Short-Term Hyperhomocysteinemia-Induced Oxidative Stress Activates Retinal Glial Cells and Increases Vascular Endothelial Growth Factor Expression in Rat Retina

Insun Lee,1 Hwayoung Lee,2,3 Ji-Myung Kim,1 Eun Hye Chae,1
Soo Jung Kim,1 and Namsoo Chang1,4,5

1Department of Nutritional Sciences, Ewha Women’s University, Seoul, 120-750, South Korea
2Department of Anatomy, College of Medicine, Ewha Women’s University, Seoul, 120-750, South Korea
3Medical Research Center, Ewha Women’s University, Seoul, 120-750, South Korea
4Asia Food and Nutrition Research Institute, Ewha Women’s University, Seoul, 120-750, South Korea

Received November 21, 2006; Accepted January 31, 2007; Online Publication, May 7, 2007
doi:10.1271/bbb.60657

Hyperhomocysteinemia is associated with an increase in the incidence of vascular diseases, including retinal vascular diseases. We examined the effects of high plasma levels of homocysteine on retinal glial cells and vascular endothelial growth factor (VEGF) expression. Male Sprague-Dawley rats were fed either a 3.0 g/kg homocystine diet or a control diet for 2 weeks. The homocystine-diet group had higher plasma levels of homocysteine and thiobarbituric acid reactive substances (TBARSs) and lower plasma levels of folate, retinol, α-tocopherol, and retinal expression of CuZn superoxide dismutase (SOD) than the controls. The rats fed the homocystine-diet showed an increase in vimentin, glial fibrillary acidic protein (GFAP), and VEGF immunoreactivity in the retina as compared to the controls. The increase in vimentin immunoreactivity in the hyperhomocysteinemic rats was correlated with changes in GFAP immunoreactivity in astrocytes within the ganglion cell layer. We found for the first time that short-term hyperhomocysteinemia-induced oxidative stress activates retinal glial cells and increases VEGF expression in the retina.

Key words: hyperhomocysteinemia; retina; vimentin; glial fibrillary acidic protein; vascular endothelial growth factor

Hyperhomocysteinemia is associated with an increase in the incidence and progression of arterial occlusive disease,1 atherosclerosis,2 and retinal vascular diseases, including the development of diabetic retinopathy,3 ocular venous occlusion,4 and neovascular age-related macular degeneration.5 In addition, increased concentrations of plasma homocysteine in patients with open-angle glaucoma have been implicated in the pathogenesis of glaucomatous optic neuropathy.6 Intraocular injection of homocysteine in mice stimulated N-methyl-D-aspartate receptors and caused apoptotic cell death in the retinal ganglion cell layer (GCL) in a manner that resembled early diabetic degenerative processes.7 However, the mechanism by which elevated homocysteine concentrations might contribute to the development of retinal disease is obscure.

High plasma concentrations of homocysteine have been associated with various vascular-based diseases that are mediated by oxidative stress.8,9 The retina is particularly vulnerable to oxidative stress due to a high demand for oxygen, a high content of long-chain polyunsaturated fatty acids, and constant exposure to high-energy photons in visible light.10 In a line of cultured human retinal pigmented epithelial cells, the addition of homocysteine to the culture medium stimulated the expression of VEGF via ATF4-dependent activation of VEGF transcription.11

There is mounting evidence that VEGF plays multiple repair roles within the nervous system, which include angiogenesis, permeabilization of the blood-brain barrier, and neurotrophic, gliotrophic, and antiapoptotic actions. Changes in VEGF expression are associated with many angiogenesis-driven pathologies, including diabetic retinopathy (which is characterized by a loss of retinal capillaries that leads to progressive retinal ischemia), increased retinal vascular permeability, and the growth of new retinal vessels.12,13 Müller cells and astrocytes, which are the major types of glial cell within the retina, are responsible for...
modulating the microenvironment of individual neurons by maintaining tissue integrity and ion homeostasis, and these cells also regulate the transport, uptake, and metabolism of neurotransmitters.14) Even though there is evidence that suggests that glial cells might play a crucial role in development, injury repair, and regeneration in the nervous system and retina,15–17) the effects of hyperhomocysteinemia and homocystine diet-induced oxidative stress on retinal glial cells and retinal injury have not been studied to date.

Recently, we reported that folic acid reduced the cerebrovascular endothelial damage caused by a homocystine diet in rats.18,19) Several studies have focused on exploration of retinal injury prevention strategies through food substances with antioxidant properties8) or nutrients such as B vitamins.4) For this, the development of a proper animal model for retinal injury should be quite useful.

The purpose of the present study was to determine the effects of hyperhomocysteinemia induced by a 2-week-long homocystine diet on oxidative status in the plasma and retina, retinal glial cell activation, and VEGF expression in the rat.

Materials and Methods

Animals. The experimental protocol conformed to the guidelines of the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996). Male Sprague-Dawley rats (n = 24, 8 weeks old, 278.8 ± 6.4 g initial weight; Orient Company, Seoul, Korea) were housed individually in stainless steel cages under controlled conditions (25 °C, 12:12 h light:dark cycle). Eight rats were sacrificed to obtain baseline data. The remaining 16 rats were allocated to either the control group (group C, n = 8), which was fed the AIN-93 maintenance diet,20) or the experimental group (group H, n = 8), which was fed a diet that contained 3.0 g/kg homocystine, vitamin B-12, and folic acid. All the rats were allowed free access to food and water.

Measurement of plasma concentrations of homocysteine, vitamin B-12, and folic acid. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium. Samples of blood withdrawn from the heart were centrifuged at 1,750 × g for 15 min at 4 °C. The plasma concentration of homocysteine was measured by the high-performance liquid chromatography (HPLC; Waters 474; Waters, Milford, MA) fluorescence-based detection method developed by Araki and Sako.21) Plasma concentrations of vitamin B-12 and folic acid were measured using Dualcount Solid Phase No Boil radioimmunoassay kits (Diagnostic Products, Los Angeles, CA) based on detection of 57Co vitamin B-12 and 125I folic acid.

Measurement of plasma concentrations of retinol and α-tocopherol. Plasma retinol and α-tocopherol concentrations were measured using the HPLC (Waters 474; Waters) ultraviolet detection method developed by Bieri et al.22) First, 100 μl each of plasma and ethanol were mixed vigorously using a vortex mixer for 20 s. The solvent was evaporated under a stream of nitrogen gas. The lipid fraction that remained was dissolved in methanol and acetonitrile for injection into the chromatograph. Retinol and α-tocopherol was detected at wavelengths of 325 and 292 nm respectively.

Measurement of plasma concentrations of thiobarbituric acid-reactive substances. The concentration of thiobarbituric acid-reactive substances (TBARSs) in the plasma was measured using the method described by Yagi et al.23) A sample of plasma was mixed with 2.34 ml of sulfuric acid, 100 g/l of phosphotungstic acid, and 6.7 g/l of thiobarbituric acid. This mixture was incubated for 1 h at 95 °C before TBARSs were extracted with 3 ml of 1-butanol. After centrifugation at 1,750 × g for 10 min, the fluorescence of the butanol layer was measured at an emission/excitation wavelength of 553/515 nm using a luminescence spectrophotometer (Perkin-Elmer LS50, Boston, MA).

Immunohistochemistry. Five rats each from groups C and H were anesthetized with ether and perfused through the heart with 0.05 M phosphate-buffered saline (PBS), followed by 4 mM paraformaldehyde in PBS. Immunohistochemical staining of vimentin, glial fibrillary acidic protein (GFAP), and VEGF was carried out separately on 12-μm-thick cryostat sections of retina. The tissue sections were dried at room temperature, immersed in distilled water for 20 min, and then incubated with 0.1 mM hydrogen peroxide in absolute methanol for 20 min. The sections were then incubated with a blocking solution that contained normal goat serum (Vector Laboratories, Burlingame, CA) for 90 min at room temperature. For vimentin immunostaining, the sections were incubated at 4 °C overnight with a mouse monoclonal antivimentin antibody (BioGenex, San Ramon, CA) diluted to 1:500 in PBS that contained 0.001 M normal goat serum. Negative control sections were processed as described above, except that no primary antibody was used. The sections were then washed with PBS and then incubated with the secondary antibody (biotinylated antimouse IgG, 1:500 dilution; Vector Laboratories) for 1 h at room temperature. This was followed by incubation with avidin–biotin–peroxidase complex (1:100 dilution; Vector Laboratories). Finally, vimentin immunoreactivity was visualized with diaminobenzidine tetrachloride (Sigma-Aldrich, St. Louis, MO). The aforementioned method was used to immunostain GFAP and VEGF using a rabbit polyclonal anti-GFAP (1:500...
dilution; BioGenex) and anti-VEGF antibodies (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) respectively, and a biotinylated antirabbit secondary antibody (1:100 dilution; Vector Laboratories).

Western blot analysis. Samples of retinas from three rats each from groups C and H were snap-frozen in liquid nitrogen and then homogenized in lysis buffer, as described previously.18 Equal amounts of protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis. Immunoblotting was performed using a polyclonal antibody raised against LOX-1, CuZn superoxide dismutase (SOD) and VEGF (Santa Cruz Biotechnology). Immunoblotted membranes were then probed with a secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences, Arlington Heights, IL) and then developed using chemiluminescence. Densitometric measurements of protein concentrations were made using the Gel Doc 2000 gel documentation system and Quantity One software (Bio-Rad, Hercules, CA).

Statistical analysis. Data are presented as means ± SEM values. Data from different treatment groups were compared by Student’s t-test. A Wilcoxon signed-rank test was used to analyze the densitometric data from different treatment groups. Differences were considered to be significant at P < 0.05. Pearson correlation coefficients were used to assess associations among plasma concentrations of homocysteine, vitamins, and TBARSs. Data were analyzed using commercially available software (SAS Institute, Cary, NC).

Results

Weight gain, food intake, and plasma concentrations of homocysteine, folate, and vitamin B-12

There was no difference in weight gain or food intake between the control rats and the rats that consumed the homocysteine-rich diet (data not shown). Rats that consumed the homocysteine-rich diet developed hyperhomocysteinemia, and plasma concentrations of homocysteine in those rats were up to 2.5-fold higher than those in the control group after 2 weeks (Fig. 1). Plasma folate concentrations were significantly lower in the hyperhomocysteinemic rats than in the control group. By contrast, there was a significant increase in plasma concentrations of TBARSs in the hyperhomocysteinemic rats after 2 weeks (Fig. 2). There was a significant negative correlation between plasma concentrations of homocysteine and retinol (r = -0.733, p = 0.001, data not shown) and between plasma concentrations of homocysteine and α-tocopherol (r = -0.493, p = 0.038, data not shown). By contrast, plasma concentrations of TBARSs were correlated positively with concentrations of homocysteine (r = 0.579, p = 0.01, data not shown).

Fig. 1. Plasma Concentrations of Homocysteine (A), Folate (B), and Vitamin B-12 (C).
C, control group. H, homocystine-diet group. Values are the mean ± SEM of eight rats per group. ***Significantly different compared to control group, P < 0.001.

Retinal expression of LOX-1 and CuZn SOD

To examine homocysteine-induced oxidative stress in the retina, LOX-1 and CuZn SOD levels were determined by western blot analysis. Even though LOX-1 protein expression in the retina did not show any differences as between the hyperhomocysteinemic rats and the control group, retinal expression of the CuZn SOD decreased significantly in the hyperhomocysteinemic rats as compared with control (Fig. 3).

Responses to retinal injury: Upregulation of intermediate filament protein expression

Vimentin and GFAP are retinal intermediate filament proteins. Changes in the pattern of vimentin and that of GFAP immunoreactivity were very similar (Fig. 4). In the hyperhomocysteinemic rats, there was a substantial increase in the intensity of vimentin immunoreactivity in
the filaments of Müller cells that extended from the 
nerve fiber layer (NFL) into the outer nuclear layer 
(ONL) of the retina (Fig. 4B, arrowhead) as compared to 
a normal retina (Fig. 4A). Weak vimentin immunoreac-
tivity was observed in Müller cell bodies in the inner 
nuclear layer (INL) (Fig. 4B, arrow). GFAP immunor-
reactivity in the hyperhomocysteinemic rats was also 
substantially more intense than in the control group 
(Fig. 4C, D). The intensity of GFAP immunoreactivity 
in the hyperhomocysteinemic rats was also 
substantially more intense than in the control group 
(Fig. 4C, D). The intensity of GFAP immunoreactivity 
in the hyperhomocysteinemic rats increased in the 
processes of astrocytes and Müller cells in the inner 
plexiform layer, the INL, and the ONL (Fig. 4D, 
arrowhead). GFAP immunoreactivity in ganglion cells 
(Fig. 4D, arrowheads) and Müller cell bodies (Fig. 4D, 
arrow) was weak.

**Responses to retinal injury: VEGF expression in glial 
cells and retinal neurons**

VEGF immunoreactivity in the retina was signifi-
cantly more intense in the hyperhomocysteinemic rats 
than in the control group (Fig. 5A, B). VEGF immu-
moreactivity was detected predominantly within the GCL 
(Fig. 5B, arrowhead) and INL (Fig. 5B, arrow). Western 
blot analysis of VEGF protein expression in the retina 
revealed significantly more VEGF protein expression in 
the retinas of hyperhomocysteinemic rats than in the 
control group (Fig. 5C).

**Discussion**

The present study was designed to determine whether 
hyperhomocysteinemia in rats induces retinal dysfunc-
tion *via* an increase in oxidative stress, activation 
of retinal glial cells, and upregulation of the retinal 
vascular system. We found that a diet-induced increase 
in plasma concentrations of homocysteine caused retinal 
abnormalities that were reflected by increases in 
vimentin, GFAP, and VEGF. However, due to limited 
amounts of retinal samples, we could not confirm the 
immunohistochemical data for vimentin or GFAP with 
Western blots.

We found that a homocysteine-rich diet that caused 
hyperhomocysteinemia was associated with a decrease 
in plasma concentrations of folate and antioxidant 
vitamins such as retinol and *α*-tocopherol, while the 
plasma concentration of TBARSs increased. We also 
found that hyperhomocysteinemia caused a significant 
decrease in the expression level of retinal CuZn SOD. 
These findings suggest that there was an increase in 
oxidative stress within the retina due to a decrease in the
availability of antioxidants as well as to the antioxidant defense system in the retina itself.

We also identified LOX-1, a main mediator of endothelial dysfunction, as another oxidative stress marker in the retina. Even though CuZn SOD expression was attenuated in the retina, a 2-week-long homocysteine-rich diet did not increase the expression levels of LOX-1. We are not aware of any study in which the retinal expression of LOX-1 varies with changes in plasma homocysteine levels. Further studies are needed to clarify these associations.

 Müller cells are the predominant type of glial cell within the vertebrate retina. The cell bodies of Müller cells lie close to the middle of the INL, and these cells extend processes vertically across the full thickness of the retina. Astrocytes are another major type of glial cell within the retina, and these cells are distributed throughout the entire retina, except in nonvascular areas. In addition to providing structural support to the nervous system, retinal glial cells play an important role in the metabolism of neurotransmitters, modulating synaptic neurotransmission and maintaining homeostasis of the microenvironment of neurons.

There is mounting evidence that glial cells are important in the maintenance of normal neuronal activity, and that they protect the integrity of the nervous system by regulating bidirectional communication.

Glial cells within the nervous system are cited increasingly frequently as participants in the patho-

---

**Fig. 4.** Immunohistochemical Staining for Vimentin (A, B) and Glial Fibrillary Acidic Protein (GFAP) (C, D) in the Retinas of Control Rats (A, C) and Hyperhomocysteinemic Rats (B, D).

A, Normal vimentin immunoreactivity in control rats. B, Vimentin immunoreactivity in the processes of Müller cells that extended from the nerve fiber layer (NFL) to the outer nuclear layer (ONL) (arrowhead) and in Müller cell bodies within the inner nuclear layer (INL) (arrow). C, Normal GFAP immunoreactivity in control rats. D, GFAP immunoreactivity in the processes of astrocytes and Müller cells (arrowhead), ganglion cells (arrowheads), and Müller cell bodies (arrow). GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; IS/OS, inner segment/outer segment. Original magnification ×400. Scale bars = 1 μm.

**Fig. 5.** Immunohistochemical Staining for Vascular Endothelial Growth Factor (VEGF) (A, B) and Immunoblotting Analysis of VEGF Protein Expression (C) in Retina.

A, Normal VEGF immunoreactivity in control rats. B, VEGF immunoreactivity in the GCL (arrowhead) and INL (arrow). Abbreviations are as in the legend to Fig. 4. Original magnification ×400. Scale bars = 1 μm. C, Densitometric analysis of immunoblots of VEGF expression. Data are normalized to the level of β-actin expression. All data are the mean ± SEM values for three rats from each group. **Significantly different as compared to the control group, *P* < 0.01.
ical course of neuronal damage due to mechanical, ischemic, and various other types of injury. A characteristic feature of nerve tissue injury or stress is the activation of glial cells, which is characterized by an increase in the size and number of glial cells and the upregulation of intermediate filament proteins within the affected tissue.\(^{33-35}\) As a consequence of retinal degeneration, expression of the two major intermediate filament proteins in the retina, vimentin and GFAP, increases dramatically in Müller cells and astrocytes.\(^{36}\) In the present study, we observed vimentin expression predominantly in the filaments of Müller cells that extended from the NFL to the ONL. In addition, relatively weak vimentin immunoreactivity was detected in the INL, which is where the cell bodies of Müller cells are located. The aforementioned pattern of vimentin expression was paralleled by intense GFAP immunoreactivity in the NFL and GCL, which is where astrocytes are located, and also in the NFL and ONL. In the normal retina, astrocytes act as surveillance cells that intervene during acutely destabilizing situations to repair damage due to minor trauma and to protect against changes in homeostasis.\(^{37}\) Astrocytes have many other functions, which include ensheathing the axons of ganglion cells and forming adherent junctions with other glial cells such as Müller cells.\(^{38}\) Müller cells play an important role in modulating neuronal activity and maintaining retinal homeostasis by regulating intra- and extracellular concentrations of calcium, potassium, glutamate, and \(\gamma\)-aminobutyric acid.\(^{29,38,39}\) Retinal Müller cells are believed to provide metabolic support to retinal neurons, because these cells are the principal site within which glycogen is stored in the retina. During metabolic stress caused by insults such as hypoglycemia, the breakdown of glycogen within Müller cells might provide crucial metabolites (e.g., lactic acid) to energy-deprived neurons.\(^{40}\) In our previous study,\(^{18}\) expression of glucose transporter protein was found to decrease in the brains of hyperhomocysteinemic rats, which suggests that hyperhomocysteinemia might lead to glucose deprivation via harmful effects on the glucose transporter system, which stimulates Müller cells. Therefore, the upregulation of vimentin and GFAP expression within Müller cells that we observed in hyperhomocysteinemic rats in the present study suggests that increased plasma concentrations of homocysteine might be detrimental to neuronal tissues such as the retina, which have a high demand for glucose.

Hyperhomocysteinemia is an independent risk factor for atherosclerosis and atherothrombosis, and it is associated with vascular endothelial cell injury,\(^{41}\) proliferation of vascular smooth muscle cells,\(^{42}\) and activation of the coagulation cascade.\(^{43}\) It has been proposed that VEGF plays an important role in the progression of atherosclerosis, induction of the migration and proliferation of endothelial cells, enhancement of vascular permeability, and stimulation of angiogenesis.\(^{44}\) Recently, homocysteine was reported to increase VEGF expression in macrophages\(^{45}\) and retinal pigment epithelium \textit{in vitro}.\(^{56}\) In the present study, hyperhomocysteinemia induced by a 2-week-long homocysteine-rich diet was associated with strong VEGF expression in glial cells and some neurons within the NFL, GCL, and INL. An increase in VEGF expression has been associated with hypoxia and hypoxia-induced generation of reactive oxygen species.\(^{47}\) The effects of hyperhomocysteinemia on plasma concentrations of retinol, \(\alpha\)-tocopherol, and TBARSs and the expression of retinal CuZn SOD that we observed in the present study suggest that plasma concentrations of homocysteine attenuate systemic and local antioxidant defense systems and/or induce hypoxia. We speculate that hyperhomocysteinemia-induced endothelial dysfunction can cause chronic vascular damage, subsequent breakdown of the blood-retinal barrier (BRB), and a reduction in the flow of blood to retinal neurons and glia, which would result in endothelial hypoxia.\(^{48,49}\) If the BRB is damaged due to hypoxia, there might be a decrease in the vascular supply of oxygen and nutrients to the retina, which might stimulate VEGF production in glial cells and retinal neurons. Hence, the metabolism of reactive oxygen species generated in response to hyperhomocysteinemia might induce the aforementioned retinal injuries.

In summary, we have found, to our knowledge for the first time, that short-term hyperhomocysteinemia-induced oxidative stress activated retinal glial cells and increased VEGF expression in the retina. Hyperhomocysteinemic rats appear to be a good animal model to study retinal injury. Further studies are required to determine whether nutrients such as folate ameliorate retinal dysfunction induced by hyperhomocysteinemia.

Acknowledgment

This study was supported by a grant from the Korea Research Grant Foundation funded by the Korean Government (KRF-2005-C00088-I00261).

References

4. Cahill, M. T., Stinnett, S. S., and Fekrat, S., Meta-analysis of plasma homocysteine, serum folate, serum vitamin B(12), and thermolabile MTHFR genotype as risk factors for retinal vascular occlusive disease. \textit{Am. J.


36) Lewis, G. P., and Fische, S. K., Up-regulation of glial fibrillary acidic protein in response to retinal injury: its


