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

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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 μ M homocysteine stimulated the NMDA receptor in presence of 100 μ M glycine. Homocysteine induced $59.67 \pm 4.89\%$ ganglion cell death that was reduced to $19.87 \pm 3.03\%$ 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 $\sim 40\%$ and $\sim 90\%$, respectively, after 6 hours. Homocysteine treatment elevated peroxynitrite by $\sim 85\%$ after 9 hours.

CONCLUSIONS. These experiments provide compelling evidence that homocysteine induces retinal ganglion cell toxicity through direct NMDA receptor stimulation and implicate, 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/iops.10-6870

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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 folate, B₁₂, or B₆. Recently, studies have implicated such moderate elevations of homocysteine in the impairment of cognition and the pathogenesis of age-related neurodegenerative 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 μ M DL-homocysteine induced $\sim 50\%$ 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

by prolonged exposure to moderately elevated plasma homocysteine. To further augment plasma homocysteine, *cbs*^{+/-} 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).²⁶ 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),²⁴ 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 cells²⁷; however, the experiments were performed using L-homocysteic acid, a metabolite of homocysteine, rather than L-homocysteine itself. Lipton et al.⁹ used HPLC analysis to show that when neuronal cultures were exposed to 100 μ M 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 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/6J 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.^{28,29} Cells were grown in Neurobasal medium (Invitrogen, Carlsbad, CA), which contains a concentration of 400 μ M glycine. Cells were treated as described (DL-homocysteine, L-glycine, and MK-801 were purchased from Sigma [Saint Louis, MO]).

Patch-Clamp Analysis

Whole-cell currents were measured from primary ganglion cells using the perforated patch technique.³⁰ 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 M Ω 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 μ g/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 G Ω 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 μ M) 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 ApopTAG fluorescein direct in situ apoptosis detection kit (Millipore, Temecula, CA) following our published method.²¹ Cells that were grown on coverslips were treated with homocysteine (50 μ M) 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 Axioplan 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.), 27 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 μ M homocysteine plus 100 μ M 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 μ M homocysteine plus 100 μ M 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.²⁹ They were sub-

jected 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), a polyclonal rabbit anti-AMPA 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 reprobbed 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 μ M 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 0.4 mg/mL. Cells were collected in PBS and spun down to form a pellet. PBS was removed and cells were resuspended in EPR buffer (PBS plus 5 μ M diethyldithiocarbamate [DETC, Sigma] and 25 μ M desferrioxamine [Def MOS, Sigma]). Samples were loaded into a 50- μ L capillary tube and analyzed using a MiniScope MS200 ESR (Magnetech, Berlin, 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 each sample was analyzed using ANALYSIS software (version 2.02; Magnetech) and the difference between EPR maximum and minimum spectral amplitudes was quantified as a representation of total CM-superoxide spin-trap product. Protein levels for each sample were estimated by the published method of Bradford³¹ using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The EPR readings were then normalized to protein content and expressed as a ratio to baseline EPR values (time 0 hours; ratio of EPR readings).

Media from cells that were treated with 50 μ M homocysteine for 0, 0.5, 1, 3, and 6 hours were collected and analyzed for nitrite levels as a read-out of nitric oxide. The level of nitrite in media was analyzed using NO-specific chemiluminescence; samples were refluxed in glacial acetic acid plus sodium iodide, allowing nitrite to be reduced to nitric oxide and measured after a reaction with ozone. Nitric oxide was quantified using a chemiluminescence detector and nitric oxide analyzer (Sievers; GE Analytical Instruments, Boulder, CO). Values for each time point were obtained and expressed as a ratio to baseline nitrite levels (time 0 hours). One-way ANOVA was the statistical test used to

analyze EPR and nitrite readings and was applied using the GraphPad Prism program.

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 in cerebrocortical cultures, it also acted as a partial antagonist at the glycine-binding coagonist site. Preliminary studies involving the coadministration of physiologic levels of homocysteine and low levels of glycine (50 μ M homocysteine plus 10 μ M glycine) showed no increase in inward current and confirmed these findings (data not shown). Consequently, to isolate the neurotoxic potential of homocysteine through the NMDA receptor, studies were conducted in an excess of glycine (100 μ M). Previous studies from our laboratory have shown that 50 μ M homocysteine is sufficient to induce robust primary ganglion cell death²²; therefore, we analyzed whether acute exposure of this concentration of homocysteine was also sufficient to stimulate NMDA receptors.

The application of 50 μ M homocysteine plus 100 μ M 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 depolarizing 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 μ M MK-801 plus 50 μ M homocysteine plus 100 μ M 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. 1A). Traces were analyzed to calculate the percent difference between baseline and maximal inward current, expressed as the percent increase in inward current (Fig. 1B). The administration of 50 μ M homocysteine plus 100 μ M glycine resulted in a $252.2 \pm 65\%$ change in current. The administration of 1 to 2 μ M MK-801 with 50 μ M homocysteine plus 100 μ M glycine significantly decreased this effect and resulted in only a $8.6 \pm 4.7\%$ change in current. These studies

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 μ M homocysteine plus

MK-801 for 18 hours; cells treated with MK-801 alone served as control. Percent cell death compared with control was analyzed through measurement of cytosolic LDH release (Fig. 2A). Cells that received no treatment exhibited low levels of cell death ($1.67 \pm 2.36\%$), while cells treated with MK-801 alone displayed ~10% to 20% cell death. Cells treated with 50 μ M homocysteine had $59.66 \pm 4.89\%$ 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 ($19.87 \pm 3.03\%$; $P < 0.0002$). (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 μ M 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: $1.67 \pm 2.36\%$; 25 nM MK-801: $12.49 \pm 0.80\%$; 50 nM MK-801: $10.03 \pm 1.03\%$; 100 nM MK-801: $16.31 \pm 8.80\%$; 250 nM MK-801: $20.08 \pm 9.95\%$; 500 nM MK-801: $14.60 \pm 2.43\%$. Exposure to 50 μ M homocysteine exposure alone: $59.67 \pm 4.89\%$; homocysteine plus 25 nM MK-801: $47.10 \pm 11.00\%$; homocysteine plus 50 nM MK-801: $43.48 \pm 14.93\%$; homocysteine plus 100 nM MK-801: $33.47 \pm 6.36\%$; homocysteine plus 250 nM MK-801: $19.87 \pm 3.03\%$; 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: $2.54 \pm 1.56\%$; 250 nM MK-801 alone: $20.67 \pm 1.48\%$; 50 μ M homocysteine alone: $48.87 \pm 8.07\%$; 50 μ M homocysteine plus 250 nM MK-801: $21.95 \pm 4.82\%$. (*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 cell death ($2.54 \pm 1.56\%$); cells exposed to 50 μ M homocysteine for 18 hours experienced a significantly higher percent cell death ($48.87 \pm 8.07\%$). Pretreatment with 250 nM MK-801 for 1 hour plus duration of homocysteine exposure reduced cell death significantly to $21.95 \pm 4.82\%$ ($P < 0.002$); this level of cell death was comparable to that of 250 nM MK-801 alone ($20.67 \pm 1.48\%$). Cumulatively, these data suggest that in vitro primary 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 μ M homocysteine plus 100 μ M 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 μ M homocysteine plus 100 μ M 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

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 μ M homocysteine plus 100 μ M 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 μ M homocysteine and 100 μ M glycine. (C) Quantification of fluorescence intensity data.

FIGURE 4. Homocysteine does not greatly affect protein levels of NMDA and AMPA receptors. (A) Representative western blot of NMDA receptor ($M_r \sim 120$ kDa) and AMPA receptor ($M_r \sim 95$ kDa) expression showing that protein levels remain relatively constant after homocysteine exposure. β -actin was used as the loading control ($M_r \sim 45$ kDa). (B) Densitometry analysis of protein bands showing no significant change in protein levels ($P > 0.05$).

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 $50 \mu\text{M}$ 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 β -actin also indicated no differ-

ences (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 oxide³⁵⁻³⁷; 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 $62.06 \pm 17.34\%$ of the initial levels after 0.5 hour of $50 \mu\text{M}$ 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 $139.00 \pm 21.92\%$ 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 $183.00 \pm 26.31\%$ of initial levels ($P < 0.015$). The finding that superoxide and nitric oxide production was increased significantly after exposure to $50 \mu\text{M}$ 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 $50 \mu\text{M}$ homocysteine for 0, 3, 6, 9, 12, and 15

FIGURE 5. Homocysteine induces cumulative increase in superoxide and nitric oxide production. (A) Ratio of electron paramagnetic resonance (EPR) readings compared with 0 hours of homocysteine exposure (indicative of cellular superoxide content). 0.5 hour: 0.62 ± 0.17 ; 1 hour: 0.94 ± 0.15 ; 3 hours: 1.28 ± 0.07 ; 6 hours: 1.39 ± 0.22 . (*Significantly different from lowest superoxide content, at 0.5 hour; $P < 0.05$.) (B) Ratio of nitrate levels in media compared with 0 hours of homocysteine exposure (indicative of nitric oxide production). 0.5 hour: 1.14 ± 0.14 ; 1 hour: 1.73 ± 0.06 ; 3 hours: 1.81 ± 0.01 ; and 6 hours: 1.83 ± 0.26 . (*Significantly different from time 0 hours; $P < 0.05$.)

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 $84.00 \pm 6.63\%$ 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.³⁹ Several clinical studies reported a correlation between glaucoma and elevation of homocysteine in plasma and aqueous humor.^{40–42} Indeed, up to 25% to 30% of glaucomatous patients tested had elevation in plasma homocysteine.⁴³ 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.^{44–46} The association between hyperhomocysteinemia and glaucoma, coupled with the evidence of a $\sim 20\%$ loss of cells in the ganglion cell layer of the hyperhomocysteinemic *cbs* mutant mouse,²⁴ 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.^{7–9} 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 path-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.³⁴ In addition, dysregulation of calcium homeostasis has been implicated in glaucoma-associated ganglion cell death.^{47–51} 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.⁵² We also used the TUNEL assay as an indicator of death, which has been reported to detect both apoptotic and necrotic cell death.⁵³ We have reported earlier that homocysteine-induced ganglion cell death increases the cleavage products of caspase proteins^{20,23} 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.⁵⁴ 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.

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

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 ($M_r \sim 45$ 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: 0.73 ± 0.00 . (*Significantly different from baseline [time 0 hours], $P < 0.02$.)

(10 μ M) in experimental conditions had no effect on inward current or calcium flux. In cerebrocortical cultures, homocysteine acts as a partial antagonist at the glycine binding site, as well as an agonist at the glutamate binding site and an excess of glycine is necessary for homocysteine to stimulate the NMDA receptor at physiologic concentrations.⁹ Our experiments revealed that the nature of homocysteine stimulation of NMDA receptors in retinal ganglion cells is similar to that in cerebral neurons. Our hypothesis is strengthened by the observation that levels of glycine in retina are five-fold greater than those seen in plasma.⁵⁵

The second important finding of this study is that homocysteine-induced ganglion cell toxicity involves oxidative stress. Oxidative stress has been identified as a key pathogenetic factor in glaucoma, diabetic retinopathy, and AMD.^{56–59} Our work showed quantitatively that homocysteine stimulates superoxide and nitric oxide production within 1 hour of treatment. These two reactive species can induce stress independently, but can also combine to form peroxynitrite, a potent oxidative stress molecule. Analysis revealed that, at later time points (9–12 hours), levels of peroxynitrite were elevated. While it is not certain that the level of oxidative stress seen in our experiments is sufficient to directly induce significant cell death in the primary ganglion cell culture system, the observation that superoxide, nitric oxide, and peroxynitrite levels are elevated is noteworthy. Several studies have investigated the effect of elevated reactive oxygen species on retinal health and integrity, and evidence suggests that superoxide and nitric oxide may act as second messengers, which are elevated early on in pathogenesis and initiate various cellular cascades that culminate in neuronal death.^{57–58,60–64} In addition, numerous reports have shown that inhibition of reactive oxygen species ameliorates retinal neuronal death induced by various mechanisms; in fact, some studies even suggest that retinal neuronal death is dependent on early induction of reactive oxygen species.^{65–70} In addition, the direct effect of peroxynitrite on retina has been studied using models of diabetic retinopathy and NMDA-induced retinopathy. Peroxynitrite generation in these conditions induced breakdown of the blood–retinal barrier and toxicity to retinal ganglion cells, potentially through inhibition of nerve growth factor survival signaling.^{38,71,72} Our finding that homocysteine induces peroxynitrite generation implicates oxidative stress as an additional mechanism of homocysteine toxicity to ganglion cells.

While the current work has shown that at least two mechanisms are involved in homocysteine-induced ganglion cell death, NMDA receptor stimulation and oxidative stress induction, other mechanisms of homocysteine toxicity have been proposed, including endoplasmic reticulum stress and DNA acid damage.^{73–75} Whether these mechanisms are involved in homocysteine-mediated retinal ganglion cell death is not known but warrants future investigation. It is important to recognize that the present experiments were conducted using purified ganglion cell cultures, which permits analysis of a single cell type in response to stress. The system does not examine, however, contributions from neighboring cell types (neurons, glia, and vascular cells). We cannot assume that the in vivo system will mimic the in vitro system without accounting for the role of these neighboring cells. It is not known whether the NMDA receptor stimulation and oxidative stress production observed after homocysteine treatment in vitro will also occur in the *cbs*^{+/–} HM retina, nor is it known if the levels of these stressors will be sufficient to produce the retinal ganglion cell loss seen in this in vivo model system. For example, the Müller cell is known to buffer excess extracellular glutamate in retina and may play a similar role in attenuating toxic effects of homocysteine by removing it from the extracellular milieu. Conversely, it has been suggested that the

Müller cell may mediate retinal ganglion cell death under certain conditions.^{54,76} 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.

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