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Essential Roles of the PI3 Kinase/Akt Pathway in Regulating Nrf2-Dependent Antioxidant Functions in the RPE

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Abstract

Purpose—To investigate functional interactions between the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent antioxidant system in cultured human retinal pigment epithelium (RPE) cells.

Methods—Cultured ARPE-19 cells were treated with different concentrations of PI3K inhibitors, followed by exposure to sulforaphane, an Nrf2 inducer. Akt phosphorylation was detected by Western blot analysis. Intracellular glutathione (GSH) content was measured by HPLC. Expression of genes downstream of Nrf2, including glutamate-cysteine ligase (GCL) and glutathione S-transferase, was measured by quantitative RT-PCR. Nrf2 activity was measured by a dual luciferase assay after transfection of a reporter plasmid containing the antioxidant response element (ARE). The small interference RNA approach was used to knock down Nrf2 in the RPE. Nrf2 localization was determined by subcellular fractionation and Western blot analyses.

Results—PI3K inhibitors wortmannin and LY294002 caused dose-dependent cellular and mitochondrial GSH depletion and downregulation of the modulatory subunit of GCL in cultured RPE cells. Both the basal and the induced Nrf2 activities were inhibited by wortmannin and LY294002. Overexpression of a constitutively active form of Akt potentiated Nrf2 activation, and the effect of Akt was blocked by siRNA that knocked down Nrf2. LY294002 also inhibited sulforaphane-induced Nrf2 nuclear translocation.

Conclusions—The PI3K/Akt pathway plays key roles in regulating Nrf2-ARE-dependent protection against oxidative stress in the RPE.

Cumulative oxidative injury is an important environmental factor contributing to the development and progression of age-related macular degeneration (AMD).[1-3] In animal models of chronic oxidative stress, the retina and retinal pigment epithelium (RPE) develop pathologic lesions that are characteristic of early AMD.[4-8] In the Age-Related Eye Disease Study (AREDS), supplementation with antioxidant vitamins, zinc, or both was shown to significantly reduce the risk for progression of AMD.[9] Characterizing the antioxidant defense system and its regulatory mechanisms will be essential in defining the vulnerability of the retina to oxidative injury and in developing new treatment strategies for AMD.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is commonly involved in the transcriptional regulation of genes encoding antioxidant proteins under stress conditions.[10,11] It heterodimerizes with members of the small Maf family of transcription factors and binds to

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the *cis*-acting antioxidant response element (ARE) sequence in the promoter regions of various phase 2 detoxification genes. The latter encode for a group of enzymes, such as glutamate-cysteine ligase (GCL) and glutathione S-transferase (GST), essential in the detoxification of xenobiotics and endogenous reactive electrophilic compounds.[12,13] Agents that activate Nrf2 can protect RPE cells from oxidative injury.[14-17]

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays a major role in cell survival signaling and is implicated in a plethora of biological responses such as apoptosis, cell growth, differentiation, calcium signaling, and insulin signaling.[18,19] The PI3K consists of an 85-kDa and a 110-kDa subunit; once activated, it phosphorylates phosphatidylinositol at the D-3 position of the inositol ring.[20] LY294002 and wortmannin are two highly specific inhibitors that block PI3K activity through direct interaction with the p85 subunit.[21,22] Oxidative stress has been shown to regulate the PI3K/Akt and, consequently, to alter the downstream signaling events in cultured RPE cells.[23-25]

The interactions between the Nrf2-dependent antioxidant system and other cellular signaling pathways have not been well characterized in the retina. Previous studies using several cancer cell lines reported that PI3K and Akt functions are required for Nrf2 activation by various inducers such as *tert*-butyl hydroquinone (tBH), hemin, and peroxynitrite.[26-28] In the present study, we investigated the functional interactions between the PI3K/Akt pathway and Nrf2 in cultured ARPE-19 cells. Our results indicated that exposure of the ARPE cells to PI3K inhibitors caused decreased Nrf2 activity, downregulation of GCL and GST, and the consequent reduction of cellular and mitochondrial GSH contents. On the other hand, cells overexpressing constitutively active Akt showed enhanced Nrf2 activity and increased response to sulforaphane. The data suggest that the PI3K/Akt pathway is essential in regulating the Nrf2-ARE-dependent protection against oxidative stress in the RPE.

Materials and Methods

Materials

Sulforaphane, γ-glutamyl glutamate (γGG), wortmannin, and LY294002 were purchased from LKT Laboratories (St. Paul, MN), MP Biomedicals (Irvine, CA), Upstate (Lake Placid, NY), and Promega (Madison, WI), respectively. Anti-Nrf2, anti-HSC70, and anti-PARP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt antibodies were obtained from Cell Signaling (Danvers, MA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Experimental Conditions

The ARPE-19 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco modified Eagle medium (DMEM), which contained 1 g/L glucose, 1 mM sodium pyruvate, and MEM nonessential amino acid (Mediatech, Herndon, VA) and was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich). For experiments, cells were seeded into six-well plates or 60-mm culture dishes. After reaching confluence, cells were exposed to different concentrations of PI3K inhibitors for 30 minutes. Sulforaphane was then added for an additional 6 to 16 hours.

Plasmid Construction and Stable Overexpression

The ARE reporter plasmid was described previously[29] and contained the consensus Nrf2-binding site upstream of the firefly luciferase gene. Plasmids encoding wild-type and constitutively active Akt1/PKBα were obtained from Upstate. The active Akt1 was constructed by replacing its N-terminal pleckstrin homology domain with a myristoylation signal from the Src protein.[30] To construct the Nrf2 expression vector, full-length human *Nrf2* cDNA was

cloned by RT-PCR from ARPE cells using forward primer (5′-ATG ATG GAC TTG GAG CTG CCG CCG-3′) and reverse primer (5′-AAC TAG CTC AGA AAA GGT CAA ATC CTC-3′). The PCR products were gel purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). After sequence verification, the full-length open reading frame of *Nrf2* was subcloned into pQCXIP retroviral vector (Clontech, Mountain View, CA).

To produce active virus, the pQCXIP retroviral vector containing the *Nrf2* gene was cotransfected with pCGP and pVSV (Takara, Madison, WI) into 293T viral packaging cells.[31] The supernatant containing retrovirus was collected 72 hours after transfection and was used to infect ARPE-19 cells. Transduced cells were subsequently selected by 1 μg/mL puromycin for 2 days, and survival cells were pooled together for further experiments.

Measurement of Total and Mitochondrial Glutathione

To measure total cellular glutathione (GSH), RPE cells were extracted with 5% perchloric acid/0.2 M boric acid. Acid-soluble thiols were derivatized with iodoacetic acid and dansyl chloride and were analyzed by HPLC using a propylamine column (YMC Pack, NH2; Waters, Milford, MA) and an automated HPLC system (Alliance 2695; Waters). The *S*-carboxymethyl-*N*-dansyl-GSH and *N*, *N*′-bis-dansyl-GSSG derivatives were eluted by a gradient of sodium acetate and were detected with a fluorescence detector.[32]

For mitochondrial GSH measurement, a digitonin permeabilization method was used to prepare mitochondria-enriched fractions, as previously described.[33] Cells, after different treatments, were collected by trypsinization, pelleted, and resuspended in MES buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES (pH 7.0), 0.5 mg/mL bovine serum albumin (fatty acid free), 1 mM EDTA, and 1 mM EGTA. The cells were then permeabilized with 0.12 mg/mL digitonin (Sigma) in room temperature for 2 minutes. The disruption of the plasma membrane was confirmed by trypan blue staining. After centrifugation at 12,000*g* for 15 seconds, the pellets were washed once with MES buffer, extracted with perchloric acid/boric acid, derivatized, and analyzed by HPLC.

Measurement of Reactive Oxygen Species Production

ARPE-19 cells were seeded in six-well plates. After 24 hours, cells were treated with LY294002 for 16 hours. Then the cells were stained with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen) at 5 μM for 20 minutes in 37°C incubator. Cells exposed to tBH were used as positive control. Oxidation of H_2DCFDA led to increased fluorescence, which was detected by flow cytometry using 488-nm laser excitation.

Measurement of Nrf2 Activity by Dual Luciferase Reporter Assay

Cultured ARPE-19 cells were transfected with 1 μg ARE reporter plasmid with the use of reagent (Fugene 6; Roche, Indianapolis, IN) in serum-free DMEM. To control for transfection efficiency, cells was cotransfected with 20 ng pRL-CMV vector (Promega) containing the *Renilla* luciferase gene driven by the constitutively active cytomegalovirus promoter. In Akt1/PKBα overexpressing experiments, 1 μg vector containing *Akt1/PKBα* allelic cDNA were cotransfected with the reporter plasmids. After 8 hours, medium was replaced with fresh medium containing PI3K inhibitors, sulforaphane, or both. By the end of the treatments, cells were lysed with 500 μL passive lysis buffer (Promega), and the luciferase activities were measured with a FB12 luminometer (Zylux, Pforzheim, Germany), as described.[29]

Quantitative RT-PCR Assay of GCL and GST Expression

The mRNA levels of the catalytic and modulatory subunits of *GCL* were measured by real-time RT-PCR, as described.[29] TaqMan primers for the GCL catalytic subunit (Hs00155249_m1) and the GCL modulatory subunit (Hs00157694_m1) were provided in assay kits (Gene Expression Assay; Applied Biosystems, Foster City, CA). GST M4 and GST P1 were measured by the Universal Probe Library (UPL) approach (Roche). GST M4 was amplified by forward primer (5′-ACT TCA TCT CCC GCT TTG AG-3′) and reverse primer (5′-TGT ACA GAG GTT TTG GGA GGA-3′), and the UPL probe used was no. 13. GST P1 was amplified by forward primer (5′-TCT CCC TCA TCT ACA CCA ACT ATG-3′) and reverse primer (5′-AGG TCT TGC CTC CCT GGT-3′) and quantified by UPL probe 56. All PCR reactions were performed on an ABI system (ABI 7300; Applied Biosystems). Average threshold cycle (Ct) values were used to determine the relative differences between control and treated groups and were normalized to the 18s ribosomal RNA (rRNA).[29]

Small Interference RNA-Mediated Knockdown of Nrf2 in the RPE

Transfection of Nrf2 small interference RNA (siRNA; Integrated DNA Technologies, Coralville, IA) was carried out as previously described[29] using the target sequence 5′-AAG AGT ATG AGC TGG AAA AAC-3′ for human Nrf2. Cells were transfected with Nrf2 siRNA (Lipofectamine; Invitrogen). The medium containing Nrf2 siRNA was removed 24 hours later, and the cells were further transfected with DNA constructs of ARE reporter plasmids and myristoylated Akt. Sulforaphane treatment and luciferase assay were performed as described.

Measurement of Nrf2 Localization

To determine the subcellular localization of Nrf2 in ARPE-19 cells, we used nuclear fractionation and Western blot analysis to detect the amount of Nrf2 in nuclei and cytoplasm. After LY294002 or sulforaphane treatment, cells were lysed in ice-cold hypotonic buffer (Sigma) containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 10 mM dithiothreitol (DTT), 0.6% NP40, and a cocktail of protease inhibitors (Roche).[34] After centrifugation at 4° C at 12,000*g* for 30 seconds, the supernatant was collected as the cytosolic faction. The pellet was further extracted using buffer containing 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 26% glycerol, and 300 mM NaCl, pH 7.9, at 4°C for 30 minutes. Soluble nuclear proteins were collected after centrifugation at 12,000*g* for 10 minutes.

Results

PI3K/Akt Activity Is Required for Nrf2-Dependent GSH Synthesis in ARPE-19 Cells

GSH is the principal nonprotein thiol responsible for maintaining cellular redox homeostasis.[32] To determine how changes in PI3K-Akt signaling affected GSH contents, ARPE-19 cells were treated with two different PI3K inhibitors, wortmannin and LY294002, at various concentrations, followed by GSH measurement. Treatment with wortmannin for 6 hours at concentrations higher than 10 nM resulted in a significant decrease in the phosphorylation of Akt (Fig. 1A). Under the same conditions, wortmannin caused a dose-dependent decrease in the intracellular GSH level (Fig. 1B). GSH in control ARPE cells was 99.6 ± 2.6 nmol/mg protein and decreased to 52.3 ± 5.8 nmol/mg protein after exposure to 100 nM wortmannin. Treating cells with LY294002 for 16 hours resulted in similar changes in Akt phosphorylation (Fig. 1C) and GSH depletion (Fig. 1D). Neither inhibitor of PI3K affected RPE cell viability or GSSG level (data not shown). Thus, with the use of two different chemical inhibitors, we showed that PI3K/Akt activities were required to maintain GSH levels in cultured RPE cells.

Mitochondria do not synthesize GSH but can import it from the cytoplasm by way of specific transporters.[35,36] The mitochondrial GSH and GSSG were measured in RPE cells treated with

different concentrations of LY294002. Results (Fig. 2A) showed that, as with total cellular GSH, PI3K inhibition also caused decreased mitochondrial GSH without affecting the amount of GSSG. To determine whether RPE cells had increased ROS production after exposure to LY294002, cells were stained with a ROS-sensitive dye, H_2DCFDA, and were measured by flow cytometry. Treatment with a chemical oxidant, tBH, caused oxidation and increased DCF fluorescence; however, no difference was observed between control and LY294002-treated cells (Fig. 2B). Thus, the results suggest that inhibition of the PI3K/Akt pathway led to decreased synthesis, rather than oxidation, of the GSH pool.

GCL is the rate-limiting enzyme in GSH synthesis and is under the control of the ARE-Nrf2 pathway. The Nrf2 activity was measured by a dual luciferase reporter assay (Figs. 3A, 3B). The data showed that 10 μM LY294002 suppressed Nrf2 activity to nearly 50% of control (Fig. 3A), and approximately 30% reduction was observed in cells treated with 50 or 100 nM wortmannin (Fig. 3B). When cells were cultured in media containing either 10% or 1% serum, no differences in Nrf2 activity were detected (Fig. 3C).

The mRNA levels of both the catalytic (*GCLC*) and modulatory (*GCLM*) subunits of *GCL* as well as two other phase 2 detoxification genes, *GST P1* and *GST M4*, were measured by real-time RT-PCR. In cells exposed to LY294002 for 16 hours or to wortmannin for 6 hours, significant decreases in GCLM expression were observed (Figs. 4A, 4B). However, GCLC expression remained unchanged. The expression of *GST P1* was decreased by LY294002, but that of GST M4 was not affected significantly (Fig. 4C). Thus, inhibition of the PI3K/Akt pathway caused decreased Nrf2 activity and, consequently, led to selective downregulation of certain phase 2 detoxification genes in cultured RPE cells.

PI3K/Akt Activity Is Required for Nrf2 Activation

The results so far indicated that the PI3K/Akt pathway regulates the basal activity of Nrf2. To investigate the effect of PI3K inhibitors under conditions when Nrf2 is activated, we used the reporter assay to measure the Nrf2 activity in cells treated with sulforaphane, a potent ARE inducer.[17] The data (Fig. 5) showed a dose-dependent increase in ARE activity (330%–510% of control) when cells were treated with sulforaphane at a concentration from 5 to 20 μM. Cotreatment with 10 μM LY294002 inhibited the effect of sulforaphane (Fig. 5A). The intracellular GSH level was measured by HPLC under similar conditions. At 16 hours, the GSH content increased to 160% of control after 10 μM sulforaphane treatment but remained unchanged with the addition of 10 μM LY294002 (Fig. 5B).

To further clarify the effect of PI3K inhibitor on Nrf2 activation, we generated RPE cells stably overexpressing Nrf2 by transducing a retroviral vector containing a full-length coding sequence of the *Nrf2* gene. A nearly twofold increase in ARE activity was observed in Nrf2-overexpressing cells compared with the parental cells (Fig. 6A). The response to 5 μM sulforaphane treatment was also higher in Nrf2-transduced cells, though no significant differences were observed in responses to 20 μM sulforaphane. Regardless of the expression level of Nrf2, LY294002 treatment reduced its activity to the control level with or without sulforaphane treatment (Fig. 6A). When the mRNA levels of GCLC and GCLM were measured, GCLM expression significantly increased to 160% of vector control after exposure to 5 μM sulforaphane (Fig. 6B). No significant change was found in GCLC with or without sulforaphane (Fig. 6C).

Akt Activation Is Sufficient to Increase Nrf2 Activity

To determine whether the PI3K/Akt pathway is not only required but also sufficient for Nrf2 activation, wild-type and constitutively active Akt were overexpressed in ARPE cells by transient transfection. Active Akt1 had its N-terminal pleckstrin homology domain replaced

with a myristoylation sequence for plasma membrane targeting and activation.[30] Exogenous Akt1 (also know as PKBα) had an N-terminal c-Myc tag so that its expression could be detected by Western blot analysis (Fig. 7A). When probed for total Akt, nearly a threefold increase was observed in Akt-transfected cells.

Nrf2 activities were measured by cotransfection with the ARE reporter plasmid and Akt. Results showed that, at 24 hours, Nrf2 activity increased approximately 2.8 times when compared between cells transfected with empty vector and cells transfected with the activated *Akt1* gene (Fig. 7B). Treatment with sulforaphane further elicited a robust induction of Nrf2 activity in cells overexpressing the constitutively active Akt (Fig. 7B). In contrast, cells transfected with wild-type Akt responded similarly to vector-transfected cells.

The ARE reporter plasmid used in the study contained a consensus sequence for Nrf2 binding, [29] but it also might have been influenced by other transcription factor(s). To clarify the specificity, we transfected RPE cells with siRNA-targeting Nrf2.[29] The results (Fig. 7C) showed that ARE activity was significantly reduced when Nrf2 was knocked down. Either sulforaphane treatment or Akt overexpression was unable to upregulate the reporter activity. Thus, it is unlikely that PI3K/Akt acts on ARE through transcription factors other than Nrf2.

Nuclear Localization of Nrf2 in Cultured RPE Cells

The subcellular localization of Nrf2 is one of the major determining factors for its function. To determine how PI3K inhibitor and ARE-inducer would influence Nrf2 localization in ARPE cells, we performed subcellular fractionation followed by Western blot analyses in cells exposed to sulforaphane and LY294002. Nrf2 showed a time-dependent decrease in the cytosolic faction and a concomitant increase in the nuclear fraction (Fig. 8A), indicating sulforaphane caused a nuclear translocation of Nrf2. In cells treated with LY294002, no significant changes of the levels of Nrf2 in either the cytosolic or the nuclear fraction were observed (Figs. 8B, 8C).

Discussion

Protecting the retina and the RPE from oxidative injury is one of the important considerations for the treatment of AMD. This can be achieved by direct supplementation with antioxidant vitamins or by the use of agents that boost the intrinsic antioxidant system. Previously, we and others[14-17,29] have shown that compounds such as zinc, sulforaphane, oltipraz, and dimethyl fumarate can induce the expression of the phase 2 detoxification genes, increase GSH synthesis, and protect against oxidative injury. In this study, we further demonstrated that the PI3K/Akt pathway is essential in regulating the Nrf2-ARE-dependent antioxidant functions in cultured RPE cells.

With the use of two different PI3K inhibitors, we showed that inhibition of the PI3K/Akt pathway led to decreased Nrf2 transcriptional activity (Fig. 3), decreased GCL and GST expression (Fig. 4), and GSH depletion (Figs. 1, 2). Wortmannin and LY294002 block PI3K activity through direct interaction with the regulatory p85 subunit.[18] At concentrations that inhibit Akt phosphorylation, both compounds inhibited the basal level of GSH synthesis (Fig. 1). Furthermore, when Nrf2 activity was increased by sulforaphane treatment (Fig. 5) or overexpression (Fig. 6), LY294002 was still effective in inhibiting Nrf2. Thus, consistent with the results from earlier studies that used various cancer cells,[26-28] a functional PI3K/Akt pathway is required for the basal level and the activation of Nrf2 in the RPE. Responses of genes downstream of Nrf2 were selective. Only GCLC and GST P1 were downregulated when PI3K was inhibited. No significant changes were detected for GCLM or GST M4 (Fig. 4). Depending on the treatment conditions and the genes measured, the expression of the antioxidant genes downstream of Nrf2 may show either coordinated or discordant responses.

[37-39] Nrf2 has five ECH homology domains. They interact with different transcription factors and cofactors and, therefore, exert specificities toward various promoters.[39]

In addition to being a requirement for Nrf2, increased Akt was also sufficient to elevate its basal activity and to dramatically potentiate the effects of sulforaphane (Fig. 7). Most of the previous studies were performed in cancer cell lines that often carry gain-of-function mutations in the PI3K/Akt pathway.[19,20] In nontransformed ARPE-19 cells, overexpression of a constitutively active Akt1 led to a nearly threefold increase in the basal Nrf2 activity (Fig. 7B). The response to sulforaphane was much higher when Akt was also activated (Fig. 7B). Taken together with the results from the inhibitor studies, these data strongly suggest that one of the signaling mechanisms by which PI3K/Akt promotes cell survival is to facilitate optimal induction of the Nrf2-dependent antioxidant defensive system. A schematic summary of the functional interactions of the two pathways is presented in Figure 9.

When bound to its inhibitory protein Keap1, Nrf2 is targeted for ubiquitination and thus has relatively low protein stability.[40-42] On exposure to stimuli of oxidative stress or electrophilic compounds, Nrf2 protein can be liberated from Keap1 and translocates into the nucleus.[43] Using subcellular fractionation and Western blot analyses, we found that Nrf2 translocated into nuclei within 2 hours of exposure to sulforaphane; no significant differences were observed in LY294002-pretreated RPE cells (Fig. 8). These results implicated that Nrf2 translocation was in a dynamic homeostasis in RPE cells regulated by the PI3K pathway. Our results (Fig. 7B) indicated that LY294002 inhibited the ARE activation in cells overexpressing active Akt. Such effects were partly caused by the inhibition of the endogenous Akt in cultured RPE cells. Our results also indicated that downstream of PI3K can be pathways that are either Akt dependent or independent and that contribute to Nrf2 stability and function.

The downstream protein of PI3K/Akt involved in controlling Nrf2-ARE activity in RPE cells is still unknown. It is well established that Nrf2 is under the regulation of protein kinase C and mitogen-activated protein kinase.[44] Its nuclear localization may also be related to the phosphorylation status of tyrosine 568.[45] Nrf2 has 33 serines and 5 threonines; however, it has never been reported that Akt could directly phosphorylate Nrf2. Among the protein kinases downstream of Akt, glycogen synthase kinase 3β (GSK 3β) was recently reported as capable of directly phosphorylating Nrf2.[46] Compared with cancer cells, RPE cells might have different pathways of glucose metabolism and cell survival signaling. Future studies will be needed to better address the role of the PI3K pathway in controlling Nrf2 stability, nuclear import and export, and interaction with Keap1.

Both Nrf2 and Akt show age-dependent declines in their function.[47-49] Agents such as zinc and lipoic acid can upregulate Nrf2 activities and maintain tissue antioxidant functions. To date, most of the available compounds either have pharmacokinetic properties that do not penetrate the blood tissue barrier or have low safety margins. Better understanding of the crosstalk between the cell survival pathway and the antioxidant system will facilitate the screen and design of future treatment strategies against AMD and other age-related degenerative diseases.

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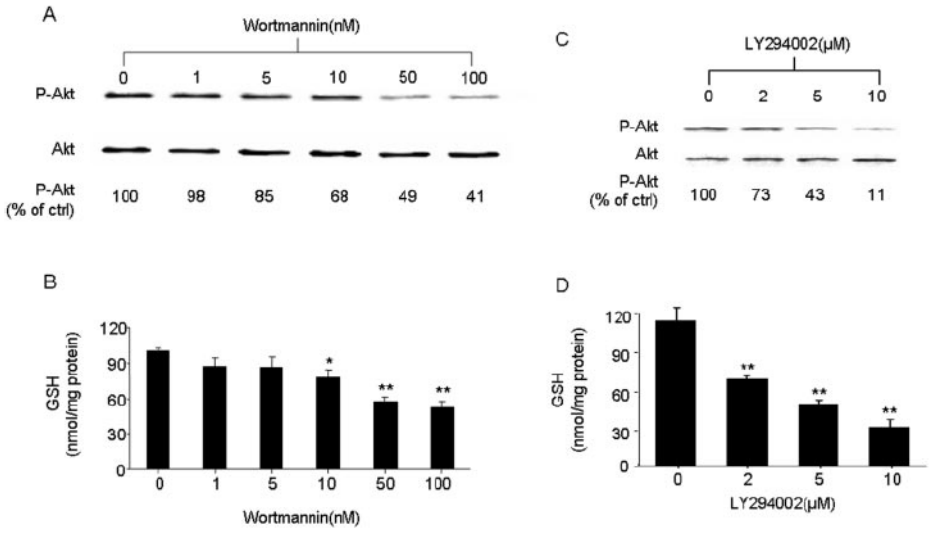

Figure 1.

Decreased intracellular GSH in ARPE-19 cells exposed to PI3K inhibitors. Cells were treated with either wortmannin for 6 hours or LY294002 for 16 hours at indicated concentrations. Akt phosphorylation was measured by Western blot analyses (**A, C**) and was calculated as the ratio of untreated control cells after normalization to the amount of total Akt. Cellular GSH was measured by HPLC (**B, D**). Data presented are the average of three separate experiments performed in duplicate (mean ± SE). ***P* < 0.01; one-way ANOVA and the Dunnett post hoc test.

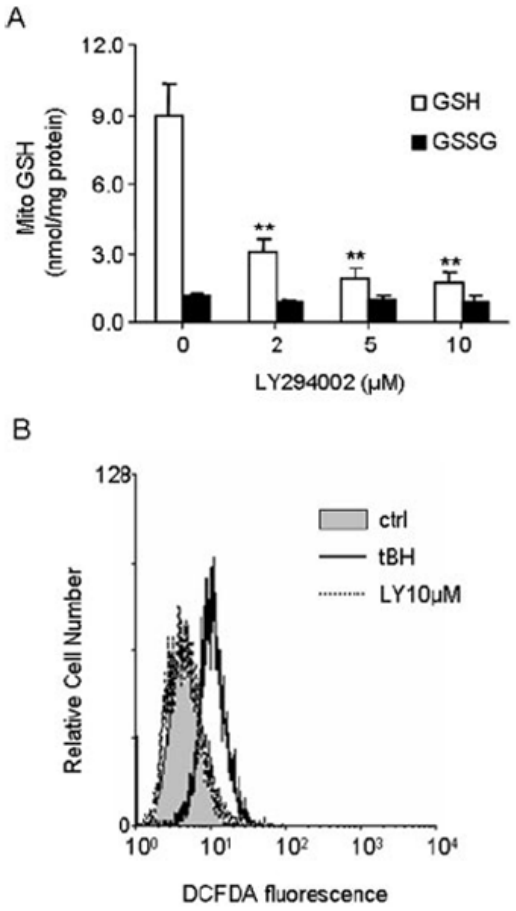

Figure 2.

Mitochondrial GSH depletion in RPE cells treated with LY294002. (**A**) Cells were treated with the indicated concentration of LY294002 for 16 hours. Mitochondrial GSH and GSSG were measured by HPLC after digitonin permeabilization. (**B**) Intracellular ROS production was measured by H_2DCFDA staining followed by flow cytometry. Treatment with 100 μM tBH was used as a positive control.

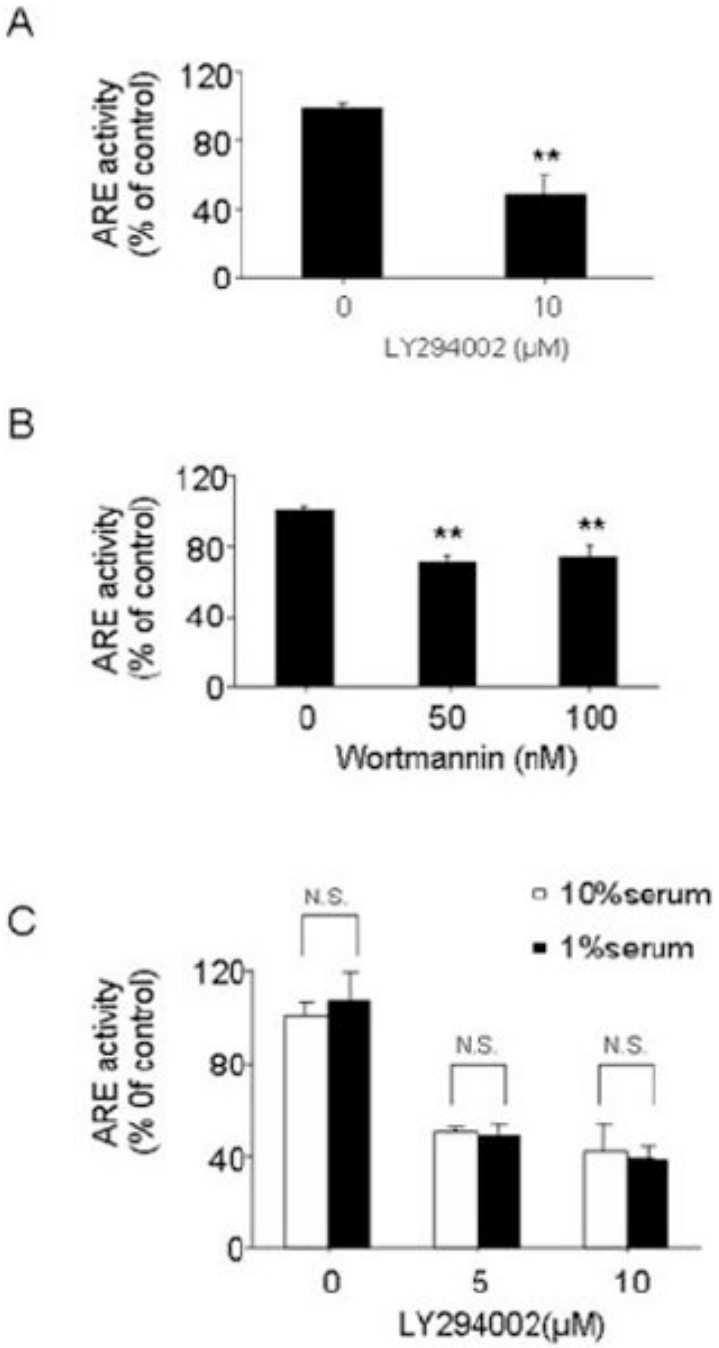

Figure 3.

Inhibition of Nrf2 activity by PI3K inhibitors. ARPE cells were treated with 10 μM LY294002 for 16 hours (**A**) or wortmannin for 6 hours (**B**) at the indicated concentrations. The Nrf2 activity was measured by transient transfection with an ARE reporter plasmid. (**C**) Cells were treated with 10 μM LY294002 in media containing either 10% or 1% fetal calf serum. Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^{**}P < 0.01$; significantly different from control untreated cells; one-way ANOVA and Dunnett post hoc test.

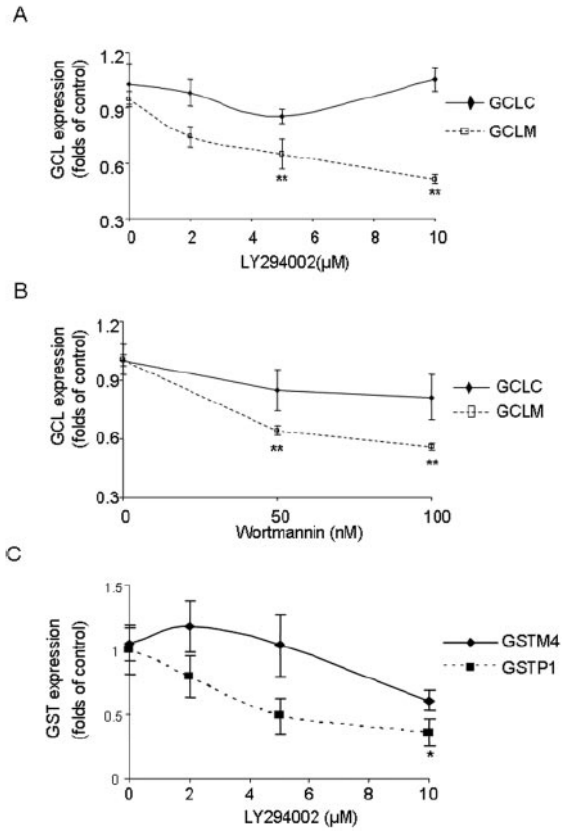

Figure 4.

Decreased expression of the antioxidant genes after PI3K inhibition. RPE cells were treated with either 10 μM LY294002 for 16 hours (**A, C**) or wortmannin for 6 hours (**B**) at indicated concentrations. mRNA levels of the catalytic (GCLC) and modulatory (GCLM) subunits of GCL, GST M4, and GST P1 were measured by quantitative RT-PCR. Differences in the average threshold cycle (ΔCt) values were determined and normalized to the expression of 18s rRNA. Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^{**}P < 0.01$; significantly different from control untreated cells; one-way ANOVA and Dunnett post hoc test.

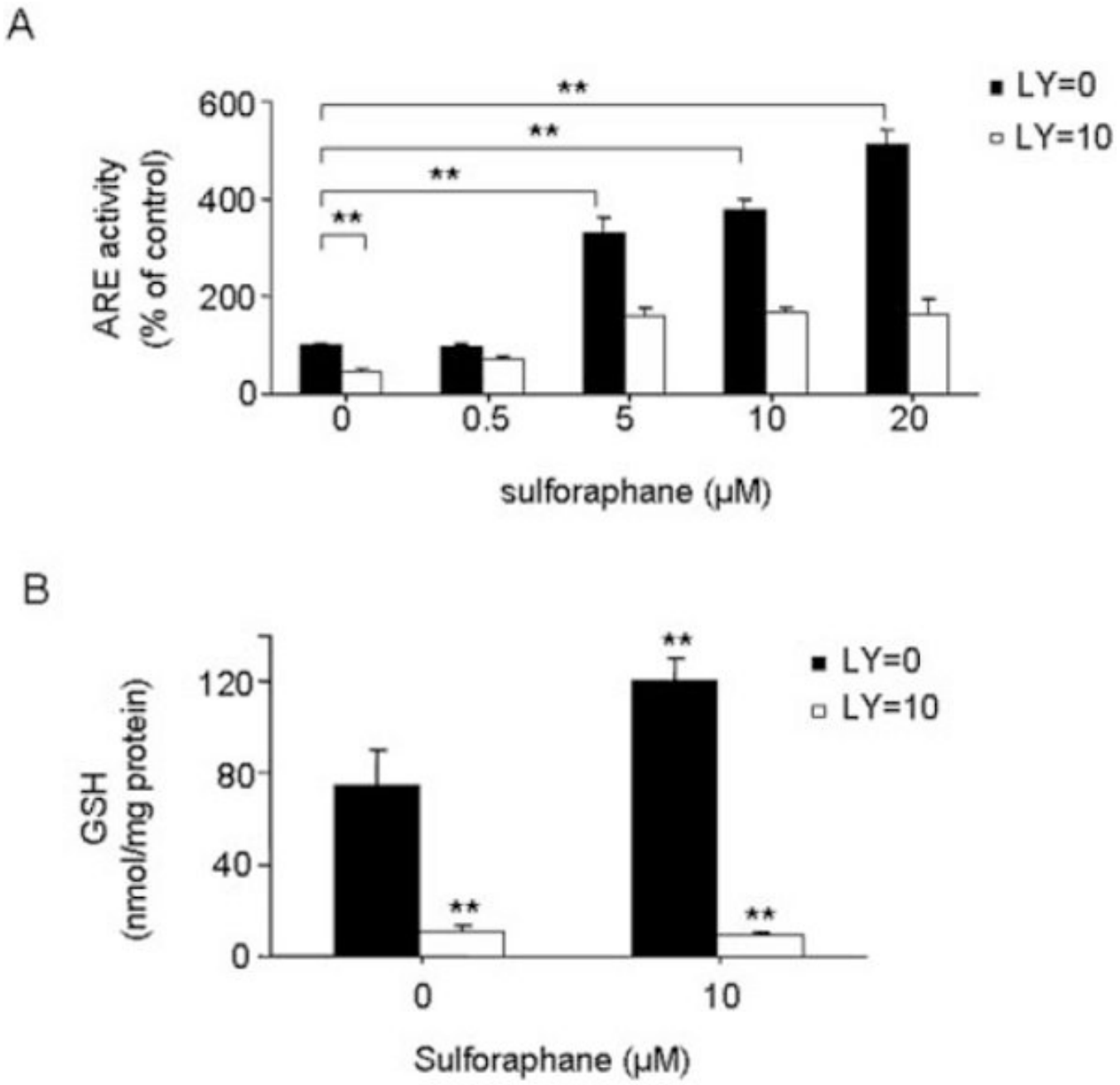

Figure 5.

LY294002 inhibited sulforaphane-induced Nrf2 activity in the RPE cells. (**A**) ARPE-19 cells were treated with sulforaphane alone (LY = 0) or sulforaphane and 10 μM LY294002 (LY = 10). Nrf2 activity was measured by the dual luciferase reporter assay. Data presented are the average of three separate experiments (mean ± SE). ***P* < 0.01 (significantly different from control untreated cells); one-way ANOVA and Dunnett post hoc test. (**B**) Measurement of intracellular GSH concentration in cells exposed to 10 μM sulforaphane with and without 10 μM LY204002. ***P* < 0.01; Student's *t*-test.

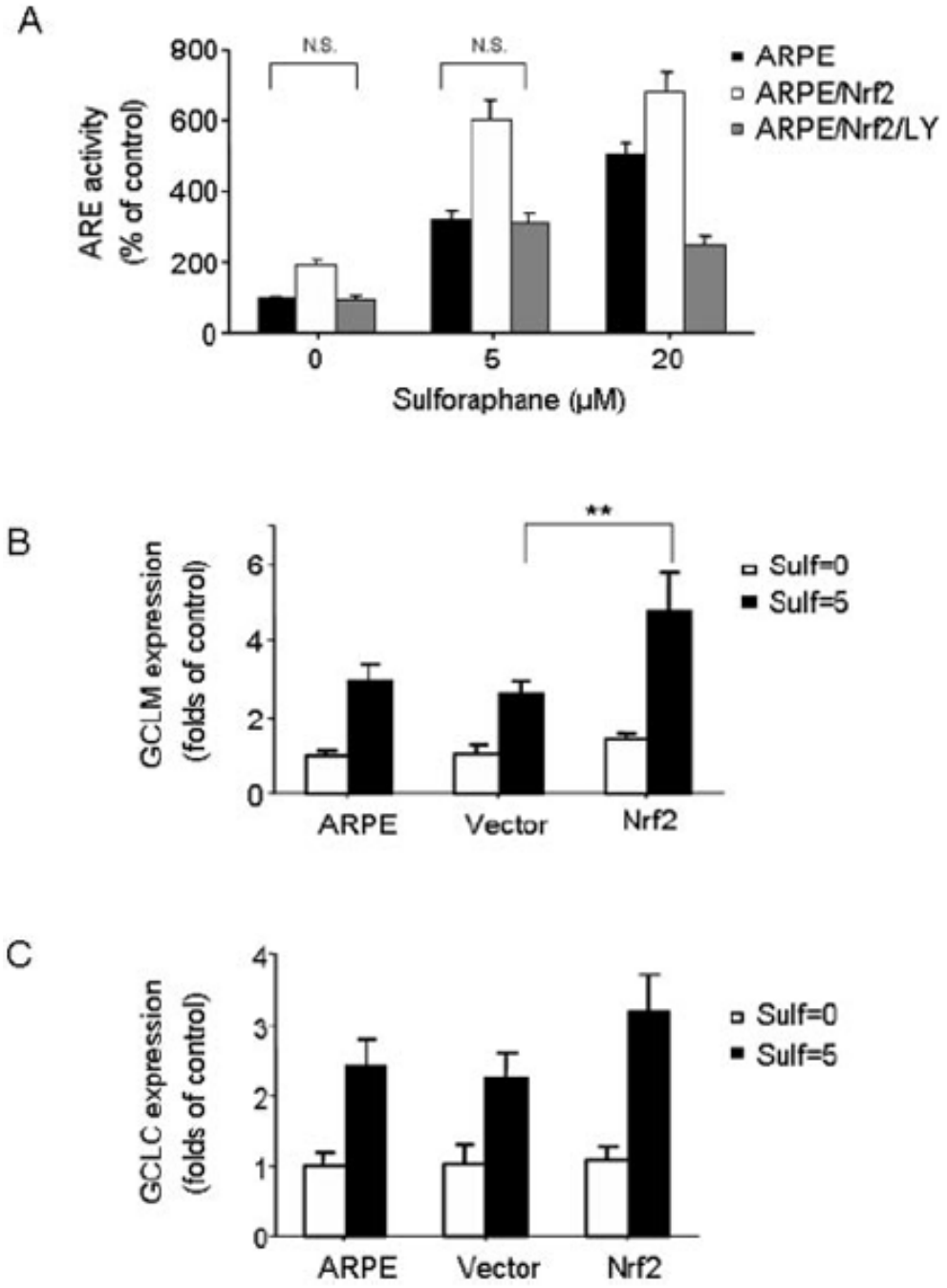

Figure 6.

Effects of LY294002 in RPE cells overexpressing Nrf2. (**A**) Nrf2 was stably overexpressed in the ARPE-19 cells. Nrf2 activities were measured after treatment with indicated concentrations of sulforaphane, with or without 10 μM LY294002. (**B, C**) mRNA levels of GCLM and GCLC were measured in parental, vector, and Nrf2-transduced RPE cells using real time RT-PCR. Differences in the average threshold cycle (ΔCt) values were determined and normalized to the expression of the 18s rRNA. 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.

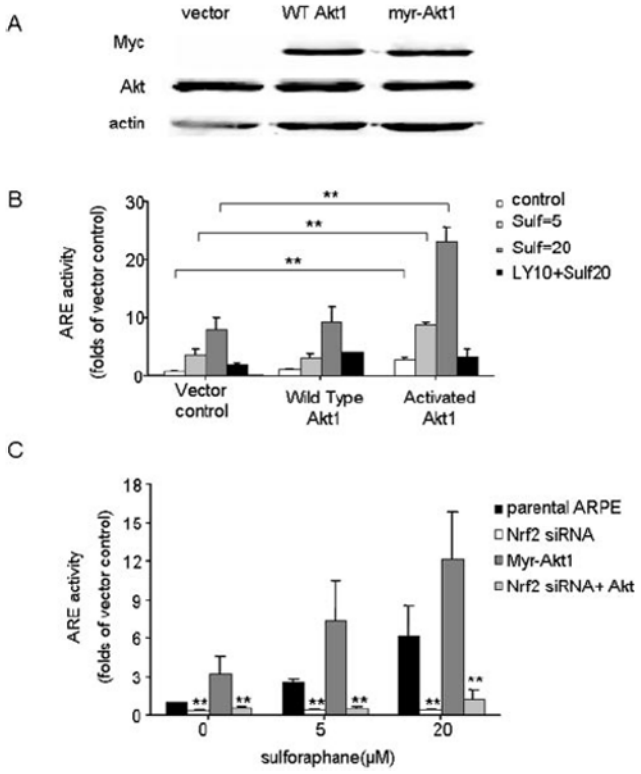

Figure 7.

Effects of Akt activation on Nrf2 function. (**A**) Western blot analysis showing the overexpression of c-Myc–tagged Akt in ARPE-19 cells. The Akt with an N-terminal myristoylation signal (myr-Akt) is constitutively active. (**B**) Measurement of Nrf2 activity in cells overexpressing Akt and treated with sulforaphane and LY294002. The ARE reporter plasmid was transiently cotransfected with the plasmid encoding either wild-type or constitutively active Akt. Sulforaphane and LY294002 were added 8 hours after transfection. Luciferase activities were measured after an additional 16 hours. Each point represents the average of three separate experiments performed in duplicate (mean ± SE). $^{**}P < 0.01$, significantly different from vector-transfected cells; one-way ANOVA and the Dunnett post hoc test. (**C**) Measurement of Nrf2 activity after Nrf2 had been knocked down. RPE cells were transfected with siRNA downregulating Nrf2. After 24 hours, cells were further transfected with the constitutively active form of Akt1 and treated with sulforaphane at indicated concentrations. Data presented are the average of three separate experiments (mean ± SE). $^{**}P < 0.01$ (significantly different from cells that did not receive siRNA).

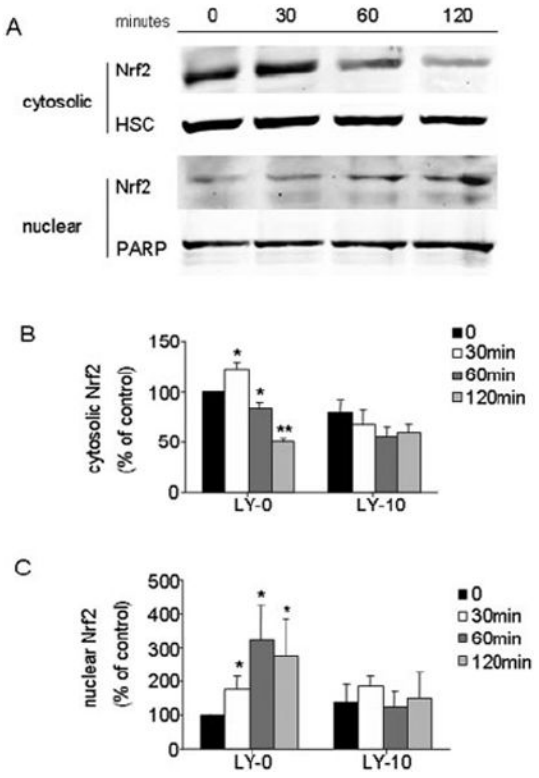

Figure 8.

Sulforaphane-induced Nrf2 nuclear translocation in ARPE-19 cells. (**A**) The amount of Nrf2 in nuclear and cytosolic fractions was measured by subcellular fractionation followed by Western blot analyses. Cells were pretreated with 10 μM LY294002 and then exposed to 20 μM sulforaphane for the indicated times. (**B, C**) Relative amounts of Nrf2 in the cytosolic and nuclear fractions, respectively. Hsc70 and poly(ADP-ribose)polymerase were used as loading controls. Each point represents the average of three separate experiments performed in duplicate (mean ± SD). *$P < 0.05$, **$P < 0.01$ (significantly different from untreated control cells); one-way ANOVA and Dunnett post hoc test.

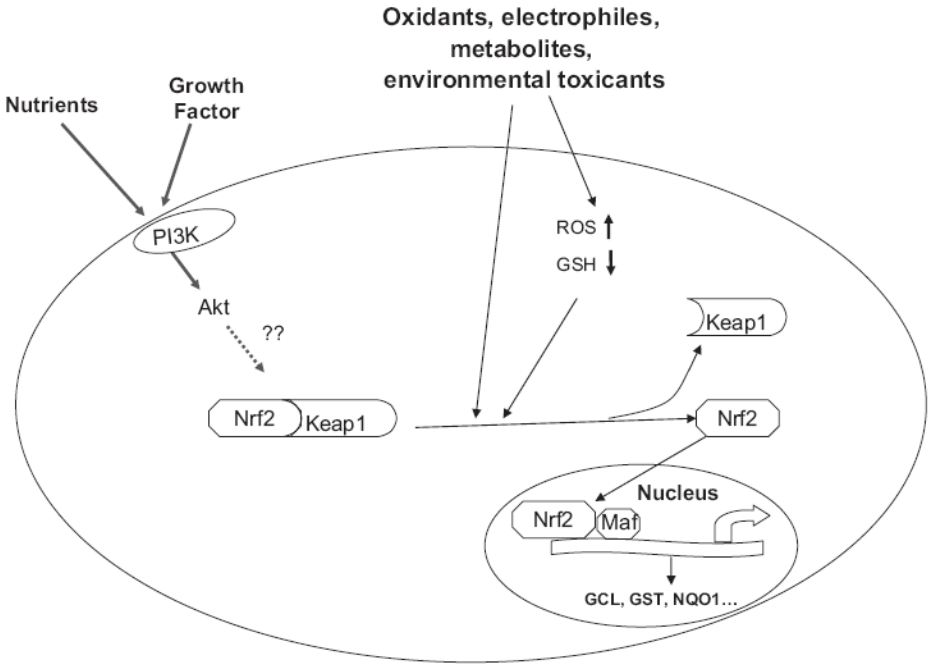

Figure 9.

Schematic presentation of the functional interaction between PI3K/Akt and Nrf2. Under various conditions of oxidative stress, Nrf2 is activated and released from Keap1 and translocates into nuclei to mediate the transcription of the antioxidant genes. PI3K and Akt modulate the Nrf2 function, though the direct mechanistic link between the two pathways remains to be elucidated.