Role of Mitochondrial Superoxide Dismutase in the Development of Diabetic Retinopathy

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PURPOSE. Apoptosis of retinal capillary cells is an early event in the pathogenesis of retinopathy in diabetes, and oxidative stress has been linked to accelerated apoptosis of retinal capillary cells. Mitochondria are the major endogenous source of superoxide, and superoxide is considered to be a causal link between elevated glucose and the major biochemical pathways postulated to be involved in the development of vascular complications in diabetes. The purpose of the present study is to determine the role of mitochondrial superoxide dismutase (MnSOD) in the development of diabetic retinopathy.

METHODS. The effect of overexpression of MnSOD on glucose-induced endothelial cell oxidative stress, nitrosative stress, and apoptosis was determined by using bovine retinal endothelial cells. Furthermore, the effect of diabetes in rats (11 months' duration) on the activity and the mRNA expression of retinal MnSOD were also determined.

RESULTS. MnSOD activity in the nontransfected control retinal endothelial cells was 20% compared with the total SOD activity and was increased to 60% in the MnSOD-transfected cells. MnSOD overexpression prevented a glucose-induced increase in oxidative stress (8-hydroxy guanosine levels), nitrosative stress (nitrotyrosine formation), and apoptosis of retinal endothelial cells. MnSOD enzyme activity and its mRNA were decreased significantly in the retina obtained from the diabetic rats, and these abnormalities were prevented by long-term lipoic acid therapy.

CONCLUSIONS. The results of this study suggest a protective role for MnSOD in retinal capillary cell death and, ultimately, in the pathogenesis of retinopathy in diabetes. Understanding the role of MnSOD to modify the course of retinopathy could elucidate important molecular targets for future pharmacological interventions. (Invest Ophthalmol Vis Sci. 2006;47: 1594–1599) DOI:10.1167/iovs.05-1276

Diabetes increases oxidative stress in the retina: the levels of lipid peroxide, thiobarbituric acid substances, and superoxide are increased in the retina.1,2 This increase in oxidative stress can be the result of several diabetes-induced abnormalities, including auto-oxidation of glucose, the formation of advanced glycation end products, and impairments in the antioxidant defense system.3–5 Reactive oxygen species (ROS) are considered to be a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications.6 ROS are closely linked to apoptosis in a variety of cell types. Underlying mechanisms involve increased membrane lipid peroxidation, increased oxidative injury to other macromolecules, or alterations in signal transduction.7

Mitochondria are the major endogenous source of superoxide, peroxynitrite, and hydroxyl radicals.8 Mitochondrial superoxide production is considered as a single unifying mechanism for diabetic complications.2 Release of cytochrome c from the mitochondria is triggered by ROS, and, in addition, increased lipid peroxidation itself damages mitochondrial membrane potential, provoking apoptosis.10,11 In diabetes, retinal mitochondria experience dysfunction; they become leaky when the duration of diabetes is such that capillary-cell apoptosis can be observed.12 Superoxide levels are increased in the retina and its capillary cells, and inhibition of superoxide prevents glucose-induced mitochondrial dysfunction and apoptosis in retinal capillary cells.2,12 The activity of superoxide dismutase (SOD), an enzyme known to scavenge superoxide, is decreased in the retina in diabetes, and its expression is downregulated.4,5 The therapy that inhibits the development of retinopathy in diabetic rats, including aminoguanidine and antioxidants, prevents a diabetes-induced decrease in superoxide accumulation and SOD activity in the retina.2,4 Overexpression of SOD is shown to reduce oxidative stress; decrease mitochondrial release of cytochrome c and apoptosis in neurons; and, in mice, prevent diabetes-induced glomerular injury, thus suggesting a major role of SOD in the regulation of apoptosis.13–15

The purpose of the present study is to determine the role of mitochondrial SOD (MnSOD) in the development of diabetic retinopathy. We have investigated the effect of overexpression of MnSOD on glucose-induced retinal endothelial cell oxidative stress, nitrosative stress, and apoptosis. Furthermore, the effect of long-term diabetes and the therapy that is shown to inhibit retinal capillary-cell apoptosis and histopathology in diabetic rats is determined on the activity and the mRNA expression of retinal MnSOD.

METHODS

Retinal Endothelial Cells

Endothelial cells were prepared from bovine retina12,16 and were cultured to 80% confluence in petri dishes coated with 0.1% gelatin. The culture medium consisted of Dulbecco’s Modified Eagle Medium containing 15% fetal calf serum (heat inactivated), 5% replacement serum (Nu-serum; BD Bioscience, San Jose, CA), heparin (50 μg/mL), endothelial growth supplement (25 μg/mL), and antibiotic/antimycotic in an environment of 95% O2 and 5% CO2. Confluent cells from the 4th to 6th passage were used to transiently transfect with MnSOD plasmids.

Transient Transfection of Retinal Endothelial Cells

Transfection complex was allowed to form by incubating MnSOD expression plasmid DNA17 or pGIL3 (luciferase control plasmid expression vectors) with superfet transfection reagent (Qiagen, Valencia, Investigative Ophthalmology & Visual Science, April 2006, Vol. 47, No. 4 Copyright © Association for Research in Vision and Ophthalmology

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CA) for 10 minutes at room temperature. Serum-free medium was added to the transfection complex and was mixed by pipetting up and down a few times. This transfection complex was added to a petri dish that contained endothelial cells (60%–80% confluency) in 1-mL medium. This medium was replaced after 8 hours with fresh medium that contained 2.5% fetal calf serum (heat inactivated), 10% Nusum serum, heparin (50 μg/mL), endothelial growth supplement (2.5 μg/mL), and antibiotic/antimycotic supplemented with 5 or 20 mM glucose. Parallel osmotic controls were run by incubating the cells in 20 mM mannitol instead of 20 mM glucose. The cells were incubated for 48 to 96 hours, washed with PBS, and lysed by using 300 μL of the lysis buffer. The cells were scraped, centrifuged, and used for biochemical measurements; the supernatant was used to determine the efficiency of the transfection by luciferase assay. The efficiency of the transfection was approximately 20% to 25%. The transfection was repeated at least four times by using three different endothelial cell preparations.

**Diabetes in Rats**

Wistar rats made diabetic with streptozotocin (55 mg/kg body weight) were divided into 2 groups: 1 group of diabetic rats received a powder diet (Purina 5001; Purina, Brentwood, MO) supplemented with lipoic acid (400 mg/kg), and the other group received a diet without any supplementation, as recently reported by us. Glycated hemoglobin was measured at 2 months of diabetes and every 5 months thereafter by using affinity columns (kit 442-B, Sigma Chemicals, St. Louis, MO). Diabetic rats who received a diet supplemented with or without lipoic acid and age-matched normal rats were killed at 11 months of diabetes by overdose of pentobarbital. The retina was isolated under a dissecting microscope and used for biochemical measurements. The treatment of the animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

**Enzyme Activity of SOD**

The enzyme activity of SOD was measured in 5 to 10 μg protein (retina or endothelial cells) with a kit from Cayman Chemical (Ann Arbor, MI). The method uses tetrathionate salt to quantify superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve was generated by using a quality-controlled SOD standard. MnSOD activity was determined by performing the assay in the presence of potassium cyanide to inhibit Cu-Zn SOD and thus measuring the residual MnSOD activity.

**Protein Expression of MnSOD**

The expression of MnSOD was determined by the Western blot technique. The cells were homogenized in 30 mM Tris-HCl buffer containing 10 mM EGTA, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 15 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 1 mM Na3VO4. Protein (30–40 μg) was separated on 12% denaturing polyacrylamide gel and then was transferred to nitrocellulose membranes. The membranes were blocked with 5% milk, followed by incubation with antibody against MnSOD (Binding Site, Birmingham, UK). The membranes were washed and incubated for 1 hour at room temperature with horseradish peroxidase linked anti-rabbit IgG (1:2500 dilution; Amersham Biosciences, Piscataway, NJ), and developed by using ECL-Plus Western peroxidase linked anti-rabbit IgG (1:2500 dilution; Amersham Biosciences). The expression of the housekeeping protein, β-actin, was determined by using mouse monoclonal antibody against β-actin (Sigma Chemicals).

**8-Hydroxy-2’-Deoxyguanosine**

8-Hydroxy-2-deoxyguanosine (8-OHdG) levels were measured by performing an ELISA with a kit from Oxis Research Laboratories (Portland, OR) (Kowluru RA, et al. IOVS 2005;46:ARVO E-Abstract 422). DNA was purified from the cells, digested with DNase, and used for the assay to enhance the accuracy and the reproducibility of the measurements. The 8-OHdG standard (0.5–40 ng/mL) was incubated for 1 hour with monoclonal antibody against 8-OHdG in a microtiter plate precoated with 8-OHdG. The final color was developed by the addition of 3,3,5,5-tetramethylbenzidine, and absorbance was measured at 450 nm.

**Nitrotyrosine**

Nitrotyrosine levels were quantified by enzyme immunoassay by using a nitrotyrosine-ELISA kit from Oxis Research according to the instructions provided with the kit. The nitrotyrosine standard or cells were incubated with nitrotyrosine antibody in the microplate for 1 hour; this was followed by incubation with streptavidin peroxidase for 1 hour. The samples were incubated with tetramethylbenzidine for 30 minutes, the reaction was stopped with 2.0 M citric acid, and the formation of the product was measured at 450 nm.

**Cell Apoptosis**

Endothelial cell apoptosis was determined by ELISA by using a Cell Death Detection ELISA PLUS kit from Roche Diagnostics (Indianapolis, IN) and was confirmed by measuring the enzyme activity of caspase-3. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified by using monoclonal antibodies directed against DNA and histones, respectively, as described by us previously. The cytoplasmatic fraction of the cells was transferred onto a streptavidin-coated microtiter plate and incubated for 2 hours at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone. The plate was washed thoroughly, incubated with 2.2-azino-di-[3-ethylbenzthiazoline sulfonate] diaminonium salt (ABTS; Roche Diagnostics), and absorbance was measured at 405 nm. After separation of the cytoplasmatic fraction, the nuclear pellet was suspended in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM NaCl, 0.05 mM NaHPO4 (pH 7.5), and was sonicated. DNA was measured in this fraction, and apoptosis was normalized to the amount of DNA.

Caspase-3 activity was measured in the cells by measuring the cleavage of Ac-DEVD-pNA. The absorbance of p-nitroanilide formed was measured at 405 nm (BIOMOL Research Laboratories, Plymouth Meeting, PA). Each sample was measured in duplicate.

**mRNA Levels**

The mRNA levels of MnSOD and Cu-Zn SOD were determined by real-time RT-PCR in a LightCycler (Roche Diagnostics) by using SYBR Green I (Sigma) with the method previously used in our laboratory. The reaction mixture consisted of FastStart DNA SYBR Green I master mix, forward and reverse primers (MnSOD: CTG AGG AGA GCA GCG CTT GCT forward and GTC CCC ATA TTG GTA CTT forward and reverse primers; Cu-Zn SOD: GTT CCG AGG CCG GGC GTG forward and GTC CCC ATA TTG AGC GAC reverse), MgCl2, and cDNA. An additional signal acquisition step was added after the elongation phase to allow for signal acquisition specifically from the target sequence. The mRNA levels were quantified by the standard curve method by using a serially diluted standard template and was normalized to the mRNA of β-actin in each sample.

**Statistical Analysis**

Data are reported as mean ± SD. The experimental groups were compared by using the nonparametric Kruskal-Wallis test followed by the Mann–Whitney test. Similar conclusions were reached also by using ANOVA with Fisher test or Tukey test.
RESULTS

Overexpression of MnSOD in Retinal Endothelial Cells

The protein expression of MnSOD, as determined by Western blot technique, was increased by 60% in the retinal endothelial cells that were transfected with MnSOD compared with the nontransfected control cells (Fig. 1a). In the same transfected cells, MnSOD activity contributed to approximately 60% of the total SOD activity compared with 20% in the nontransfected control cells (Fig. 1b).

Effect of Overexpression of MnSOD on Oxidative Stress

As shown in Figure 2, incubation of retinal endothelial cells with 20 mM glucose increased 8-OHdG levels by 75% compared with the cells incubated in 5 mM glucose medium; but this glucose-induced increase in 8-OHdG levels was prevented when the cells were overexpressed with MnSOD. 8-OHdG levels were similar in the normal control cells incubated in 5 mM glucose medium and the MnSOD transfected cells incubated in 5 or 20 mM medium.

The effect of 20 mM glucose on oxidative stress was not caused by osmotic changes, because the cells incubated in 20 mM mannitol had similar levels of 8-OHdG as obtained from the cells incubated in 5 mM glucose.

Effect of Overexpression of MnSOD on Nitrosative Stress

When retinal endothelial cells were incubated with 20 mM glucose for 3 days, nitrotyrosine levels were elevated by 55%. However, transfection of the cells with MnSOD prevented a glucose-induced increase in the nitrotyrosine levels (Fig. 3). Nitrotyrosine levels obtained from the cells incubated in 20 mM mannitol were similar to those from the cells incubated in 5 mM glucose.

Protection of Apoptosis by MnSOD Overexpression

Cell apoptosis, as determined by ELISA, was increased by 60% in the endothelial cells incubated in 20 mM glucose medium (Fig. 4a). In the same cells, caspase-3 activity was increased by 30% compared with the cells incubated in 5 mM glucose (Fig. 4b). MnSOD overexpression in the endothelial cells inhibited a glucose-induced increase in cell apoptosis as determined by both ELISA and caspase-3 activity; caspase-3 activity was not significantly different in the normal control cells incubated in 5 mM glucose medium and the MnSOD transfected cells incubated in 5 or 20 mM medium.

Effect of Diabetes and Lipoic Acid Therapy on Retinal MnSOD Activity

Activity of MnSOD was decreased significantly in the retina obtained from the diabetic rats (11 months’ duration) compared with the age-matched normal control rats (Fig. 5). Furthermore, mRNA level of MnSOD was subnormal in the retina.
of the diabetic rats compared with the age-matched normal rats, while that of cytosolic SOD (Cu-Zn SOD) was similar in both normal and diabetic rats (Fig. 6).

Long-term administration of lipoic acid to the rats, which we have shown to inhibit capillary cell apoptosis and development of diabetic retinopathy, inhibited a diabetes-induced decrease in the activity and mRNA levels of retinal MnSOD. These beneficial effects of lipoic acid were not because of amelioration of the severity of hyperglycemia; glycated hemoglobin (GHb), body weight, and urine volumes were comparable between the diabetes and diabetes plus lipoic acid groups, as reported by us previously.

**DISCUSSION**

This is the first report suggesting that MnSOD provides protection to the retinal endothelial cells from a glucose-induced increase in oxidative stress, nitrosative stress, and apoptosis. In diabetes, the activity of MnSOD and its mRNA remain subnormal in the rat retina at a duration when capillary-cell apoptosis and histopathology can be seen. Furthermore, diabetes-induced decreases in the activity of retinal MnSOD and its mRNA are inhibited by long-term administration of lipoic acid; the same therapy that is shown by us to inhibit diabetes-induced retinal capillary-cell apoptosis and retinopathy.

Superoxide is among the most abundant reactive oxygen species produced by the mitochondria that can damage cellular macromolecules and is postulated to be involved in cellular signaling pathway. Under normal conditions, the mitochondrial electron transport chain is a major source of superoxide, converting up to perhaps 5% of molecular O₂ to superoxide, but, its levels are elevated in pathologic conditions. Mitochondrial superoxide levels are increased in rat retina in diabetes and in retinal cells incubated in high glucose, and downregulation of superoxide inhibit glucose-induced apoptosis in both endothelial cells and pericytes. Superoxide is considered to be a causal link between elevated glucose and the major biochemical pathways postulated to be involved in the development of vascular complications in diabetes. SOD catalyzes the breakdown of superoxide into hydrogen peroxide scavenging superoxide, and, because of its mitochondrial localization, MnSOD is considered as the first line of defense against oxidative stress. Here we show that the overexpression of MnSOD in retinal endothelial cells prevents high-glucose-induced apoptosis of the cells, and the process is mediated via activation of caspase-3. Because retinal capillary cells are shown to be lost via apoptosis before other histopathology is detectable and the therapies that inhibit the development of retinopathy also inhibit apoptosis and caspase-3 activation, this suggests that superoxide plays an important role in the pathogenesis of diabetic retinopathy.

8-OHdG is a sensitive indicator of oxidative damage to DNA, and this could lead to subsequent mutations of mitochondrial DNA. We have shown that 8-OHdG levels are increased in the retina in diabetes, and this increase is inhibited by the
administration of lipoic acid to diabetic rats, suggesting that oxidative modification of DNA might be playing an important role in the pathogenesis of retinopathy in diabetes. We now show that MnSOD overexpression prevents glucose-induced increase in 8-OHdG levels in retinal endothelial cells, thus, MnSOD could be important in preventing oxidative modification of retinal capillary-cell DNA in diabetes. In support, others have shown that diabetes-induced endothelial dysfunction and impaired wound healing can be improved by the overexpression of MnSOD.

Superoxide reacts with nitric oxide to form peroxynitrite, and the reaction of nitric oxide with superoxide is 3 times faster than dismutation of superoxide by SOD. Nitration of proteins plays a role in the apoptosis of retinal cells by disrupting the protein assembly and functions, with possible pathologic consequences, and peroxynitrite itself can indirectly produce additional increases in superoxide by nitrating MnSOD. Our previous studies showed that nitrosative stress in the retina that is increased early in the course of development of retinopathy in diabetes appears to contribute to the progression of retinopathy after reconstitution of good glycemic control, and the therapies that inhibit the activation of apoptosis execution enzyme development of retinopathy in diabetic rats decrease nitrosative stress in the retina. Here we show that the overexpression of MnSOD prevents glucose-induced nitrosative stress in retinal endothelial cells: the levels of nitrotyrosine were not increased when endothelial cells overexpressing MnSOD were incubated in a high-glucose medium. In support, hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain is postulated to activate the major pathways of hyperglycemic damage in aortic endothelial cells.

Thus, our in vitro results clearly show that MnSOD protects retinal endothelial cells from glucose-induced oxidative stress and apoptosis; the level of oxidative stress and apoptosis in a high-glucose condition is significantly lower in MnSOD transfected cells than in nontransfected cells when these cells are incubated under identical culture conditions and glucose concentrations. In support, we have shown that a SOD mimic can inhibit high-glucose-induced apoptosis of retinal endothelial cells.

The data presented here show that the activity of mitochondrial SOD remains inhibited and that its mRNA decreased in the retina at a duration of diabetes when retinopathy is developing in diabetic rats. This clearly suggests that the mitochondrial defense system remains impaired in diabetes when the pathology is developing in the retina. Because retina is a complex tissue, our in vivo results in diabetic conditions are supported by in vitro data from the isolated retinal endothelial cells, representing specific cell type of the retina. Thus, it is plausible to suggest that endothelial cells are one of the targets of diabetes-induced changes. Furthermore, lipoic acid administration, the therapy that inhibits retinal capillary cell apoptosis and formation of acellular capillaries in diabetic rats, also prevents a diabetes-induced decrease in MnSOD activity and its mRNA levels. Previous reports from our laboratory and that of others have shown that SOD activity is subnormal in the retina of rats at 6 to 8 weeks of diabetes, and lipoic acid prevents such short-term diabetes-induced changes. Here, for the first time, we show that the mitochondrial enzyme responsible for scavenging superoxide remains inhibited during the apoptosis of capillary cells and the appearance of histopathology characteristic of retinopathy in diabetes. The role of mitochondria in the pathogenesis of retinopathy is supported by our previous reports that show that retinal mitochondria experience dysfunction in diabetes; diabetes of 8 months in rats (a duration when capillary-cell apoptosis is seen in the retina) increases the release of cytochrome c into the cytosol and Bax into the mitochondria. In isolated retinal capillary cells, incubation in high-glucose medium results in the release of cytochrome c in the cytosol and Bax in the mitochondria, and these abnormalities are accompanied by increased cell apoptosis. Thus, this is the first report that shows a significant role for MnSOD in the development of retinopathy in diabetes when using both in vivo (retina from diabetic rats) and in vitro (retinal endothelial cells incubated in high glucose) models. Our results strongly suggest that mitochondrial SOD plays a protective role in the progression of retinopathy.
the development of diabetic retinopathy; overexpression of MnSOD in isolated retinal endothelial cells protects retinal capillary cells from glucose-induced oxidative and nitrative stress and increased capillary cell apoptosis. Further, lipoic acid therapy that inhibits diabetes-induced retinal capillary cell apoptosis and histopathology in rats also prevents inhibition of MnSOD in the retina. Understanding the role of dismutation of superoxide by mitochondria to modify the course of retinopathy is expected to elucidate important molecular targets for future pharmacological interventions.

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