Overexpression of Manganese Superoxide Dismutase Suppresses Increases in Collagen Accumulation Induced by Culture of Mesangial Cells in High-Media Glucose

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Superoxide has been implicated in the cellular signalling pathways, which regulate growth of mesangial cells (MC) and vascular smooth muscle cells. Manganese $(Mn)^{2+}$-dependent superoxide dismutase (SOD-2) is primarily responsible for metabolism of superoxide produced in mitochondria by respiratory chain activity during aerobic metabolism of glucose and other substrates. In the current studies, we examined the role of superoxide in the stimulation of collagen accumulation induced in MC by culture in media containing a high concentration of glucose. Aconitase, an iron sulfur enzyme whose activity is inhibited by superoxide, was used as an index of cellular superoxide production and action. SV-40–transformed mouse MC were cultured in media containing 100 (low) or 500 (high) mg/dL D-glucose and infected with a recombinant adenoviral (Ad) vector encoding either human mitochondrial Mn^{2+} SOD-2 or green fluorescent protein (GFP). In cells infected with SOD-2 (SOD-2-Ad) and cultured in low glucose, SOD-2 activity was 5-fold higher than in cells infected with GFP (GFP-Ad), whereas Cu^{2+}/Zn^{2+} cytoplasmic SOD (SOD-1) did not differ; culture in high-glucose media did not alter SOD-2 or SOD-1 activity in either GFD-Ad or SOD-2-Ad. In GFP-Ad, high glucose suppressed aconitase activity and increased collagen accumulation compared with corresponding values in low glucose. In SOD-2-Ad, high glucose failed to suppress aconitase activity or increase collagen accumulation. Addition of exogenous (presumably extracellular) SOD to GFP-Ad had no effect on the stimulation of collagen accumulation by high glucose. Analogous to the effects of SOD-2-Ad, diphenylene iodonium (DPI), a nonspecific inhibitor of the production of superoxide by mitochondrial respiration and other nicotinamide adenine dinucleotide (phosphate) (NAD)(P)H oxidase activities, reduced collagen accumulation in GFP-Ad cultured in low glucose and blocked stimulation of collagen accumulation induced by culture in high glucose. These results support a role for increased cellular superoxide production derived from NAD(P)H oxidase activity in the stimulation of collagen accumulation induced in MC by high glucose and demonstrate that an increase in mitochondrial SOD-2 activity suppresses this response.

NUMEROUS PREVIOUS STUDIES in experimental animal models of diabetes,[1-5] in patients with diabetes,[6-8] and in cells cultured in media containing a high concentration of glucose[9-15] have implicated oxidant stress as a key component of vascular dysfunction and cellular injury in the glomerulus in diabetes. Enhanced permeability to albumin,[1,2,5,8] increased transforming growth factor-β (TGFβ) production,[5,12] mesangial deposition of extracellular matrix proteins[2,12-14] resulting in mesangial expansion, and reductions in glomerular filtration rate (GFR) have all been proposed as potential consequences of oxidant radical-induced injury in the kidney.

Studies in cultured mesangial cells (MC) have implicated O_2^-. in the mediation of angiotensin II (AII)-induced increases in cell growth and hypertrophy.[16] There is evidence that O_2^-. production by vascular tissue in response to AII is derived from activation of plasma membrane NADPH oxidase activities.[17,18] MC in culture contain components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system[19] and synthesize O_2^-. in response to AII[20] and phorbol ester activators of protein kinase C.[21] MC,[11] endothelial cells,[22] and vascular smooth muscle cells[15] also generate increased quantities of O_2^-[15,22] or H_2O_2[11] in response to culture in high glucose. Recently, overexpression of mitochondrial manganese $(Mn)^{2+}$ superoxide dismutase (SOD-2) in endothelial cells cultured in high glucose was shown to block several metabolic responses linked to hyperglycemic cell injury, including increases in protein kinase C activity, expression of TGFβ1, formation of advanced glycosylation end products, and flux through the aldose reductase pathway.[22] These results support a role for mitochondrial O_2^-. in the mediation of these cellular responses to glucose. SOD-2 is primarily responsible for metabolism of O_2^-. generated by respiratory chain activity during aerobic metabolism of glucose and other substrates.[23]

In the present study, we assessed the influence of infection of MC with a recombinant adenoviral vector encoding the human SOD-2 transgene on increases in collagen accumulation induced by culture of cells in high-media glucose. The feasibility of these studies was suggested by the preliminary finding that culture of MC in high-glucose media per se did not alter endogenous SOD-2 activity (see below), consistent with previous observations in endothelial cells cultured in high glucose.[24] The activity of the [4Fe-4S] protein aconitase, which is suppressed by O_2^-., was used to monitor cellular O_2^-. production.[25,26] The influence of diphenylene iodonium (DPI), a nonspecific inhibitor of NAD(P)H oxidases, including mitochondrial NADH-ubiquinone oxidoreductase (complex I),[27,28] on collagen accumulation was also assessed. The results support a key role for O_2^-., produced via NAD(P)H oxidases in the increase in collagen accumulation induced by culture of MC in high-media glucose.

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Submitted September 12, 2000; accepted March 26, 2001.

Supported by a grant from the American Diabetes Association and by the General Medical Research Service of the Department of Veterans Affairs.

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0026-0495/01/5009-0025$35.00/0

doi:10.1053/meta.2001.25802

MATERIALS AND METHODS

Culture of SV-40 Transformed Mouse MC

Transformed mouse mesangial cells (MMC) mesangial (MES)-13 cells were obtained from the ATCC (Rockville, MD) and maintained in RPMI 1640, containing 5% fetal bovine serum (FBS), penicillin 100 U/mL, streptomycin 100 μg/mL, and 100 mg/dL glucose at 37°C in an atmosphere of 5% CO_2, 95% air. Cells were passed 3 times/week. Where indicated in the text, the concentration of glucose in the medium was raised to 500 mg/dL for 2 weeks prior to study.

Culture of Mouse MC

Glomeruli were prepared from mouse renal cortex under sterile conditions by graded sieving as previously described in detail.[29] MC were cultured from collagenase-treated glomeruli as previously reported.[29] MC had typical stellate morphology, stained positively for smooth muscle actin and thy1.1, and were negative for Factor VIII. MMC were maintained in RPMI 1640 containing 15% FBS, penicillin 100 U/mL, streptomycin 100 μg/mL, insulin transferrin and sodium selenite (ITS) 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, and 100 mg/dL glucose at 37°C in 5% CO_2, 95% air. Where indicated in the text, the concentration of glucose in the medium was raised to 500 mg/dL for 2 weeks prior to study. MC were passed at weekly intervals and media changed at 3- to 4-day intervals or when 80% confluent.

Adenovirus Infections

A recombinant adenoviral vector driven by the cytomegalovirus (CMV) promoter, and encoding human SOD-2 was obtained from Gene Transfer Vector Core, University of Iowa, Iowa City, IA. An adenoviral vector, also driven by the CMV promoter and encoding green fluorescent protein (GFP) was a kind gift of Dr Paul Robbins, Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA. Virus preparations contained 10^{12} particles/mL. Infectivity of each of the preparations was estimated to be 1%. The virus preparations were diluted 10 times in 10 mmol/L TrisCl pH 7.5, 0.15 mol/L NaCl, 10 mmol/L $MgCl_2$, and 3% sucrose just before use. Cells were seeded in 6-well plates in complete media and allowed to reach 50% confluency. Media were then changed to serum-free and 40 μL of the diluted virus preparations added to 1 mL of media in each well. Multiplicity of infection (infective particles/cell) was estimated to be 80 to 100. After 1.5 hours, the media was replaced with complete media containing serum and the incubations continued for 3 days.

Preparation of Homogenates for Determination of Enzyme Activities

The culture medium was aspirated and cells were scraped into 0.5 mL/well of 0.05 mol/L sodium phosphate buffer, pH 7.4 containing 10^{-4} mol/L EDTA and sonicated with a Fisher Model 300 probe sonicator (Pittsburgh, PA) at the highest setting for three 1-second bursts on ice. Homogenates were centrifuged at $700\times g$ for 10 minutes and supernatants used for assays described below.

Assay of SOD Activity

Total SOD activity was determined by its ability to inhibit the initial rate of 6-hydroxydopamine auto-oxidation, as previously described.[30] A standard curve was generated in the range of 25 to 200 ng/mL bovine erythrocyte SOD. Enzyme activity in the MC extracts was calculated by comparison with the standard curve. SOD activity was expressed as the amount (μg) of bovine erythrocyte SOD, which gave an equivalent inhibition of 6-hydroxydopamine auto-oxidation. The proportion of total activity, which was inhibited by 5 mmol/L cyanide, was taken as SOD-1 activity; SOD-2 activity is not cyanide-sensitive.[30]

Assay of Aconitase Activity

The linear absorbance increase due to reduction of $NADP^+$ was followed for 60 minutes at 340 nm at 25°C in a 1.0-mL reaction mixture containing 50 mmol/L Tris Cl pH 7.4, 5 mmol/L sodium citrate, 0.6 mmol/L $MnCl_2$, 0.2 mmol/L $NADP^+$, 1 to 2 U isocitrate dehydrogenase, and 25 to 75 μg of extract protein.[24] A unit of enzyme activity is defined as formation of 1 nanomole of NADPH/min/mg protein.

Collagen Accumulation

Collagen accumulation was determined as previously described.[31] Briefly, cells were seeded in 6-well plates and harvested 4 days later. Additions were made for the times indicated in the text. A total of 50 μg/mL L-ascorbic acid was added to the cells 24 hours before harvest. Media was changed to fresh complete media containing 50 μg/mL L-ascorbic acid, 80 μg/mL β aminoproprionitrile, and [^{3}H] proline 1 μCi/well for the final 6 hours of incubation. At the end of the incubation, media was removed and cells lysed by addition of 1 mL H_2O. Cells were then scraped and sonicated 3 times for 1 second each. Proteins were precipitated at a final concentration of 10% trichloroacetic acid, and the samples were centrifuged at 4,000 rpm in a microcentrifuge. The pellets were washed with cold 10% trichloroacetic acid until the wash was free of radioactivity. They were then dissolved in 1.5 mL of 0.1 N NaOH. The pH of the samples was adjusted to 7.4 with 1 mol/L HEPES. The resuspended pellet was then mixed with an equal volume of 0.1 mol/L Tris, 15 mmol/L $CaCl_2$, 4 mmol/L N-ethylmaleimide, and incubated in the presence and absence of 40 U/mL collagenase VII for 90 minutes at 37°C. At the end of the incubation, carrier albumin was added 0.5%, proteins were precipitated with 5% trichloroacetic acid, and the supernatants counted. Counts released into the supernatant in the absence of collagenase were subtracted from the counts released by collagenase digestion. Results shown represent labeled proline incorporation into collagenase-sensitive protein as a function of total cellular protein.

Statistics

Significance of differences was determined by analysis of variance followed by the Fisher multiple comparison test using Statview software (SAS Institute, Cary, NC).

RESULTS

Virtually all of the cells infected with GFP-Ad express GFP (Fig 1). In other studies, the infection efficiency of primary MC cultures was analogous to that seen with MES-13 cells and culture in high glucose did not affect infection efficiency.

Figure 2 illustrates the influence of the SOD-2 transgene on SOD-2 activity in MES-13 cells cultured in 100 or 500 mg/dL glucose. In these studies, cells were cultured in high or low glucose for 2 weeks. They were then infected with SOD-2-Ad or GFP-Ad and studied 3 days later. As shown, infection with SOD-2-Ad increased SOD-2 activity 5-fold compared with that observed in cells, which had been infected with GFP-Ad or noninfected cells (not shown). Infection with SOD-2-Ad had no effect on SOD-1 activity (not shown). As is also shown in Fig 2, culture in high-media glucose for 2 weeks had no effect on SOD-2 activity in either SOD-2-Ad or GFP-Ad–infected cells.

Figure 3 illustrates the influence of SOD-2 transgene on aconitase activity in MES-13 cells cultured in 100 versus 500 mg/dL glucose. Culture in high glucose significantly reduced aconitase activity in cells, which had been infected with GFP-Ad. In low-glucose media, aconitase activity of cells infected

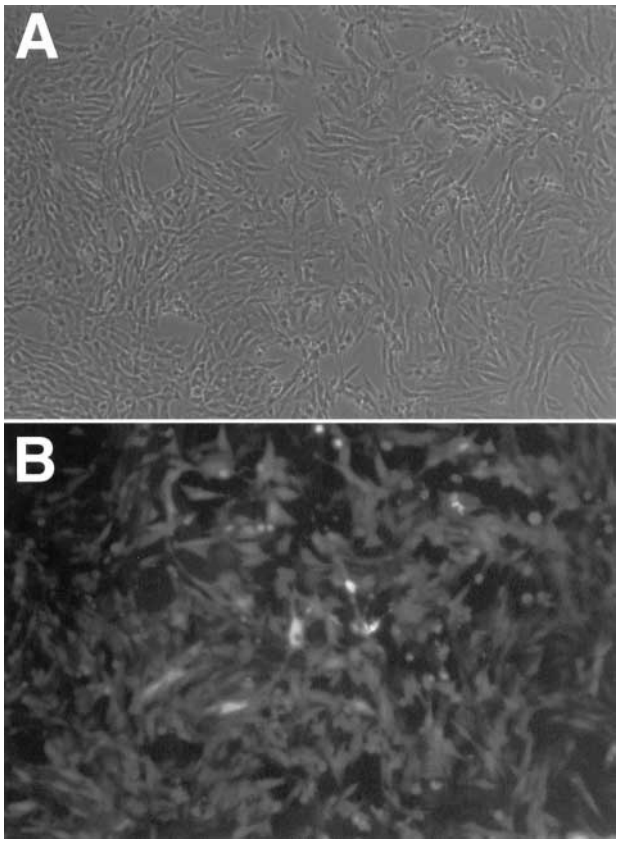

Fig 1. Fluorescent microscopy of MES-13 cells infected with GFP-Ad. MES-13 cells were infected with GFP-Ad and observed 3 days after infection. Cells in the same field were illuminated with visible (A) or ultraviolet (UV) (B) light (350X).

with SOD-2-Ad was higher than that observed in cells, which had been infected with GFP-Ad. No decrease in aconitase activity was observed in cells infected with SOD-2-Ad and cultured in high glucose.

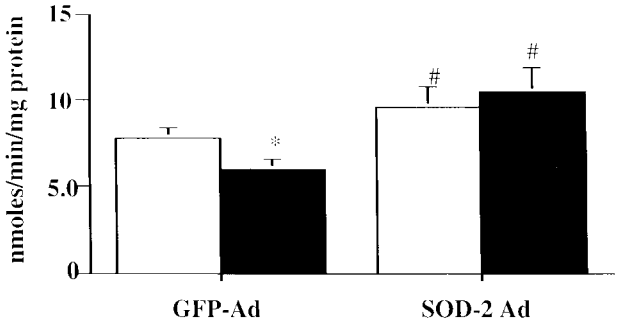

Fig 3. Influence of SOD-2 transgene on aconitase activity in MC cultured in high and low glucose. MES-13 cells were treated as described in the legend to Fig 2. Results shown are means ± SE of aconitase activity determinations on 6 separate cell extracts from 3 separate experiments. *$P < .05$ *v* 100 mg/dL glucose; #$P < .05$ *v* GFP-AdV.

Figure 4 illustrates changes in collagen accumulation, as assessed by [^{3}H] proline incorporation into collagenase-sensitive protein, in MES-13 cells cultured in high and low glucose. As shown, culture in high glucose significantly increased collagen accumulation in cells, which had been infected with GFP-Ad. Infection with SOD-2-Ad had no influence on collagen accumulation in cells cultured in 100 mg/dL glucose, but completely prevented the increases induced by culture in high glucose. In other studies (not shown), infection of primary MC cultures with SOD-2-Ad also prevented increases in collagen accumulation in response to high glucose. Addition of exogenous SOD-1 (presumably extracellular) activity had no effect on collagen accumulation in MES-13 cells cultured in 100 or 500 mg/dL glucose (not shown).

Figure 5 illustrates the effects of DPI on collagen accumulation in MES-13 cells cultured in 100 and 500 mg/dL glucose. Prior exposure to DPI reduced collagen accumulation by 65% in cells, which had been cultured in 100 mg/dL glucose and prevented the increase in collagen accumulation induced by culture in high glucose. DPI was not cytotoxic, as reflected by

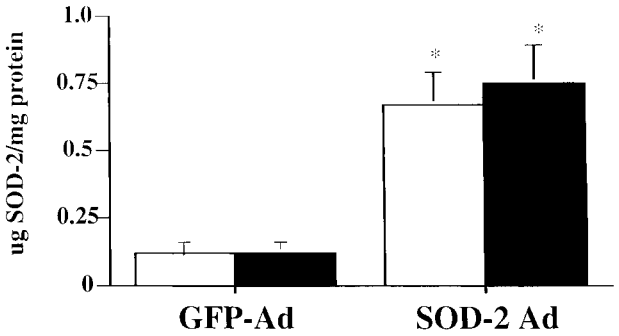

Fig 2. Influence of SOD-2 transgene on SOD-2 activity in MC cultured in high and low glucose. MES-13 cells were cultured for 2 weeks in complete media, which contained 100 or 500 mg/dL glucose. They were then infected with GFP-Ad or SOD-2-Ad and harvested 3 days later. Results shown are means ± SE of SOD-2 activity of determinations on 6 separate cell extracts from 3 different experiments. *$P < .05$ *v* GFP-Ad.

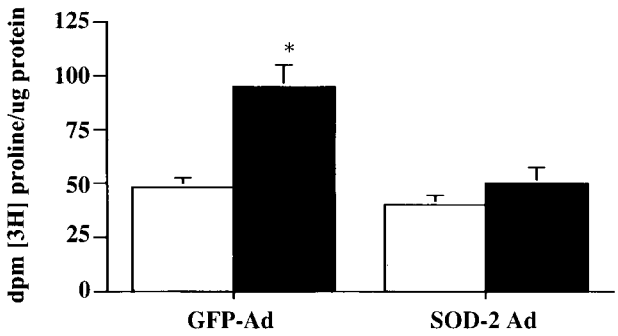

Fig 4. Influence of SOD-2 transgene on collagen accumulation in MC cultured in high and low glucose. MES-13 cells were treated as described in the legend to Fig 2. [^{3}H] Proline incorporation into collagenase-sensitive protein was determined during the final 6 hours of culture as described in the Methods section. Results shown are means ± SE of determinations on 6 separate wells from 3 different experiments. *$P < .05$ *v* 100 mg/dL glucose.

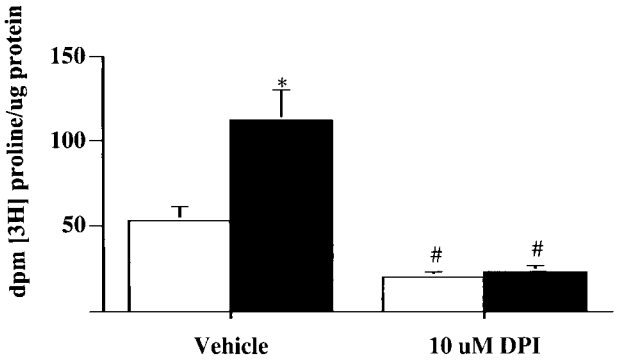

Fig 5. Effects of DPI on collagen accumulation in MC cultured in high and low glucose. MES-13 cells were cultured for 2 weeks in 100 or 500 mg/dL glucose. DPI (10 μmol/L) or vehicle (0.1% dimethyl sulfoxide [DMSO]) was added 24 hours before cells were harvested. [^{3}H] Proline incorporation into collagenase-sensitive protein was determined during the final 6 hours of culture as described in the Methods. Results shown are means ± SE of determinations on 6 wells from 3 separate experiments. *P < .05 *v* 100 mg/dL glucose; #P < .05 *v* vehicle.

its lack of effect on trypan blue exclusion, release of lactate dehydrogenase (LDH) into the media, or cell morphology.

DISCUSSION

Results of the present study show that culture of MC in high-media glucose significantly decreases aconitase activity, an indirect indicator of O_2^-. production.[26] O_2^-. reversibly inactivates [4Fe-4S] containing dehydrases, resulting in loss of activity of several enzymes, including aconitase.[25,26] Aconitase is present in both mitochondrial and cytoplasmic compartments.[25,26] In the present study, total cellular aconitase activity was measured. Overexpression of mitochondrial SOD-2 in MC for the final 3 days of culture resulted in an increase in aconitase activity in cells cultured in 100 mg/dL glucose and prevented the decrease in aconitase activity induced by culture for 2 weeks in high-glucose media. SOD-2 activity was increased approximately 5-fold in MC, which overexpressed SOD-2. In agreement with a previous study in cultured endothelial cells,[24] culture of MC in high glucose for 2 weeks did not influence endogenous SOD-2 activity. Moreover, in the present study, culture in high glucose did not alter activity of this enzyme in cells, which overexpressed the SOD-2 transgene.

Consistent with previous results from our own and numerous other laboratories,[31-34] culture of MC in high-glucose media increased collagen accumulation. MC in culture synthesize primarily type I collagen and to a much lesser extent type IV collagen.[34] Culture in high glucose increases mRNA for both collagen α1(IV) and α2(I) chains,[34] implying an effect of high glucose on synthesis of collagens I and IV. Culture in high glucose has also been demonstrated to decrease mesangial matrix degradation.[35] In the present study, overexpression of SOD-2 prevented the increase in collagen accumulation otherwise observed in response to culture in high glucose. By contrast, addition of exogenous SOD-1, which presumably remained extracellular, had no effect on collagen accumulation in MC cultured in 100 or 500 mg/dL glucose. These findings support a role for increased intracellular generation of O_2^-., possibly produced in mitochondria, in the stimulation of collagen accumulation seen in response to culture of MC in high glucose. They are also consistent with results of the earlier report of Jaimes et al,[20] which found no effect of exogenous SOD on AII-induced increases in protein synthesis in cultured MC, and with the findings of Nishikawa et al,[22] which found that overexpression of SOD-2 prevents several metabolic responses of endothelial cells induced by culture in high glucose, which have been linked to cell injury.

Results of the present study further implicate NAD(P)H oxidase activities in the generation of O_2^-. in MC cultured in high glucose. Thus, DPI, a nonspecific inhibitor of flavin-containing NAD(P)H oxidases,[27] including mitochondrial NADH-ubiquinone oxidoreductase (complex I),[28] prevented the increase in collagen accumulation induced by culture of MC in high glucose. NAD(P)H oxidase activity has been previously implicated as the major source of O_2^-. produced in vascular tissue.[17,18] Recently, DPI was also shown to inhibit basal- and AII-induced increases in MC hypertrophy.[20]

SOD converts O_2^-. to H_2O_2. Whether an increase in SOD confers protection against or susceptibility to oxidant injury may be dependent on the magnitude of the increase in SOD and also the isozyme altered. Previous studies have indicated that marked increases in SOD-1 activity can exacerbate oxidant stress and increase oxidant-mediated cell injury,[36,37] possibly by increasing H_2O_2 levels. However, studies in hemizygous mice, which modestly overexpress either SOD-1 or SOD-2 have demonstrated protection from various forms of oxidant-induced injury. Thus, hemizygous mice, which overexpress SOD-1 or SOD-2, are highly resistant to reperfusion injury after focal cerebral ischemia[38,39] and are protected against traumatic brain injury.[40] They are also resistant to pulmonary oxygen toxicity,[41] myocardial ischemia,[42] adriamycin-induced acute cardiac toxicity,[43] and diabetes-associated embryopathy.[44] Macrophage induction of NF-κB and macrophage function were also reduced in the hemizygous SOD-1 transgenic mouse.[45] By contrast, mice homozygous for the transgene express higher levels of SOD-1 and are more susceptible to reperfusion brain injury[36] and have decreased survival in association with evidence of oxidant cell injury.[37] Thus, at least in vivo, there may be an important dose effect relationship in the determination of protection or susceptibility to oxidant injury with increases in SOD activity.[46]

Subcellular localization or other unique properties of 2 major intracellular SOD isozymes may also be determinants of the influence of SOD on protection against specific types of oxidant injury. Thus, intratracheal injection of adenovirus encoding the SOD-2, but not the SOD-1 transgene, protected athymic nude mice from irradiation-induced alveolitis and prevented radiation-induced increases in TGFβ mRNA and fibrosis in the lungs.[23] By contrast, in the current and a previous study,[46] overexpression of either SOD-1 or SOD-2, but not addition of exogenous SOD to culture media, prevented increases in collagen accumulation in response to high glucose in MC. These results suggest that the 2 intracellular SOD isoforms play a more important role than the

extracellular SOD in attenuating cellular responses to high glucose, at least in MC. Recent studies from our laboratory demonstrate that hemizygous transgenic mice, which overexpress SOD-1, are protected from early diabetic renal injury, including the development of albuminuria and glomerular accumulation of TGFβ and collagen IV.[46] Whether SOD-2 overexpression confers similar protection from diabetic renal injury in vivo remains to be determined.

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