The Role of N-Methyl-D-Aspartate Receptor Activation in Homocysteine-Induced Death of Retinal Ganglion Cells

Preethi S. Ganapathy,1,2 Richard E. White,7,5 Yonju Ha,1,2 B. Renee Bozard,1,2 Paul L. McNeil,1 R. William Caldwell,5 Sanjiv Kumar,4 Stephen M. Black,4 and Sylvia B. Smith1,2,5

PURPOSE. Elevated plasma homocysteine has been implicated in glaucoma, a vision disorder characterized by retinal ganglion cell death. The toxic potential of homocysteine to ganglion cells is known, but the mechanisms are not clear. A mechanism of homocysteine-induced death of cerebral neurons is via N-methyl-D-aspartate (NMDA) receptor overstimulation, leading to excess calcium influx and oxidative stress. This study examined the role of the NMDA receptor in homocysteine-mediated ganglion cell death.

METHODS. Primary mouse ganglion cells were used for these experiments. NMDA receptor stimulation by homocysteine was determined by patch clamp analysis and fluorescent detection of intracellular calcium. NMDA receptor involvement in homocysteine-mediated cell death was determined through assessment of lactate dehydrogenase release and TUNEL analysis. These experiments used the NMDA receptor blocker MK-801. Induction of reactive species superoxide, nitric oxide, and peroxynitrite was measured by electron paramagnetic resonance spectroscopy, chemiluminescent nitric oxide detection, and immunoblotting for nitrotyrosine, respectively.

RESULTS. 50 µM homocysteine stimulated the NMDA receptor in presence of 100 µM glycine. Homocysteine induced 59.67 ± 4.89% ganglion cell death that was reduced to 19.87 ± 3.03% with cotreatment of 250 nM MK-801. Homocysteine elevated intracellular calcium ~7-fold, which was completely prevented by MK-801. Homocysteine treatment increased superoxide and nitric oxide levels by ~40% and ~90%, respectively, after 6 hours. Homocysteine treatment elevated peroxynitrite by ~85% after 9 hours.

CONCLUSIONS. These experiments provide compelling evidence that homocysteine induces retinal ganglion cell toxicity through direct NMDA receptor stimulation and implicates, for the first time, the induction of oxidative stress as a potent mechanism of homocysteine-mediated ganglion cell death. (Invest Ophthalmol Vis Sci. 2011;52:5515–5524) DOI:10.1167/iovs.10-6870

Homocysteine is a nonproteinogenic amino acid that is an intermediate in methionine and cysteine metabolism. Severe elevations in plasma homocysteine (hyperhomocysteinemia) are rare and are caused by homozygous mutations in regulatory enzymes involved in homocysteine metabolism.1 Moderate elevations are much more prevalent and are caused by heterozygous mutations in these regulatory enzymes or by nutritional deficiencies in the vitamins folic acid, B12, or B6. Recently, studies have implicated such moderate elevations of homocysteine in the impairment of cognition and the pathogenesis of age-related degenerative disorders, particularly Alzheimer and Parkinson diseases.2–4 The mechanism of this homocysteine-induced neuronal stress appears to be via an increase in oxidative stress.5–6 In the brain, extracellular elevation in homocysteine is known to stimulate the N-methyl-D-aspartate (NMDA) receptor and induce an increase in intracellular calcium and oxidative stress.7–9

While much research has been conducted on the effects of excess homocysteine on cerebral and hippocampal neurons, much less is known about the effect of hyperhomocysteinemia on retinal neurons. Several clinical studies have implicated homocysteine in retinal degenerative disorders, including maculopathy, open-angle glaucoma, and diabetic retinopathy.10–18 In response to mounting clinical evidence associating hyperhomocysteinemia with retinal neurodegeneration, our laboratory has explored the effect of homocysteine on the viability of retinal ganglion cells. Our initial in vitro studies exploited a retinal neuronal cell line (RGC-5) that was recently determined to be derived from mouse.19 Using this cell line, we showed that millimolar concentrations of homocysteine were sufficient to induce cell death20 and, when the cells were chemically differentiated, they were susceptible to even lower levels of homocysteine.21 More recently, using freshly isolated ganglion cells (primary ganglion cells), we found that direct exposure of 50 µM DL-homocysteine induced ~50% to 60% cell death within 18 hours.22 Direct intravitreal injection of micromolar concentrations of homocysteine induced abundant cell death in the ganglion cell layer,23 providing the first in vivo report of ganglion cell death caused by homocysteine.

Subsequent in vivo work used a mutant mouse model of hyperhomocysteinemia to examine the effect of elevated plasma homocysteine on retinal morphology and ganglion cell viability.24 The mouse model was developed in the laboratory of Nobuyo Maeda,25 and harbors a deletion of the gene encoding cystathionine β-synthase (cbs), an enzyme responsible for the conversion of homocysteine to cysteine. As a result, the homozygous mice (cbs−/−) have a ~30-fold increase in plasma homocysteine and heterozygous mice (cbs+/−) have a ~4-fold increase in plasma homocysteine. The cbs−/− mice have a markedly shortened lifespan of 3 to 5 weeks and reportedly die of liver toxicity; cbs+/− mice, however, have a normal lifespan and are ideal for the investigation of ganglion cell loss caused

From the 1Department of Cellular Biology and Anatomy, 2Vision Discovery Institute, 3Department of Pharmacology, 4Vascular Biology Center, and 5Department of Ophthalmology, Georgia Health Sciences University, Medical College of Georgia, Augusta.

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Corresponding author: Sylvia B. Smith, Georgia Health Sciences University, Medical College of Georgia, Department of Cellular Biology and Anatomy, 1120 15th St, CB 2901, Augusta, GA 30912-2000; sbsmith@georgiahealth.edu.
by prolonged exposure to moderately elevated plasma homocysteine. To further augment plasma homocysteine, mice were fed a diet supplemented with 0.5% methionine, resulting in plasma homocysteine levels ~7-fold that of wild type mice (cbs/−/− HM, to signify heterozygous mice that were maintained on a high methionine diet). The retinas of cbs/−/− HM mice were subjected to systematic morphometric analysis at 5, 15, and 30 weeks after the onset of a high methionine diet; analysis revealed a ~20% loss of cells in the ganglion cell layer of mice as early as 5 weeks after the onset of a high methionine diet (8 weeks of age). describing for the first time the capability of endogenous elevation in plasma homocysteine to induce ganglion cell loss.

The mechanisms of homocysteine-induced ganglion cell toxicity are not clear. There has been only one study to date of the effect of homocysteine on the NMDA receptor of retinal ganglion cells; however, the experiments were performed using L-homocysteic acid, a metabolite of homocysteine, rather than L-homocysteine itself. Lipton et al. used HPLC analysis to show that when neuronal cultures were exposed to 100 μM of homocysteine for 6 days, only a negligible amount was oxidized to homocystic acid (well below levels needed to significantly stimulate the NMDA receptor). Therefore, in this study, we explore the mechanisms by which L-homocysteine induces retinal ganglion cell toxicity. To dissect the response of retinal ganglion cells apart from the complex retinal network (and the retinal ganglion cell toxicity. To dissect the response of retinal ganglion cells apart from the complex retinal network (and the contribution of other glial and neuronal cells) and examine specifically their behavior in the presence of excess homocysteine, we use the primary ganglion cell culture system and test the hypothesis that homocysteine acts directly on the NMDA receptor, resulting in increased oxidative stress and eventual cell death.

**METHODS**

**Isolation and Culture of Primary Ganglion Cells**

Ganglion cells were isolated from the retinas of 2- to 4-day-old C57BL/6 mice that were the offspring of breeding pairs purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Care and use of the mice adhered to the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Immunopanning procedures and verification of purity of the cells have been described in detail..

**Patch-Clamp Analysis**

Whole-cell currents were measured from primary ganglion cells using the perforated patch technique. This technique was used because perforated patch recordings provide accurate current measurement with minimal loss of soluble cytoplasmic components caused by cellular dialysis. In summary, cells were placed in a recording solution of the following composition: 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 20 mM HEPES, and 20 mM glucose (pH 7.2). The recording solution lacked Mg²⁺ because this ion blocks the NMDA receptor channel at depolarizing potentials. Patch pipettes with a resistance of 3 MΩ or less were made from capillaries by using a P-2000 laser pipette puller (Sutter Instrument Company, Novato, CA). To measure calcium currents, the tip of a patch pipette was filled with a solution containing 70 mM Cs₂SO₄, 10 mM HEPES, 2 mM MgCl₂, and 2 mM CaCl₂ (pH 7.4 with CsOH). The remainder of the pipette was back-filled with a similar solution to which 200 μg/ml amphotericin B was added. Cells were studied if the voltage drop across the series resistance was reduced to < 5 mV within 10 to 20 minutes after forming a GΩ seal. Voltage-clamp generation was controlled with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Sunnyvale, CA). Data were analyzed using pCLAMP 10.0 (Axon Instruments) and statistically analyzed using ANOVA. All drugs were diluted into fresh bath solution and perfused into an 0.5-mL recorder chamber (Warner Instruments Corp., Hamden, CT).

**Cell Death Assays**

Cell death was analyzed by measuring cytosolic LDH release and performing TUNEL analysis on homocysteine-treated cells in the presence and absence of MK-801. LDH analysis was performed using the Cytotoxicity Detection Kit (LDH) supplied by Roche Applied Sciences (Mannheim, Germany). In brief, cells were grown in 24-well plates and treated for 18 hours with homocysteine (50 μM) and varying doses of MK-801 (0–250 nM) in cell culture media; cells that were cotreated with MK-801 were also pretreated with the same dose of MK-801 for 1 hour LDH release into the supernatant and cellular LDH content were analyzed per the manufacturer’s instructions, and percent LDH release was calculated ([LDH] in supernatant/[LDH] in supernatant + cellular [LDH]).

TUNEL analysis was performed using the ApoTag fluorescein direct in situ apoptosis detection kit (Millipore, Temecula, CA) following our published method. Cells that were grown on coverslips were treated with homocysteine (50 μM) in the presence and absence of MK-801 (250 nM) for 18 hours. After TUNEL analysis, cells were counterstained with Hoechst 33,342 stain (Invitrogen) and viewed by epifluorescence using a Zeiss Axiosplan 2 microscope (Carl Zeiss Inc., Munich, Germany) equipped with the Axiovision program (version 4.6.3.0) and a high resolution microscopy (HRM) camera. Images were photographed (5 fields per coverslip) and examined for the presence of green fluorescent cells, indicative of cell death, and data were expressed as the number of dead cells per total number of cells counted. All experiments were performed in triplicate, and one-way ANOVA was the statistical test of significance (GraphPad Prism, La Jolla, CA).

**Intracellular Calcium Measurement**

Primary ganglion cells grown on coverslips were incubated with Fluo-4 AM using the Fluo-4 Direct Calcium Assay Kit (Invitrogen) for 30 minutes at 37°C in the absence of light. Coverslips were transferred to a 10-mm culture dish containing 2 mL of Mg²⁺-free recording solution. Using an Argon 488 excitation laser and an inverted laser scanning microscopy (LSM) 510 Meta confocal microscope (Carl Zeiss, Inc.). Images were taken every 15 seconds for a total of 405 seconds; these images served as controls. Subsequently, 1 mL of solution was removed and replaced with drug solution so that the final concentration was 50 μM homocysteine plus 100 μM glycine. Imaging was repeated as before. Individual cells were identified and the fluorescence intensities for each time point in treatment and control groups were measured using the Zeiss LSM 510 program (Carl Zeiss, Inc.). The difference in cellular fluorescence intensity between treatment and control groups was calculated (change in fluorescence) for each time point and expressed as a ratio compared to cellular fluorescence at time 0 seconds (baseline fluorescence). Data were plotted and one-way ANOVA was applied using the GraphPad Prism program.

For experiments using MK-801 as an inhibitor of NMDA receptor function, primary ganglion cells were grown on coverslips as stated previously. During incubation with Fluo-4, cells were also pretreated with 250 nM MK-801 for 30 minutes. Cells were mounted on the microscope in 10-mm culture dishes, and control images were taken. Cells were then treated with 250 nM MK-801 plus 50 μM homocysteine plus 100 μM glycine, images were taken, and fluorescence intensities were measured. The change in fluorescence/baseline fluorescence was then calculated and plotted. Data were statistically analyzed in comparison to the homocysteine plus glycine treatment data using two-way ANOVA (GraphPad Prism).

**Immunoblotting Analysis**

Protein extracted from primary ganglion cells was subjected to Western blot analysis following our published method. They were sub-
ject to SDS-PAGE and transferred to nitrocellulose membranes. These membranes were incubated with either a polyclonal rabbit anti-NMDA receptor antibody (1:1000; Cell Signaling, Beverly, MA), or a polyclonal rabbit anti-AMP receptor antibody (1:500; Abcam, Cambridge, MA), or a polyclonal rabbit anti-nitrotyrosine antibody (1:500; Alpha Diagnostics, San Antonio, TX) at 4°C overnight, followed by an HRP-conjugated goat anti-rabbit IgG antibody (Sigma) (1:3000). Proteins were visualized with the ECL Western blot detection system. Membranes were reprobed with mouse monoclonal anti-β-actin antibody (1:5000; Sigma) as a loading control. Band densitometry analysis was performed using Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and one-way ANOVA was used to statistically analyze the results (GraphPad Prism).

**Superoxide and Nitric Oxide Measurement**

Superoxide levels were measured directly in primary ganglion cells using electron paramagnetic resonance (EPR) analysis. Cells were grown in 6-well culture dishes and treated with 50 μM of homocysteine for 0, 0.5, 1, 3, and 6 hours. One hour before harvest, cells were treated with the spin-trap compound 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCL (CMH) at a final concentration of 200 μM, and the difference between EPR maximum and minimum was quantified as a representation of total CMH-SO2-. Magnetic strength of 333.95 to 3339.94 mT. The EPR spectrum for superoxide was collected in a Bio-Rad Spectrometer ESR (Magnettech) and the difference between EPR maximum and minimum for each sample was analyzed using ANALYSIS software (version 2.02; Magnettech, Germany). A capillary tube and analyzed using a MiniScope MS200 ESR (Magnettech, Germany) at a microwave power of 40 mW, modulation amplitude of 3000 mG, and modulation frequency of 100 kHz with a magnetic strength of 333.95 to 3339.94 mT. The EPR spectrum for superoxide was analyzed using ANALYSIS software (version 2.02; Magnettech). Band densitometry analysis was performed using Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). One-way ANOVA was used to statistically analyze the results (GraphPad Prism).

**RESULTS**

**Electrophysiologic Analysis of Homocysteine on Ion Flux**

Using freshly isolated retinal ganglion cells from mouse, we used whole-cell patch-clamp to determine whether homocysteine would stimulate NMDA receptor function and induce inward currents. A report from Lipton et al.9 indicated that while homocysteine possessed an affinity for the glutamate-binding site of the NMDA receptor, studies were conducted in an excess of glycine (100 μM). Previous studies from our laboratory have shown that 50 μM homocysteine is sufficient to induce robust primary ganglion cell death;22 therefore, we analyzed whether acute exposure of this concentration of homocysteine was also sufficient to stimulate NMDA receptors.

The application of 50 μM homocysteine plus 100 μM glycine was sufficient to induce a reproducible inward current in primary ganglion cells (Fig. 1A). Current kinetics were similar to previous reports32; the current contained a rapid depolarization component, followed by a slow desensitization component. To ensure that the mechanism of current generation was through NMDA receptor activation, the NMDA receptor channel blocker MK-801 was used. After administration of the agonist and coagonist (and visualization of inward current), current was allowed to return to baseline and 1 to 2 μM MK-801 plus 50 μM homocysteine plus 100 μM glycine was applied to the same cell. Application of the channel blocker concurrently with homocysteine plus glycine acutely inhibited NMDA receptor function and successfully abolished the inward current (Fig. 1B). The administration of 50 μM homocysteine plus 100 μM glycine resulted in a 252.2 ± 65% change in current. The administration of 1 to 2 μM MK-801 with 50 μM homocysteine plus 100 μM glycine significantly decreased this effect and resulted in only a 8.6 ± 4.7% change in current. These studies indicate that the inward current was generated by NMDA receptor activation.

![Figure 1](http://iovs.arvojournals.org/article-pdfaccess.asashx?url=/data/journals/iovs/933462/ on 04/05/2017)
show that homocysteine is able to induce an inward current in primary ganglion cells when in the presence of excess glycine, and that this inward current occurs through NMDA receptor activation.

**Analysis of NMDA Receptor Involvement in Homocysteine-Induced Cell Death**

Although our data supported the potential of homocysteine to activate NMDA receptor function, we were not certain that the mechanism of homocysteine-induced cell death was through NMDA receptor overstimulation. To determine the contribution of NMDA receptor in homocysteine-induced primary ganglion cell toxicity, we asked whether cell death could be inhibited by cotreatment with MK-801. Retinal ganglion cells immunopanned from mice were pretreated with MK-801 (0–250 nM) for 1 hour, then exposed to 50 μM homocysteine plus MK-801 for 18 hours; cells treated with MK-801 alone served as control. Percent cell death compared with control was analyzed by measurement of cytosolic LDH release (Fig. 2A). Cells that received no treatment exhibited low levels of cell death (1.67 ± 2.36%), while cells treated with MK-801 alone displayed ~10% to 20% cell death. Cells treated with 50 μM homocysteine had 59.66 ± 4.89% cell death after a period of 18 hours, consistent with previous findings. Cells exposed to homocysteine that were cotreated with MK-801 showed a dose-dependent decrease in percent cell death; cotreatment with 250 nM MK-801 significantly decreased homocysteine-induced cell death to levels comparable to that of MK-801 alone (19.87 ± 3.03%; P < 0.0002). (Cotreatment with 500 nM MK-801 resulted in increased cell death [data not shown].) To confirm this finding, additional primary ganglion cells were exposed to 50 μM homocysteine in the presence and absence

![FIGURE 2. Block of NMDA receptor inhibits homocysteine-induced primary ganglion cell death. (A) Percent LDH release compared with no treatment, representing percentage of total cell death. No treatment: 1.67 ± 2.36%; 25 nM MK-801: 12.49 ± 0.80%; 50 nM MK-801: 10.05 ± 1.03%; 100 nM MK-801: 16.51 ± 8.80%; 250 nM MK-801: 20.08 ± 9.95%; 500 nM MK-801: 14.60 ± 2.43%. Exposure to 50 μM homocysteine exposure alone: 59.67 ± 4.89%; homocysteine plus 25 nM MK-801: 47.10 ± 11.00%; homocysteine plus 50 nM MK-801: 43.48 ± 14.93%; homocysteine plus 100 nM MK-801: 33.47 ± 6.36; homocysteine plus 250 nM MK-801: 19.87 ± 5.03; homocysteine plus 500 nM MK-801: 49.56 ± 12.84 (P < 0.0002). (*Significantly different from homocysteine plus glycine treatment alone, P < 0.01; **P < 0.001; ***P < 0.0001). (B) Cell death as determined by TUNEL analysis; bright white (originally green) fluorescence indicates TUNEL positive cell, darker gray (originally blue) fluorescence indicates nuclear staining. (C) Quantification of TUNEL-positive cells, expressed per total cell number. No treatment: 2.54 ± 1.56%; 250 nM MK-801 alone: 20.67 ± 1.48%; 50 μM homocysteine alone: 48.87 ± 8.07%; 50 μM homocysteine plus 250 nM MK-801: 21.95 ± 4.82%. (Significantly different from homocysteine plus glycine treatment alone; P < 0.002).
of 250 nM MK-801; cell viability was assessed by analyzing percent TUNEL-positive cells (Figs. 2B, 2C). Control cells that received no treatment displayed very low levels of cell death (2.54 ± 1.56%); cells exposed to 50 μM homocysteine for 18 hours experienced a significantly higher percent cell death (48.87 ± 8.07%). Pretreatment with 250 nM MK-801 for 1 hour plus duration of homocysteine exposure reduced cell death significantly to 21.95 ± 4.82% (P < 0.002); this level of cell death was comparable to that of 250 nM MK-801 alone (20.67 ± 1.48%). Cumulatively, these data suggest that in vitro ganglion cell death induced by homocysteine is mediated, at least in part, by the stimulation of NMDA receptors.

Intracellular Calcium Measurement

When an agonist binds to its site on the NMDA receptor, the calcium channel within the receptor opens and allows a subsequent influx of calcium; this elevation in intracellular calcium triggers various downstream effects, including an increase in oxidative stress and eventual apoptosis.32–34 Patch-clamp analysis indicated that inward current increased after the administration of homocysteine, and that the inward current was abolished when homocysteine was coadministered with MK-801. However, this inward current could be attributed to any number of positive ions; it was not certain that calcium was involved. To investigate the involvement of calcium in homocysteine-mediated NMDA receptor stimulation, we used confocal microscopy to detect changes in a calcium-sensitive fluorescent dye (Fluo-4 AM) as a direct measure of intracellular calcium. After incubation with Fluo-4, primary ganglion cells were exposed to 50 μM homocysteine plus 100 μM glycine, and fluorescence was measured temporally over a 405-second period. Calcium levels increased after treatment with homocysteine and glycine (Fig. 3A), whereas treatment with 250 nM MK-801 plus 50 μM homocysteine plus 100 μM glycine produced no detectable increase in fluorescence (Fig. 3B).

To further analyze these results, the fluorescence intensity of six ganglion cell bodies per treatment group was evaluated and the change in fluorescence/baseline fluorescence was quantified for each time point (Fig. 3C). Calcium levels increased steadily after the administration of homocysteine and glycine; levels were significantly different from baseline as early as 75 seconds after treatment (P < 0.001). Maximal intracellular calcium levels were reached ~105 seconds after homocysteine exposure and were ~7-fold greater than baseline. Calcium levels remained ~7-fold greater than baseline for the duration of the experiment. Analysis of fluorescence intensity of cells treated with MK-801 plus homocysteine plus glycine indicated that there was no increase in fluorescence compared to baseline (P > 0.05). Statistical application of two-way ANOVA revealed that cells treated with homocysteine and glycine alone had significantly higher levels of intracellular calcium.

**FIGURE 3.** Homocysteine produces increased intracellular calcium levels via stimulation of the NMDA receptor. (A) Fluorescence intensity of calcium-sensitive dye at time 0 seconds and time 405 seconds after treatment with 50 μM homocysteine plus 100 μM glycine. (B) Fluorescence intensity of calcium-sensitive dye at time 0 seconds and time 405 seconds after treatment with 250 nM MK-801 plus 50 μM homocysteine and 100 μM glycine. (C) Quantification of fluorescence intensity data.
calcium than cells treated with MK-801 plus homocysteine plus glycine at 45 to 405 seconds after treatment (\(P < 0.0001\)), indicating that homocysteine-mediated stimulation of NMDA receptor in primary ganglion cells results in a significant increase in intracellular calcium.

**Immunoblotting Analysis of NMDA Receptor and AMPA Receptor Expression**

To determine whether the homocysteine-induced increase in intracellular calcium was caused by an increase in protein levels of receptors that mediate calcium entry (NMDA and AMPA receptors), immunoblotting was performed. Primary ganglion cells were exposed to 50 \(\mu\)M homocysteine for 0, 3, 6, 9, and 12 hours and protein was isolated. Results of immunoblotting using antibodies specific for NMDA receptor or AMPA receptor showed minimal changes in either protein over the time period studied (Fig. 4). Densitometry evaluation of these proteins compared to \(\beta\)-actin also indicated no differences (data not shown). It is unlikely that homocysteine-mediated increase in intracellular calcium involves increased protein expression of receptors that mediate calcium entry.

**Superoxide and Nitric Oxide Detection**

Overstimulation of the NMDA receptor is frequently accompanied by an increase in superoxide and nitric oxide\(^{35-37}\); therefore, we asked whether treatment of primary ganglion cells with homocysteine would alter the levels of these reactive species. Primary ganglion cells were exposed to homocysteine for 0, 0.5, 1, 3, and 6 hours. The cells were analyzed for superoxide levels using electron paramagnetic resonance spectroscopy, and the media was assayed for the presence of nitrites as a read-out of nitric oxide levels using chemiluminescence. Values generated were compared to baseline superoxide and nitric oxide content at 0 hours.

Superoxide production decreased initially; levels were 62.06 ± 17.54% of the initial levels after 0.5 hour of 50 \(\mu\)M homocysteine exposure. At time points after 0.5 hour, however, superoxide generation steadily increased through the period observed, such that levels at 6 hours were 139.00 ± 21.92% of baseline levels (time 0 hours). This increase in superoxide was significant when compared to levels at 0.5 hour (\(P < 0.039\); Fig. 5A). Nitric oxide production, on the other hand, increased immediately (at 0.5–1 hour) and levels remained elevated through the period examined (Fig. 5B); levels at 6 hours were significantly elevated to 185.00 ± 26.31% of initial levels (\(P < 0.015\)). The finding that superoxide and nitric oxide production was increased significantly after exposure to 50 \(\mu\)M homocysteine supported our hypothesis that primary ganglion cells are subjected to increased oxidative stress in response to an excess of the excitatory amino acid.

**Immunoblotting Analysis of Nitrotyrosine Expression**

Superoxide and nitric oxide combine to form peroxynitrite, a potent reactive species that is known to elicit ganglion cell death in retina.\(^{38}\) Although peroxynitrite is an extremely unstable molecule, it is known to nitrate protein tyrosine residues, creating nitrotyrosine. To determine the cumulative effect of increased superoxide and nitric oxide, and eventual peroxynitrite production on cellular health, immunoblotting against nitrotyrosine was performed. Primary ganglion cells were exposed to 50 \(\mu\)M homocysteine for 0, 3, 6, 9, 12, and 15
hours and the resulting protein was subjected to Western blotting analysis. Nitrotyrosine expression increased within 3 hours exposure to homocysteine, peaked at 9 hours after treatment, and decreased markedly by 12 hours (Fig. 6A). Densitometry analysis confirmed that peak nitrotyrosine expression was at 9 hours (Fig. 6B); the relative band density of nitrotyrosine compared to β-actin showed an increase of 84.00 ± 6.63% compared to baseline levels (time 0 hours; P < 0.02).

**DISCUSSION**

Hyperhomocysteinemia has been implicated in glaucoma, which is characterized by ganglion cell loss and optic neuropathy and is a leading cause of vision loss in the elderly. Several clinical studies reported a correlation between glaucoma and elevation of homocysteine in plasma and aqueous humor. Indeed, up to 25% to 30% of glaucomatous patients tested had elevation in plasma homocysteine. The etiology of glaucoma is multifactorial, and several mechanisms of glaucoma-associated ganglion cell death have been proposed including excitotoxicity, oxidative stress, ischemic stress, mitochondrial dysfunction, and neighboring glial activation. The association between hyperhomocysteinemia and glaucoma, coupled with the evidence of a ~20% loss of cells in the ganglion cell layer of the hyperhomocysteinemic cbs mutant mouse, prompted the current exploration of mechanisms of homocysteine-induced retinal ganglion cell death. In cerebral neurons, a primary action of homocysteine is to stimulate NMDA receptors and induce oxidative stress. Whether these mechanisms account for homocysteine-induced toxicity in retinal neurons is not known. In the present study, we analyzed comprehensively the involvement of the NMDA receptor and oxidative stress in homocysteine-mediated retinal ganglion cell death.

This study provides the first evidence that homocysteine mediates retinal ganglion cell death through its direct action on the NMDA receptor. This evidence was obtained using perforated patch-clamp analysis, measurement of intracellular calcium, and inhibition studies using the NMDA receptor channel blocker MK-801. The studies were performed in freshly isolated ganglion cells using L-homocysteine, the predominant form of plasma homocysteine in vivo. Physiologic concentrations of L-homocysteine were sufficient to act on the NMDA receptor acutely (within 10 seconds), inducing the opening of its ion channel, such that an inward current was detected by patch-clamp analysis. The inward current was completely abolished by cotreatment with MK-801. Fluorescent detection of intracellular calcium after exposure to physiologic levels of homocysteine showed an increase, which was inhibited by cotreatment with MK-801. Increased intracellular calcium is a hallmark of neuronal toxicity induced by excess NMDA receptor stimulation. Evidence suggests that short-term activation of the NMDA receptor (10–30 minutes) with its concomitant increase in intracellular calcium is sufficient to induce a cellular cascade, which includes mitochondrial dysfunction and induction of oxidative stress, resulting in neuronal death. In addition, dysregulation of calcium homeostasis has been implicated in glaucoma-associated ganglion cell death. These data demonstrated the ability of homocysteine to activate the NMDA receptor of retinal ganglion cells acutely, but provided no information on the potential of sustained stimulation of the NMDA receptor by homocysteine to induce retinal ganglion cell death. Therefore, we performed inhibition studies to ask whether NMDA receptor activation by homocysteine was a mechanism of inducing ganglion cell death over a period of 18 hours. Our data showed that homocysteine-mediated retinal ganglion cell death did involve the NMDA receptor because blockage of the ion channel ameliorated ganglion cell death. At the same time, not all homocysteine-induced ganglion cell death was prevented by cotreatment with MK-801, indicating that NMDA receptor overstimulation is not likely the sole mechanism of homocysteine-induced toxicity to ganglion cells. The present study did not attempt to distinguish between apoptotic mechanisms of cell death versus necrotic death. One of the measures we used to detect death, release of LDH, is characteristic of necrosis in vivo; however, in vitro cells undergo secondary necrosis after apoptosis. We also used the TUNEL assay as an indicator of death, which has been reported to detect both apoptotic and necrotic cell death. We have reported earlier that homocysteine-induced ganglion cell death increases the cleavage products of caspase proteins, which is relevant to the apoptotic death described in models of glaucoma. Our studies revealed that homocysteine activated the NMDA receptor, triggering increased intracellular calcium acutely. Interestingly, one study reported that ganglion cell death related to NMDA receptor activation in vivo occurred indirectly, because of an increase in AMPA receptor expression. To determine whether homocysteine acted through direct stimulation of the NMDA receptor or by altering NMDA and AMPA receptor expression, we examined protein levels of these receptors after homocysteine treatment over a period of 12 hours. Immunoblotting analysis of these receptors showed no alteration in protein levels. Taken together, these data implicate direct stimulation of the NMDA receptor as a mechanism of homocysteine-mediated retinal ganglion cell death.

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It is noteworthy that treatment of isolated ganglion cells with micromolar levels of homocysteine required the presence of elevated glycine (100 μM), a coagonist of the NMDA receptor, to stimulate the receptor. Inclusion of low levels of glycine

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933462/)

**Figure 6.** Homocysteine increases levels of nitrotyrosine. (A) Representative Western blot of nitrotyrosine levels showing that levels increase immediately after homocysteine treatment, peak at 9 hours, and decrease. β-actin was used as the loading control (Mr ~ 45 kDa). (B) Densitometry analysis of protein bands. 0 hours: 1.00 ± 0.00, 3 hours: 1.23 ± 0.08, 6 hours: 1.24 ± 0.30, 9 hours: 1.84 ± 0.07, 12 hours: 1.07 ± 0.18, and 15 hours: 0.75 ± 0.00. (*Significantly different from baseline [time 0 hours], P < 0.02.)
Müller cell may mediate retinal ganglion cell death under certain conditions. It is possible that in the presence of sustained retinal hyperhomocysteinemia, the Müller cell may enhance ganglion cell death. Additional work in our laboratory will analyze the contribution of other retinal cells to homocysteine-induced ganglion cell toxicity.

References


Homocysteine-Induced Retinal Ganglion Cell Loss 5523


