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Phosphatidylinositol 3 Kinase Pathway and 4-Hydroxy-2-Nonenal-Induced Oxidative Injury in the RPE

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Abstract

Purpose—4-Hydroxy-2-nonenal (4-HNE) is a major lipid peroxidation product in the retina and the retinal pigment epithelium. The purpose of the present study was to investigate how NF-E2-related factor-2 (Nrf2) and phosphatidylinositol 3 (PI3K) pathways affect the responses of cultured human retinal pigment epithelial (RPE) cells to 4-HNE.

Methods—Cultured ARPE-19 cells were treated with different concentrations of 4-HNE and a PI3K inhibitor, LY294002. Intracellular glutathione (GSH) was measured by high-performance liquid chromatography (HPLC). The transcriptional activity of Nrf2 was measured by dual luciferase assay after transient transfection with reporter plasmids. The mRNA level of glutamate cysteine ligase (GCL) was quantified by real-time RT-PCR. Formation of HNE adduct on heat shock cognate protein 70 (Hsc70) was measured by immunoprecipitation and Western blot analyses.

Results—Treatment with 4-HNE increased Nrf2 activity and GSH synthesis in a dose-dependent manner in cultured RPE cells. The modulatory subunit of GCL was upregulated by 4-HNE. Antioxidant responses were largely abolished by pretreatment with LY294002. The modification of Hsc70 by 4-HNE was increased when PI3K was inhibited.

Conclusions—The Nrf2-dependent antioxidant response protects against 4-HNE toxicity, and this protective mechanism is dependent on the functions of the PI3K pathway.

The retina has a rich content of polyunsaturated fatty acid (PUFA).^{1–3} PUFAs such as docosahexaenoic acid (DHA) and arachidonic acid are important for visual phototransduction.⁴ However, under conditions of oxidative stress such as excessive light exposure,⁵ hyperglycemia,⁶ and vitamin E deficiency,⁷ PUFAs react with free radicals to form lipid peroxidation products.⁸ 4-Hydroxynonenal (4-HNE) is one of the major reactive aldehydes and is a product from oxidation of n-6 PUFA.

Lipid peroxidation contributes to oxidative injury associated with aging and age-related diseases of the retina. A number of previous studies have demonstrated that 4-HNE can covalently modify and affect functions of proteins^{9,10} and DNA.¹¹ 4-HNE reacts with glutathione (GSH) and is mainly detoxified by glutathione-S-transferase.^{12,13} In retinal cells, nuclear factor erythroid 2-related factor 2 (Nrf2) plays key roles in controlling GSH synthesis and protecting against 4-HNE-induced toxicity by regulating the expression of antioxidant and

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detoxification genes.^{14,15} In addition to Nrf2, other transcription factors, such as NFκB and cFOS, can be activated by 4-HNE and can exert cytoprotective effects.¹⁶

Nrf2 is a basic region-leucine zipper transcription factor and can be activated by reactive intermediates and electrophilic compounds.¹⁷ It binds to the *cis*-acting antioxidant response element (ARE) and controls the expression of a number of genes encoding phase 2 detoxifying enzymes and antioxidant proteins.^{18,19} Nrf2 is sequestered normally in the cytoplasm by Kelch-like ECH-associated protein (Keap)-1.^{20,21} Under oxidative stress, it dissociates from Keap1 and translocates to the nucleus and controls ARE-mediated antioxidant gene expression.¹⁷

It has been shown that the Nrf2-mediated gene induction can be regulated by other signaling pathways, including extra-cellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K).²²⁻²⁴ In the present study, we measured the effects of 4-HNE on the antioxidant responses in cultured ARPE-19 cells. Results showed that 4-HNE activated Nrf2 and increased GSH synthesis. Inhibition of PI3K decreased the antioxidant response and potentiated oxidative protein modification by 4-HNE. The interaction between the two pathways underlay an important regulatory mechanism of antioxidant defense against lipid peroxidation and oxidative injury in RPE cells.

Materials and Methods

Cell Culture and Experimental Conditions

ARPE-19 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco modified Eagle (DMEM)/Ham F12 (50/50) medium (Mediatech, Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and non-essential amino acid (Mediatech). When they reached 90% confluence, cells were treated with 4-HNE (Cayman Chemicals, Ann Arbor, MI) with or without pretreatment with 10 μM LY294002 (Cell Signaling Technology, Beverly, MA) for 1 hour.

Measurement of Nrf2 Activity by Dual-Luciferase Reporter Assay

ARPE-19 cells were seeded in six-well plates at 2×10^5 /well. Twenty-four hours later, cells were transfected with 1 μg ARE reporter plasmid, as previously described,^{24,25} in serum-free medium using transfection reagent (FuGENE 6; Roche, Indianapolis, IN). Twenty nanograms of pRL-CMV vector containing the *Renilla* luciferase (Promega, Madison, WI) was cotransfected as a control to normalize transfection efficiency. After 8 hours, the medium was replaced with fresh DMEM containing 10 μM LY294002 for 1 hour, followed by exposure to HNE at concentrations from 0.5 to 20 μM. After an additional 16 hours, the cells were lysed with 0.5 mL buffer (passive lysis buffer; Promega), and luciferase activity was measured using a luminometer (model FB12; Zylux, Pforzheim, Germany), as described previously.^{24,25}

Measurement of Glutamate Cysteine Ligase Expression by Quantitative RT-PCR

ARPE-19 cells were treated with 20 μM of 4-HNE for 16 hours, with or without LY294002 pretreatment. Total RNA was isolated (TRIzol Reagent; Invitrogen, Carlsbad, CA), and cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI) and random hexamer (Applied Biosystems, Foster City, CA). The mRNA level of glutamate cysteine ligase (GCL) was measured by a Universal Probe Library (UPL) approach (Roche). The catalytic subunit of GCL (GCLC) was amplified by the forward primer (5'-ATG CCA TGG GAT TTG GAA T-3') and the reverse primer (5'-AGA TAT ACT GCA GGC TTG GAA TG-3') with UPL probe no. 80. The modulatory subunit of GCL (GCLM) was amplified by the forward primer (5'-GAC AAA ACA CAG TTG GAA CAG C-3') and the reverse primer (5'-

CAGTCAAAT CTG GTG GCA TC-3') with UPL probe no. 18. Quantitative PCR was performed on a real time-PCR system (ABI 7300; Applied Biosystems). Average threshold cycle (C_T) values were used to determine the relative difference between control and treated groups and were normalized to the 18S ribosomal RNA.^{24,25}

GSH Measurement by HPLC

After treatment with 4-HNE for 16 hours at indicated concentrations, cells were extracted with 5% perchloric acid solution containing 0.2 M boric acid and 10 μ M γ -glutamylglutamate (γ EE, internal standard). Acid-soluble thiols were derivatized with iodoacetic acid and dansyl chloride and were analyzed by HPLC as described.²⁴ The HPLC method does not measure protein thiols, and the amount of GSH was calculated based on the ratio between the peak areas of GSH and γ EE.

Cell Viability Assay by Flow Cytometry

ARPE-19 cells in six-well plates were pretreated with LY294002, followed by exposure to 4-HNE at final concentrations from 0 to 40 μ M. Live and dead cells were stained with 2 μ M ethidium homodimer (EthD)-1 and 1 μ M Calcein-AM (LIVE/DEAD Viability/Cytotoxicity Assay Kit, Invitrogen) and were analyzed by flow cytometry.²⁶

Measurement of 4-HNE-Modified Proteins by Western Blot Analyses

Cells were exposed to 4-HNE at 50 μ M for 1 hour and were lysed in a modified radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and a cocktail of protease inhibitors (Roche). Protein concentrations were quantified by Bradford assay (Bio-Rad, Laboratories, Hercules, CA). One milligram of the cell lysate was incubated overnight with 20 μ L anti-Hsc70 antibody (200 μ g/mL; Santa Cruz Biotechnology, Santa Cruz, CA) or 15 μ L anti-HNE antibody (200 μ g/mL; R&D Systems, Minneapolis, MN). Protein G-agarose beads (Upstate, Lake Placid, NY) were used to pull down the antibody and the bound proteins.²⁶ After stringent washes, the immunoprecipitates were boiled in 2 $\times$ Laemmli sample buffer (Bio-Rad), separated by 12% SDS-polyacrylamide gel, and subjected to Western blot analyses. The dilution of the primary and second antibodies was 1:1000.

For dot blots, 2 μ L cell lysate at the protein concentration of 1 mg/mL was mixed with equal volume of 2 $\times$ Laemmli sample buffer (Bio-Rad), loaded onto nitrocellulose membrane (Bio-Rad), and air dried. The HNE-protein adduct was blotted by anti-4-HNE antibody (1:1000; R&D Systems). Membranes were further stained with fluorescence-conjugated secondary antibodies (1:1000; Rockland, Gilbertsville, PA) and scanned with an infrared imaging system (Odyssey; LI-COR, Lincoln, NE).

Results

Effects of PI3K Inhibitor on ARE Activation and GSH Synthesis Induced by 4-HNE

As a potent electrophilic agent, 4-HNE can significantly alter cellular redox status and activate signaling pathways associated with oxidative stress.²⁷ When cultured ARPE-19 cells were exposed to different concentrations of 4-HNE, dose-dependent increases in ARE activity (Fig. 1A) and GSH levels (Fig. 1B) were observed. At concentrations lower than 5 μ M, no significant changes were detected, which suggested that low-dose 4-HNE could be effectively detoxified by the RPE cells. With exposure to 4-HNE at the concentration from 5 to 20 μ M, ARE activity increased from 252% to 425% of control (Fig. 1A), whereas GSH content increased from 247% to 451% of control (Fig. 1B). Pretreatment with the PI3K inhibitor LY294002 inhibited basal and 4-HNE-induced ARE activity (Fig. 1A) and resulted in decreased cellular GSH (Fig. 1B).

Incubating 100 μ M LY294002 with cell lysates in vitro for 30 minutes did not affect luciferase activity (data not shown), suggesting LY294002 is not a direct inhibitor of fly luciferase.

GCL is the rate-limiting enzyme for de novo synthesis of GSH in the RPE cells.^{24,28} GCL mRNA was quantified by real-time PCR after ARPE-19 cells were treated by 20 μ M of 4-HNE with or without LY294002 pretreatment. 4-HNE increased the mRNA expression of GCLM to threefold of control, and upregulation was inhibited by LY294002 (Fig. 2). There was no significant difference in GCLC expression after 4-HNE and LY294002 treatment. Thus, consistent with previous reports,^{14,22} 4-HNE activated the Nrf2-dependent pathway in RPE cells, and the antioxidant responses required PI3K.

Effects of PI3K on 4-HNE-Induced Protein Modification

4-HNE can modify the lysine, cysteine, and histidine residues of various cellular proteins and subsequently impair their functions.²⁹⁻³¹ 4-HNE-protein adducts were measured by Western blot analyses using anti-4-HNE antibody. On Western blot analysis, lysates from ARPE-19 cells treated by 20 μ M of 4-HNE for 1 hour showed numerous proteins modified by 4-HNE (Fig. 3A), and LY294002 pretreatment increased protein adduct formation. When quantified by semiquantitative dot blot analyses, results showed a 1.54 ± 0.07 -fold increase of total protein adduct in a LY294002-cotreated sample compared with 4-HNE treatment alone (Fig. 3C).

Previous proteomic study identified that heat shock cognate protein 70 (Hsc70) is a selective target of 4-HNE in the RPE.⁹ This was confirmed by our results from immunoprecipitation and Western blot analyses (Fig. 4A). HNE-modified proteins were pulled down from RPE cell lysates using an anti-HNE antibody. Subsequent Western blot analysis using anti-Hsc70 antibody clearly showed the modification. Under the same experimental conditions, modification of cathepsin D, another potential target of 4-HNE,³² was not detected (data not shown). To quantify the relative amount of Hsc70 modified by HNE, reciprocal immunoprecipitation was carried out (Fig. 4B). Hsc70 was immunoprecipitated, and the membrane from Western blot was probed with anti-4-HNE antibody. The relative amount of the modified Hsc70 protein was increased by 40% when PI3K was inhibited by LY294002 (Figs. 4C, 4D). Thus, 4-HNE selectively modified cellular proteins in the RPE, and such effect was potentiated by PI3K inhibition.

The effects of 4-HNE on RPE cell viability were measured by flow cytometry after exposure to 4-HNE at different concentrations for 48 hours, with or without LY294002 pretreatment. As shown in Figure 5, viability decreased to 47% of control after exposure to 4-HNE at 40 μ M. However, there was no significant difference between LY294002-pretreated and untreated samples. The data suggested that HNE-induced cell death was not affected by inhibition of PI3K.

Effects of PI3K Inhibitor on Other Inducers of Nrf2

To further characterize the interaction between PI3K and Nrf2, we tested the effects of LY294002 on Nrf2 activation stimulated by several groups of structurally different inducers that have been used in previous studies. Some of the compounds, including sulforaphane,³³ oltipraz,³⁴ and ZnCl₂,²⁵ have protective effects on the RPE. Arsenite (NaAsO₂),³⁵ cadmium (CdCl₂),³⁶ redox cycling agents (hydroquinone and t-butylhydroquinone),^{36,37} GSH-depleting agent (diethyl maleate),^{36,37} and oxidants (paraquat and menadione)³⁷ are toxic compounds and induce oxidative stress. All the agents increased ARE activity (Fig. 6). Pretreatment with LY294002 consistently attenuated the Nrf2 activation caused by these inducers (Fig. 6). Thus, the interaction between the PI3K and Nrf2 pathways is a general mechanism for controlling the antioxidant response in the RPE.

In addition to PI3K, mitogen-activated protein kinases (MAPKs) have been reported to play important roles in regulating the cellular responses to 4-HNE.^{21,22} Using the ARE reporter assay, the effects of two MAPK inhibitors on 4-HNE-induced Nrf2 activation were measured (Fig. 7). PD98059 inhibits MEK1, which is upstream of ERK. SD203580 inhibits p38 MAPK. Results (Fig. 7) showed that both PD98059 and SD203580 were able to inhibit Nrf2 activation in ARPE cells exposed to 20 μ M 4-HNE, though neither affected the basal activity of Nrf2.

Discussion

The retina is particularly susceptible to lipid peroxidation because of its high concentration of easily oxidized PUFAs, high oxygen tension, and high metabolic rate.¹⁻³ 4-HNE is one of the major toxic products of lipid peroxidation. It reacts with nucleophilic sites in proteins and nucleic acids and generates a wide range of adducts to exert its cytotoxicity.^{5,6} 4-HNE-protein adducts have been found in vivo in many diseases, including atherosclerosis, Parkinson's disease, and Alzheimer disease.³⁸⁻⁴⁰ Recent studies reported that 4-HNE-modified proteins might be involved in oxidative damage of the retina.^{9,41} 4-HNE can also be conjugated with GSH through Michael addition.¹² Results of our present study and of previous reports^{14,42,43} suggest that RPE cells use the Nrf2-dependent antioxidant system to detoxify 4-HNE.

The ARE activity and intracellular GSH level were increased dose dependently in ARPE-19 cells treated with 4-HNE (Fig. 1). GCLM, which regulates the rate of GSH synthesis,^{44,45} was selectively induced by 4-HNE (Fig. 2). Compared with other types of cells, such as astrocytes¹⁶ and neurons,⁴² RPE cells appear to be more resistant to 4-HNE. At 10 and 20 μ M, 4-HNE increased GSH synthesis in the RPE; in other cell types, the same concentration caused dramatic cell death.⁴² Efficient detoxification of 4-HNE through the Nrf2-dependent system will ensure the RPE cells are protected from lipid peroxidation products under in vivo conditions in which they are active in the phagocytosis of photoreceptor outer segments and the metabolism of lipid membranes with high PUFA content.

Activation of Nrf2 is a tightly controlled process subjected to multiple levels of regulation.¹⁷ Electrophiles such as 4-HNE may modify the reactive cysteine residues of Keap1 and release Nrf2 from inhibition.⁴⁶⁻⁴⁸ Direct modification of Keap1 protein by 4-HNE has been demonstrated by immunoprecipitation and Western blot analysis¹⁵; however, the sites of modification have not been mapped out. In addition, parallel signaling pathways, including PKC, ERK1/2, p38 MAPK, JNK and PI3K, are important in regulating Nrf2 localization and function.^{22-24,49}

The specific PI3K inhibitor LY294002 effectively inhibited Nrf2 activity (Fig. 1A), GSH synthesis (Fig. 1B), and GCLM upregulation (Fig. 2). It also significantly increased protein adduct formation (Figs. 4, 5). In our previously study, we showed that the PI3K/Akt pathway was required in sulforaphane-induced Nrf2 activation.²³ Similar effects were observed when RPE cells were treated with a number of different inducers with diverse chemical structure and signaling mechanisms (Fig. 6). Taken together, our results suggest that the PI3K-mediated cell survival pathway plays important roles in protection against cumulative oxidative injury in the RPE. Treatment with LY294002 did not affect 4-HNE-induced cell death (Fig. 5), suggesting apoptosis caused by high-dose HNE resulted from pathways distinct from the PI3K. When compared with the PI3K inhibitor, PD98059 and SD203580 only inhibited 20 μ M HNE-induced Nrf2 activation but had no effects on basal activity (Fig. 7). PI3K and MAPK may have implications in signaling events that resulted from exposure of the retina and RPE to different levels of oxidative stress.

The antioxidant proteins that function downstream of Nrf2 are involved in a number of cellular processes, such as metabolism of reactive intermediates and regulation of apoptosis and

inflammation, which can protect against retinal dysfunction and age-related degeneration.¹⁷ Nrf2 is one of the transcription factors controlling the production of cytosolic thioredoxin 1 (Trx1) in the retina, and the neuroprotective functions of Trx1 have been reported by several independent studies.^{14,33} GCL and GST perform GSH-dependent detoxification functions.⁵⁰ Heme oxygenase (HO)-1 may be involved in neuroprotection and inflammation.^{51,52} Therefore, Nrf2 inducers, such as sulforaphane,³³ are promising candidate compounds in protecting against oxidative injury and retinal degeneration.

Modification of Hsc70 by 4-HNE has been reported from proteomic studies.⁹ Results of our Western blot analyses indicate the Hsc70 can be modified by 4-HNE and the adduct formation was increased when the PI3K was inhibited (Fig. 4). Hsc70 has a number of potential roles in signal transduction, such as modulating response to insulin and sensitivity to apoptosis,^{53,54} interacting with cyclin D-dependent kinase⁵⁵ and translation initiation factor 2.⁵⁶ A recent study using proteomic approaches also showed that heat shock proteins appeared to be preferential targets of HNE modification.⁵⁷ Future studies will be needed to further characterize the involvement of Hsc70 in protecting RPE cells from oxidative injury.

In summary, we have shown that the PI3K plays key regulatory roles in Nrf2 function and protection against oxidative injury in the RPE. Interactions between the PI3K and Nrf2 pathways will be a key consideration in designing pharmacologic intervention to increase the antioxidant capacity of the retina and RPE.

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Figure 1.

Changes in Nrf2 activity and cellular GSH content in APRE-19 cells treated with 4-HNE. Cells were treated with 4-HNE at indicated concentrations for 16 hours, either with or without 1-hour pretreatment of 10 μM LY294002. (A) Nrf2 activity was measured by a dual luciferase ARE reporter assay. (B) GSH was measured by HPLC. Data presented are the average of 3 to 5 experiments. ** $P < 0.01$, significantly different from control; one-way ANOVA and Dunnett post hoc test.

Figure 2.

Changes in the expression of glutamate cysteine ligase in ARPE cell treated with 4-HNE. Cells were treated by 20 μ M of 4-HNE with or without LY294002 pretreatment. mRNA levels of modulatory (GCLM) and catalytic (GCLC) subunits of GCL were measured by real-time RT-PCR. The difference between average threshold cycles (ΔC_T) was determined and normalized to expression of 18S rRNA. Data presented are the average of three separate experiments.

* $P < 0.05$, significantly different from control; one-way ANOVA and Dunnett post hoc test.

Figure 3.

Quantification of HNE-protein adduct formation in APRE-19 cells. Cells were treated with 20 μ M of 4-HNE for 1 hour, with or without pretreatment of 10 μ M LY294002. HNE-induced protein modification was detected by Western blot (**A**) and dot blot (**B**) analyses using anti-HNE antibody. HNE-protein adduct formation affected by LY294002 was semiquantified by dot blot analysis, presented as a ratio of LY pretreated to untreated (C). Data presented are the average of four separate experiments. * $P < 0.05$; Student's t -test.

Figure 4.

Inhibition of the PI3K resulted in increased HNE adduct formation on HSC70 protein in ARPE cells. Immunoprecipitation (IP) experiments on ARPE cells after treatment with 4-HNE and LY294002, using antibodies as indicated. (A) IP by anti-HNE antibody and Western blot by anti-HSC70. (B) IP by anti-HSC70 and Western blot by anti-HNE. (C) IP by anti-HSC70 and Western blot by anti-HSC70. HNE-HSC70 adduct formation affected by LY294002 was semiquantified and presented as a ratio of LY pretreated to untreated (D). Data presented are the average of three separate experiments. * $P < 0.05$; Student's t -test.

Figure 5.

Changes in RPE cell viability by 4-HNE exposure. Cells were exposed to 4-HNE at indicated concentrations for 48 hours, with or without LY294002 pretreatment for 16 hours. Viable cell percentage was measured by flow cytometry. Data presented are the average of three separate experiments performed in duplicate (mean $\pm$ SE).

Figure 6.

Changes of ARE activity in ARPE cells treated with various Nrf2 inducers. Cells were exposed to sulforaphane (10 μ M), oltipraz (50 μ M), ZnCl₂ (100 μ M), sodium arsenate (2.5 μ M), cadmium chloride (10 μ M), diethylmelaimide (100 μ M), hydroquinone (25 μ M), t-hydroxylquinone (2.5 μ M), paraquate (100 μ M) and menadione (5 μ M). ARE activity was measured by luciferase reporter assay. Data presented are the average of three experiments performed in duplicate. * P < 0.05, ** P < 0.01, *** P < 0.001; Student's t -test.

Figure 7.

Effects of MAPK inhibitors on ARE activity. Cultured ARPE cells were pretreated with 1 μM SB203580 and 10 μM PD98059, followed by exposure to 4-HNE at indicated concentrations for 16 hours. Dimethyl sulfoxide (DMSO) was used as vehicle control. ARE activity was measured by luciferase reporter assay. Data presented are the average of four experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, significantly different from DMSO-treated sample; one-way ANOVA and Dunnett post hoc test.