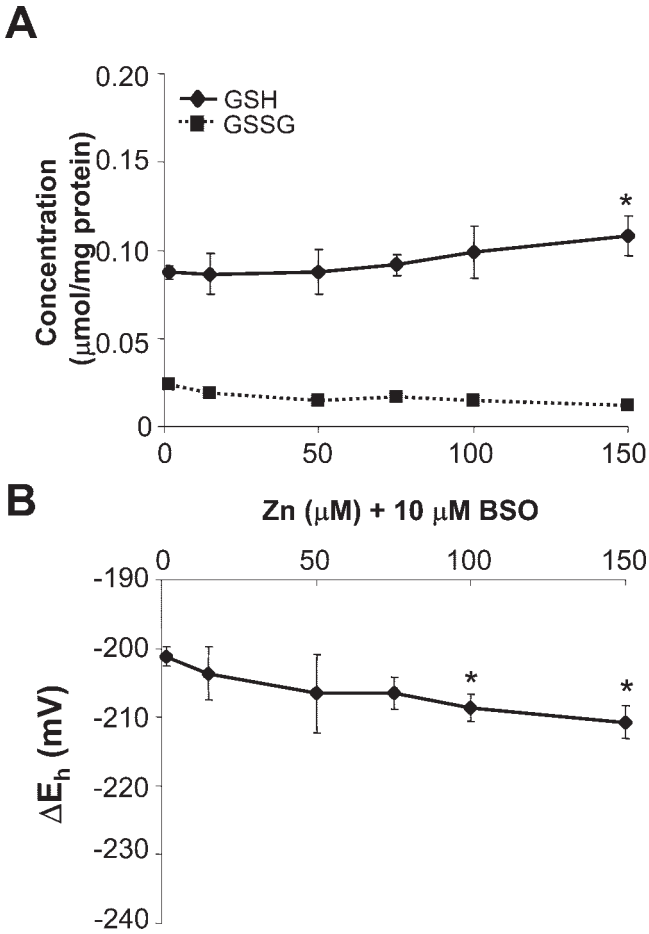

FIGURE 3. BSO diminished zinc's effects. ARPE-19 cells were treated with various concentrations of zinc in the presence of 10 μM of BSO, a selective inhibitor of glutamate-cysteine ligase (GCL). There were dose-dependent changes in the intracellular GSH and GSSG levels (**A**) accompanied by reduction of the GSH redox potential (ΔE_h) (**B**). Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^*P < 0.05$; one-way ANOVA and Dunnett post hoc test.

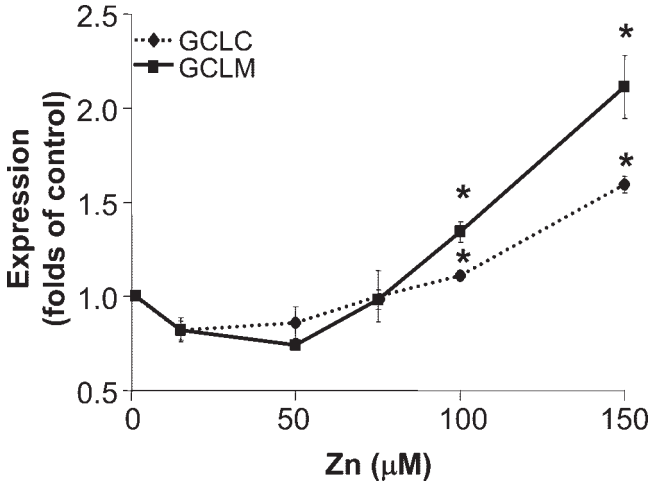

FIGURE 4. Zinc treatment upregulated GCL mRNA in ARPE-19 cells. ARPE-19 cells were treated with indicated concentrations of $ZnSO_4$ for 4 hours. Total RNA was isolated, and the expression of the catalytic (GCLC) and modulatory (GCLM) subunits of GCL were measured by using quantitative RT-PCR. Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^*P < 0.05$; significantly different from control untreated cells; one-way ANOVA and Dunnett post hoc test.

Bio-Rad). For each sample, the intensity of the experimental band was normalized to the intensity of the α-tubulin band to control for loading variation. In double transfection experiments, medium with Nrf2 siRNA was removed after 24 hours, replaced with fresh medium, and transfected with the pGL3-wtARE vector for 6 hours before zinc treatment, as described earlier.

RESULTS

Zinc-Induced GSH Synthesis in RPE Cells

Glutathione is the principal cellular nonprotein thiol responsible for maintaining redox homeostasis. We measured the GSH level in ARPE-19 cells treated with different zinc concentrations to determine the effects of zinc on this important antioxidant. Treatment of cells with $ZnSO_4$ for 24 hours resulted in a statistically significant increase in GSH level—30% and 70% at 100 and 150 μM zinc, respectively (Fig. 2A)—without significant changes in the GSSG level. There were significant increases in the negativity (reduction) of the GSH redox potential at these concentrations (Fig. 2B). Treating cells with $ZnSO_4$, $ZnCl_2$, or zinc acetate resulted in similar GSH and GSSG level profiles (data not shown), demonstrating that the observed effects were solely due to zinc and not to the anions.

Cellular GSH level is maintained by the de novo synthesis and the salvage pathways. To determine which pathway was responsible for the increase in the GSH level, we measured the GSH and GSSG concentrations in the presence of BSO, a selective inhibitor of GCL. The addition of a low concentration of BSO (10 μM) significantly inhibited the zinc-induced increase in GSH synthesis (Fig. 3A) and partially diminished zinc-induced reduction of the GSH redox potential (Fig. 3B). Furthermore, if the salvage pathway was responsible for the increase in the GSH level, one would expect to observe a comparable decrease in the GSSG level; however, no significant changes in the GSSG level were seen with zinc treatment (Fig. 2A). Thus, the data suggest that zinc stimulated the de novo synthesis of GSH in RPE cells.

Zinc-Induced GCL Expression through an ARE-Nrf2–Dependent Pathway

Having established that zinc induced the de novo synthesis of GSH mediated by GCL, we next measured the GCL expression as a surrogate of ARE-Nrf2 activity in ARPE-19 cells. Time course experiments in which we harvested cells treated with 100 μM zinc at different time points and performed quantitative RT-PCR indicated that GCL expression peaked at 4 hours after treatment (data not shown). Using this time point, we then treated cells with various concentrations of zinc and measured a dose-dependent increase in the expression of both the catalytic (GCLC) and the modulatory (GCLM) subunits of GCL with increasing zinc concentrations (Fig. 4). There was a slight decrease in GCL expression at lower zinc concentrations, followed by significant increases in the expression of both the GCLC and GCLM subunits at 100 and 150 μM zinc concentrations.

To confirm that zinc activates the ARE-Nrf2 pathway in ARPE-19, we used a dual luciferase reporter assay to measure

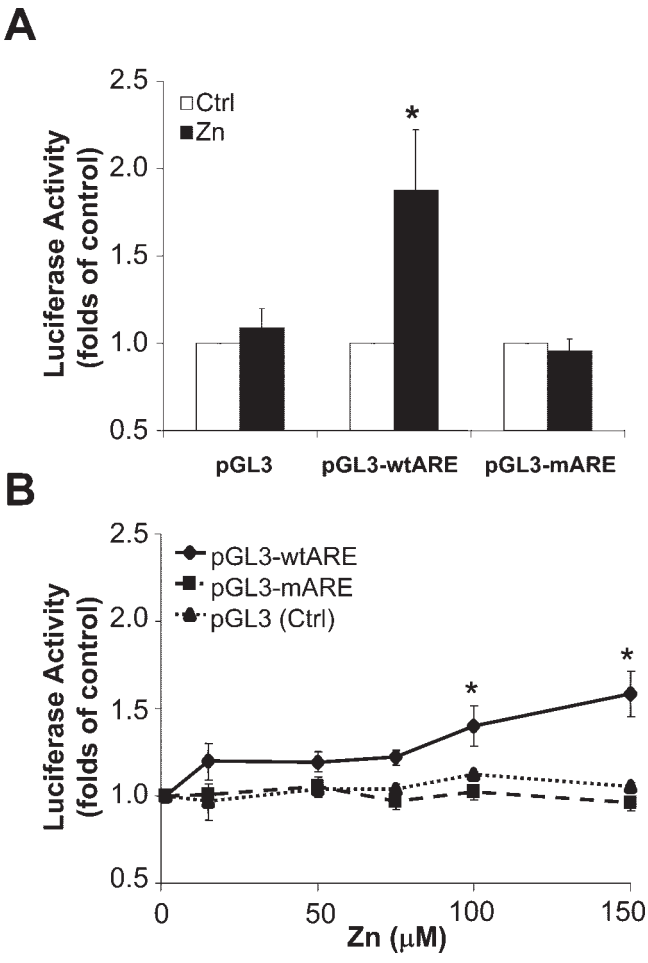

FIGURE 5. Dual luciferase reporter assays of the ARE activity in ARPE-19 cells 24 hours after treatment with zinc. ARPE-19 cells were transfected with the pGL3 vector, the pGL3-wtARE vector, or the pGL3-mARE vector and treated with 100 μM zinc for 24 hours before luciferase activity was measured (**A**). Cells were transfected with the pGL3 vector, pGL3-wtARE vector, or pGL3-mARE vector and treated with various concentrations of zinc for 24 hours before measurement of luciferase activity (**B**). Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^*P < 0.05$; significantly different from control untreated cells; one-way ANOVA and Dunnett post hoc test.

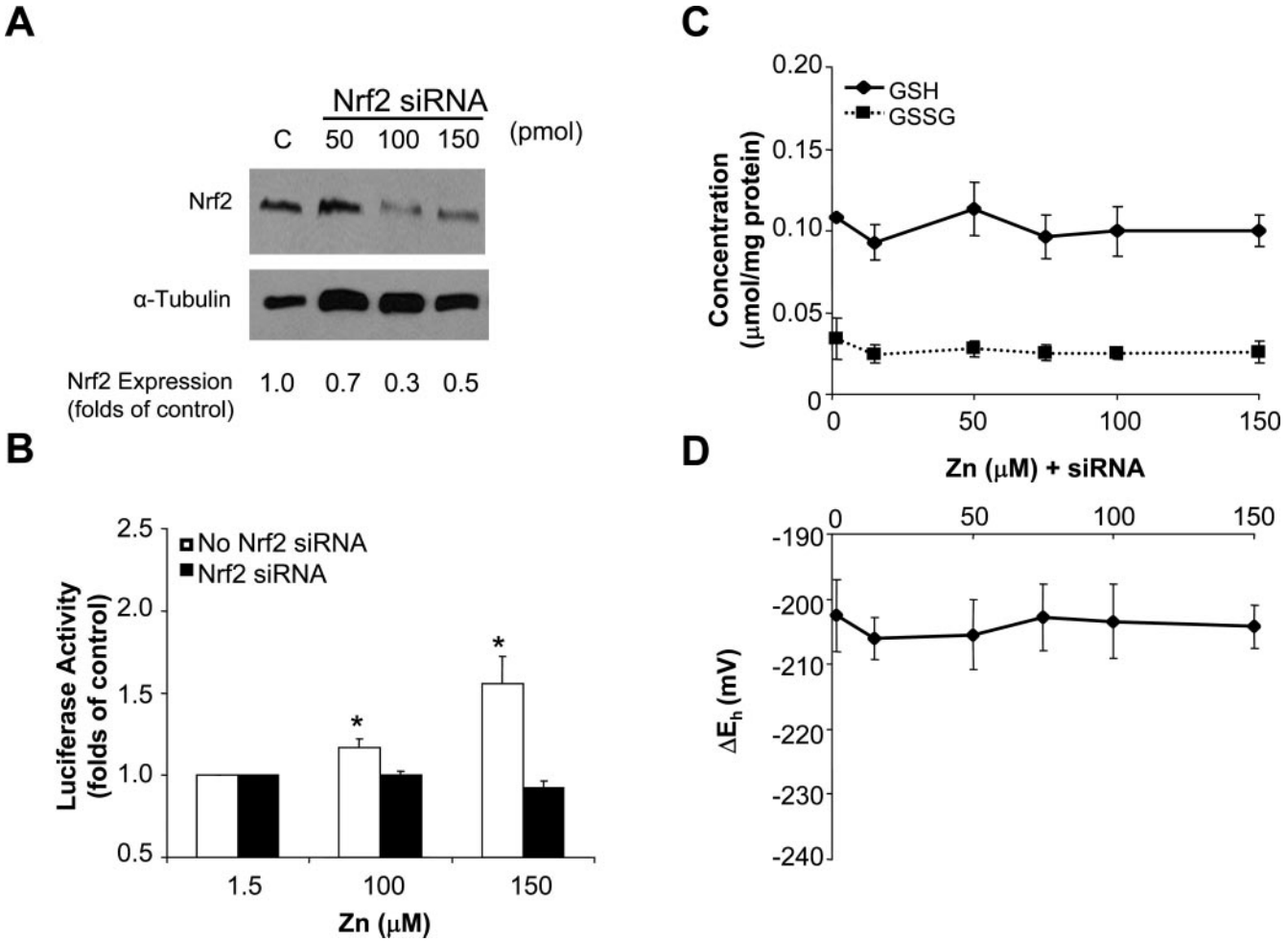

FIGURE 6. Knockdown of Nrf2 expression abolished zinc-induced ARE activation and GSH elevation in ARPE-19 cells. ARPE-19 cells were first transfected with Nrf2 siRNA and then with the pGL3-wtARE vector. (**A**) Western blot analysis of Nrf2 expression after transfection with 50, 100, or 150 pmol Nrf2 siRNA. Cell lysates were prepared from cells 24 hours after transfection with Nrf2 siRNA. Proteins were separated on a 12% SDS-polyacrylamide gel and probed with anti-Nrf2 antibody. α-Tubulin was used as a loading control. Data are representative of three separate experiments. Relative Nrf2 expression to control (no zinc) was calculated after normalizing to the level of tubulin in each sample. (**B**) Decreased Nrf2 expression inhibited zinc-induced ARE activation. Cells were transfected first with 100 pmol Nrf2 siRNA for 24 hours then with the pGL3-wtARE reporter plasmid for 6 hours and treated with different concentrations of $ZnSO_4$ for 24 hours. ARE activity was measured with a dual luciferase assay. (**C**) Inhibition of the ARE-Nrf2 pathway with Nrf2 siRNA abolished the effects of zinc (100 μM) on GSH synthesis. Cells were transfected with 100 pmol Nrf2 siRNA and treated with different concentrations of $ZnSO_4$ for 24 hours. Cellular GSH and GSSG were measured by HPLC. (**D**) Decreased Nrf2 expression also inhibited zinc-induced reduction of the GSH redox potential, ΔE_h. Each point represents the average of three separate experiments performed in duplicate (mean ± SE). No significant differences were observed between control and zinc-treated groups; one-way ANOVA.

luciferase activity in cells previously transfected with the pGL3 vector, pGL3-wtARE vector, or pGL3-mARE vector in the presence and absence of zinc. Using 100 μM, we showed that zinc moderately activated ARE in cells transfected with the pGL3-wtARE but not in cells transfected with the pGL3 or the pGL3-mARE negative controls (Fig. 5A). Compared with treatments with 50 μM oltipraz and 5 μM sulforaphane (data not shown), treatment with 100 μM zinc had less effect on ARE activation. We further established that zinc activated ARE in a dose-dependent manner, whereas there was no significant dose-dependent response to zinc in the negative control experiments (Fig. 5B).

Knockdown of Nrf2 Expression Eliminated the Effects of Zinc

To confirm further that the ARE-Nrf2 pathway is the mechanism by which zinc induces GSH synthesis, we blocked this pathway and measured zinc's ability to induce GSH synthesis. Western blot analysis showed that 100 pmol of Nrf2 siRNA significantly reduced the expression of Nrf2 (Fig. 6A). No apparent cytotoxicity was observed in ARPE-19 cells transfected with this amount of siRNA. To measure the ARE activity in Nrf2 knockdown, cells were doubly transfected, first with Nrf2 siRNA, and then with the pGL3-wtARE vector. They were then treated with various zinc concentrations. Dual luciferase reporter assay studies showed that knockdown of Nrf2 expression abolished zinc-induced activation of the ARE-Nrf2 pathway (Fig. 6B). Furthermore, knockdown of the ARE-Nrf2 pathway abolished zinc-induced increase in GSH synthesis (Fig. 6C) and reduction of the GSH redox potential (Fig. 6D). There was an overall increase in the GSSG level and oxidation of cells transfected with siRNA.

DISCUSSION

Age-related macular degeneration is the leading cause of blindness in individuals aged 55 years and older in the United States

and Western Europe.[30] Currently, intake of antioxidants and zinc is the only established treatment for those with the atrophic or "dry" form, although there has been a recent increase in treatment options for those with the neovascular or "wet" form.[31–34] However, even these treatments are largely ineffective in reversing existing visual impairment. Thus, the development of prevention strategies for AMD assumes great importance.[34] With oxidative stress thought to play a key role in the pathogenesis of AMD,[4–8] current prophylactic treatment centers on reducing or preventing oxidative damage. Zinc, with direct and indirect antioxidant properties, has been shown to delay significantly the progression of AMD and reduce moderate vision loss.[1]

Our present study provides evidence that zinc stimulates GSH synthesis through the upregulation of GCL expression in an ARE-dependent pathway in RPE cells. Cellular GSH level is maintained by the de novo and the salvage pathways. In our study, induction of GSH synthesis by zinc was not accompanied by a comparable decrease in the GSSG level, resulting in a reduction of the GSH redox potential. Furthermore, the increase in GSH level was diminished by the addition of BSO, a selective GCL inhibitor. These findings support the presumption that the increase in the GSH level is mediated through the de novo synthesis pathway. Furthermore, an increase in the GSH level correlated with the increases in both GCL expression and ARE activity. To confirm that this pathway is responsible for zinc's induction of GSH synthesis, we blocked the pathway at the transcriptional and posttranslational level of GCL expression. We showed that Nrf2 siRNA blocked ARE-Nrf2 activation and blunted zinc's induction of GSH synthesis. Similarly, BSO also diminished the effects of zinc on GSH synthesis. Zinc has long been recognized to have both direct and indirect antioxidant effects. In the current study, we showed that zinc can activate the transcription of phase II genes and afford powerful and prolonged antioxidant effects.

Various groups have investigated the effects of zinc on RPE cells, and their data are consistent with our current findings. Wood and Osborne[35] studied the effects of zinc on the viability of human RPE cells prepared from donor eyes. In their study these cells tolerated up to 110 μM zinc, similar to our finding that ARPE-19 cells tolerated up to 150 μM zinc. In addition to the ARE-Nrf2 pathway, previous studies have also suggested that zinc induces GSH synthesis through the activation of the metallic response element-MTF-1 (metal response element-binding transcription factor 1) pathway.[36] Prestera et al.,[19] using a luciferase assay to study ARE activation by various compounds, including $ZnCl_2$, in hepatoma cells, showed that these compounds activate ARE up to slightly more than one- to fourfold. The magnitude of induction is similar to our observations with 100 and 150 μM of zinc (Fig. 5). Zinc deficiency has been reported to result in decreased plasma GSH levels in rats.[37] Treatment with very high doses of zinc (>200 μM) induces RPE cell death, probably by chelation and depletion of cellular GSH.[38] Similarly, we observed GSH depletion by 200 μM zinc in ARPE-19 cells (data not shown). However, the safety of the AREDS recipe has been validated in clinical trial. It is unlikely that the current clinically prescribed dosage of zinc will result in drastic increases of its local concentration in the retina.

In previous studies, other ARE inducers have been evaluated for their role in the protection of RPE cells from various sources of oxidative injury. Nelson et al.[39] have shown that oltipraz is effective in protecting hRPE from oxidative damage from *tert*-butylhydroperoxide (tBH). Gao and Talalay[40] have shown that sulforaphane is effective in protecting ARPE-19 cells from oxidative damage from photosensitization. Tanito et al.[28] showed that sulforaphane supplementation is effective in protecting rat RPE and photoreceptors from photooxidative damage. In this report, while demonstrating the induction of ARE by zinc, we also found that oltipraz and sulforaphane are much more potent and efficacious ARE inducers than is zinc in cultured RPE. The clinical significance of these compounds and their derivatives remains to be determined.

Our study used cultured RPE cells and has several limitations. We observed significant effects with zinc concentrations of 100 and 150 μM. The concentration of zinc in the plasma is 15 μM,[2,41] although it is thought to be much higher in the retina, particularly in the chorioretinal complex.[42] In addition, in experiments with an immortalized RPE line rather than primary cultures, there may be differences in the metabolism of zinc in ARPE-19 cells compared with hRPE cells. First, immortalized cell lines, such as ARPE-19, have less pigment than donor RPE cells. These pigments are involved in the storage of zinc.[43,44] Decreased pigments may result in reduced intracellular zinc storage and availability. Second, it has been shown that transformed prostate epithelial cells (which are normally similar to RPE in that they accumulate high intracellular zinc) have decreased ability to accumulate zinc because of the downregulation and loss of the Zrt/Irk-like protein (ZIP) involved in zinc uptake.[45]

With AREDS demonstrating a beneficial effect of zinc both alone and in concert with antioxidants, it is enticing to attribute these effects to zinc's many postulated direct antioxidant activities. However, AREDS also reported the observation that antioxidant vitamins alone were not protective against AMD progression.[1] Our data show that high-dose zinc is capable of inducing phase II enzymes, suggesting that the beneficial effects of zinc supplementation observed in AREDS may be due, at least in part, to phase II enzyme induction. In addition to increasing GCL expression, these compounds most likely increase the expression of several other phase II enzymes that are also protective of oxidative stress.[40] Thus, further study of the role of phase II enzyme inducers may be indicated for the treatment of patients with AMD.

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