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Manganese Supplementation Reduces High Glucose-induced Monocyte Adhesion to Endothelial Cells and Endothelial Dysfunction in Zucker Diabetic Fatty Rats*

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Background: Mn^{2+} levels are lower in blood of diabetic and atherosclerosis patients.
Results: Mn^{2+} supplementation reduces monocyte adhesion in endothelial cells by down-regulating ROS, ICAM-1 expression, and MCP-1 secretion, and lowers blood levels of ICAM-1 and cholesterol in ZDF rats.
Conclusion: Mn^{2+} supplementation is beneficial in lowering markers of endothelial dysfunction.
Significance: Mn^{2+} supplementation can potentially prevent or delay progression of atherosclerosis.

Endothelial dysfunction is a hallmark of increased vascular inflammation, dyslipidemia, and the development of atherosclerosis in diabetes. Previous studies have reported lower levels of Mn^{2+} in the plasma and lymphocytes of diabetic patients and in the heart and aortic tissue of patients with atherosclerosis. This study examines the hypothesis that Mn^{2+} supplementation can reduce the markers/risk factors of endothelial dysfunction in type 2 diabetes. Human umbilical vein endothelial cells (HUVECs) were cultured with or without Mn^{2+} supplementation and then exposed to high glucose (HG, 25 mM) to mimic diabetic conditions. Mn^{2+} supplementation caused a reduction in monocyte adhesion to HUVECs treated with HG or MCP-1. Mn^{2+} also inhibited ROS levels, MCP-1 secretion, and ICAM-1 up-regulation in HUVECs treated with HG. Silencing studies using siRNA against MnSOD showed that similar results were observed in MnSOD knockdown HUVECs following Mn^{2+} supplementation, suggesting that the effect of manganese on monocyte adhesion to endothelial cells is mediated by ROS and ICAM-1, but not MnSOD. To validate the relevance of our findings *in vivo*, Zucker diabetic fatty rats were gavaged daily with water (placebo) or $MnCl_2$ (16 mg/kg of body weight) for 7 weeks. When compared with placebo, Mn^{2+}-supplemented rats showed lower blood levels of ICAM-1 (17%, $p < 0.04$), cholesterol (25%, $p < 0.05$), and MCP-1 (28%, $p = 0.25$). These *in vitro* and *in vivo* studies demonstrate that Mn^{2+} supplementation can down-regulate ICAM-1 expression and ROS independently of MnSOD, leading to a decrease in monocyte adhesion to endothelial cells, and therefore can lower the risk of endothelial dysfunction in diabetes.

Manganese is an essential micronutrient that serves as a cofactor for many enzyme systems. Metalloenzymes, or manganese-containing enzymes, such as arginase, pyruvate carboxylase, and manganese superoxide dismutase (MnSOD),[2] require Mn^{2+} to function. MnSOD is the major mitochondrial antioxidant and is responsible for protecting the cell from reactive oxygen species (ROS) by scavenging mitochondrial superoxide (1). MnSOD acts by catalyzing the conversion of superoxide radicals (such as O_2) to hydrogen peroxide, which is further metabolized to water by other antioxidant enzymes such as catalase and glutathione peroxidase (2). At low concentrations, Mn^{2+} ions have been shown to have antioxidant properties with the ability to scavenge superoxide and hydroxyl radicals (3). Several studies have reported that changes in dietary Mn^{2+} induced changes in MnSOD activity and that MnSOD activity was reduced in heart and livers of Mn^{2+}-deficient animals (4–6). Previous studies report lower levels of Mn^{2+} in the plasma and lymphocytes of type 2 diabetic patients (7, 8) and in the heart and aortic tissue of patients with atherosclerosis when compared with those of healthy controls (9). Other studies have also shown beneficial effect of Mn^{2+} on lipid metabolism and a decrease in total serum cholesterol, aorta cholesterol, and regression of atherosclerosis following manganese supplementation in cholesterol-fed rabbits (10). The mechanisms by which Mn^{2+} can reduce cholesterol, however, are unknown. Also, a possible beneficial role of Mn^{2+} supplementation alone (without MnSOD) on vascular inflammation has never been investigated. Endothelial dysfunction and vascular inflammation, characterized by monocyte adhesion to endothelial cells and increased levels of MCP-1, ROS, and ICAM-1, are known to play a significant role in the development of atherosclerosis (11–13). This study examines the hypothesis that Mn^{2+} supplementation can prevent vascular inflammation and endothelial dysfunction in type 2 diabetes and that Mn^{2+} can have a beneficial role independently of MnSOD. Our results demonstrate that Mn^{2+} supplementation reduces ROS levels, MCP-1 secretion, ICAM-1 expression, and the adhesion of monocytes to endothelial cells. Furthermore, similar results were obtained

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[2] The abbreviations used are: SOD, superoxide dismutase; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cell; ZDF, Zucker diabetic fatty; HG, high glucose; NBT, nitro blue tetrazolium.

in MnSOD knockdown human umbilical vein endothelial cells (HUVECs). Further studies *in vivo* showed that Mn^{2+} supplementation lowers blood levels of ICAM-1 and cholesterol in Zucker diabetic fatty rats. These *in vitro* and *in vivo* studies demonstrate that Mn^{2+} supplementation can lower markers of oxidative stress and endothelial dysfunction, such as monocyte adhesion to endothelial cells, ICAM-1, ROS, MCP-1, and cholesterol, thereby lowering the risk of endothelial dysfunction in diabetes. We also show for the first time that Mn^{2+} supplementation can have beneficial effects on endothelial cells independently of MnSOD.

EXPERIMENTAL PROCEDURES

Human Umbilical Vein Endothelial Cells—HUVECs were purchased from Lonza Walkersville Inc., Walkersville, MD. Cells were cultured in EGM-2 medium and 5% CO_2, in a 37 °C humidified atmosphere, and grown to confluence in T75 flasks coated with gelatin. Experiments were performed within 24 h after reaching confluence, between passages 3 and 10. Cells were pretreated with Mn^{2+} (0, 5, and 10 μM as $MnCl_2$) for 24 h followed by high glucose (HG, 25 mM) or normal glucose (7 mM) exposure for another 24 h. Many previous studies have reported glucose concentrations as high as 50 mM in the blood of patients with uncontrolled diabetes (25). It is true that blood glucose levels in patients are not likely to stay as high as 25 mM for 24 h. However, tissue damage in diabetic patients occurs over many years of countless hyperglycemic episodes. Thus, the glucose concentration of 25 mM used to mimic diabetes in this cell culture study does not seem unreasonable. We did not observe any effect of on Mn^{2+} on cell viability, similar to results from previous cell culture studies (14, 15).

Silencing Studies—SOD2 siRNA was purchased from Santa Cruz Biotechnology. For every transfection, 2 μl of transfection reagent (Lipofectamine from Invitrogen) was added to 100 μl of transfection medium (from Santa Cruz Biotechnology, serum-free). 100 nM SOD2 siRNA was added to the mix. Cells were trypsinized and then resuspended in transfection medium and plated to 60-mm dishes. Cells were incubated for 3–4 h at 37 °C. Normal medium was then added to the cells and incubated overnight at 37 °C. The next day, fresh medium was added, and the cells were treated for the experiment within the next 18–30 h.

MnSOD Activity Assay—Total SOD activity was assessed using the xanthine-xanthine oxidase and nitro blue tetrazolium (NBT) diformazan method as in Ref. 16. Xanthine oxidase is used to generate O_2^- and NBT reduction is used as an indicator of O_2^- production. SOD competes with NBT for O_2^-; the percentage of inhibition of NBT reduction is a measure of the amount of SOD present. KCN was used to inhibit Cu/ZnSOD activity. Absorbance was measured at 560 nm to measure NBT reduction. Absorbance per minute was used to determine the percentage of inhibition of diformazan formation. 50% inhibition of NBT reduction equals to 1 unit of SOD activity.

ROS Assay—ROS levels were measured using the dihydrorhodamine 123 dye. Cells were incubated with the dye for 30 min after treatment (2 h HG instead of 24 h). Mean fluorescence was analyzed. After treatment, cells were washed once with PBS and then loaded with 30 μM dihydrorhodamine 123 in PBS with 10% FCS. The cells were incubated at 37 °C for 30 min in the dark and subsequently washed with PBS. The intensity of dihydrorhodamine 123 fluorescence in the supernatant was read at excitation and emission wavelengths of 485 and 528 nm, respectively, using a multidetection microplate reader (Synergy HT, Bio-Tek). The change in intracellular ROS level was plotted as mean fluorescence intensity.

Surface ICAM-1—Surface ICAM-1 was determined using flow cytometry. After treatment, cells were washed in FACS buffer (PBS without Mg^{2+} and Ca^{2+}, with the addition of 10% fetal bovine serum and 0.1% sodium azide), centrifuged, resuspended in FACS buffer, and incubated for 1 h at 4 °C with FITC-conjugated anti-ICAM-1 antibody (Santa Cruz Biotechnology, sc-107) at a 1:100 dilution in the dark. The cells were then washed twice in washing buffer for FACS (PBS containing 1% BSA and 0.1% sodium azide) and resuspended in 0.3 ml of FACS buffer. In each experiment, a minimum of 15,000 cells was analyzed (per treatment condition) by FACSCalibur flow cytometer (BD Biosciences) equipped with multicolor analysis capability. Gates were set to exclude nonviable cells, cell debris, and cells of abnormal size and shape. Results were expressed as mean fluorescence intensity per 15,000 cells.

Western Blotting Analyses of Cell Lysates—Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM $NaVO_4$). Lysates were subjected to mild sonication and centrifuged at 15,000 rpm (4 °C, 30 min), and the supernatants were collected. Total protein concentrations were determined by BCA assay (Pierce/Thermo Scientific). Equal amounts of protein from each group were loaded onto SDS-polyacrylamide gels after boiling for 5 min with or without β-mercaptoethanol as a reducing agent (ICAM-1 was determined in nonreducing conditions). The separated proteins were transferred to a nitrocellulose membrane, blocked with 1% BSA in TBS-T (0.25% Tween 20 in PBS), and incubated overnight at 4 °C with the respective primary antibodies, using 1:1000 dilutions. The next day, membranes were washed with TBS-T (8 min, four cycles) and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) in 5% nonfat milk for 30 min at room temperature. The membranes were again washed with TBS-T (8 min, 4 cycles), treated with chemiluminescence reagents for 2 min, and exposed to x-ray films developed through autoradiography. β-Actin or α-tubulin antibodies were used to assess the loading equality. Primary antibodies for MnSOD were purchased from Abcam, and antibodies for ICAM-1 were from Santa Cruz Biotechnology.

Human THP-1 Monocytes—Human THP-1 monocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For treatments, the cells were counted on a hemocytometer using trypan blue exclusion and adjusted to $\sim 1 \times 10^6$ cells/ml in complete medium. Cells

were treated with HG and Mn^{2+} in the same manner as were HUVECs.

Monocyte-Endothelial Cells Adhesion Assay—Assay was performed as described previously (17). HUVECs were plated and allowed to grow to confluent monolayers. HUVECs were treated with different concentrations of Mn^{2+} (0–10 μM) for 24 h and then exposed to either HG or MCP-1 for another 24 h. Monocytes (THP-1) were loaded with 8 μM CellTracker Green (5-chloromethylfluorescein diacetate; Invitrogen) and then treated with concentrations of Mn^{2+} matching those of the HUVECs. After treatment, 2×10^6 monocytes were added to the endothelial monolayers and incubated at 37 °C for 30 min. The nonadherent cells were washed away with PBS, and adherent cells were lysed in 0.2% Triton X-100 for quantification. The fluorescent intensity of the monocytes added to the monolayer (input) as well as the nonadherent cells was measured at excitation 485 and emission 528 nm.

Murine 3T3L1 Fibroblast Cell Line—Adipocytes and murine 3T3L1 fibroblast cell line were obtained from the ATCC. Cells were cultured in high glucose DMEM medium containing 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and maintained at 37 °C in an incubator containing 5% (v/v) CO_2. 3 days after reaching confluence, cells were incubated in high glucose DMEM medium containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and supplemented with 100 milliunits/ml insulin, 0.5 mM isobutylmethylxanthine, and 250 nM dexamethasone for 2 days to allow differentiation into adipocytes. Cells were then placed in the same medium containing insulin but lacking additional supplements for an additional 2 days. The medium was replaced every 2 days thereafter until more than 85% of the cells contained lipid droplets. 7–10 days after the induction of differentiation, 3T3L1 adipocytes were ready to be used in experiments (18). The cells were incubated with serum-free low glucose DMEM during the experimental incubation period. Cells were treated with HG and Mn^{2+} in the same manner as were HUVECs.

Animal Study—All procedures followed were in accordance with the ethical standards of the institution, and approval was obtained from the institutional Animal Welfare Committee. Male Zucker diabetic fatty (ZDF) rats were purchased at 5 weeks of age (200–220 g) from Charles River Laboratories (Wilmington, MA) and allowed 2 days for environmental and trainer handling acclimation. The rats were housed and labeled in individual cages. Rats were assigned into various groups by computer-generated randomization. Rats were fasted overnight and then weighed. The rats were tested for hyperglycemia by measuring their blood glucose concentration. Blood for blood glucose measurements was obtained via tail incision and measured using an Advantage Accu-chek glucometer (Roche Applied Science). At 6 weeks of age, rats were randomly divided into two groups: group A (diabetic controls) and group B (Mn^{2+}-supplemented diabetic rats). Each rat was supplemented with the appropriate dose of Mn^{2+} or water daily for 7 weeks by oral gavage using 20-gauge feeding needles (Popper and Sons, New Hyde Park, NY). Group B was supplemented with a 16 mg/kg of body weight dose of Mn^{2+}. Previous studies have used 0.001% Mn^{2+} in the diet as an adequate amount and 0.01% Mn^{2+} as supplementation (19, 20). Various studies have reported daily food consumption for ZDF rats to range between 35 and 50 g per day (21, 22). One study reported daily food consumption to be up to 66 g per day in ZDF rats used as their control group (23). Assuming that ZDF rats consume 50 g of food per day, 0.01% Mn^{2+} corresponds to 5 mg. Rats weigh on average 300 g; therefore, 5 mg of Mn^{2+} per rat equals 16 mg/kg of body weight. Body weights were monitored weekly to determine the dose of Mn^{2+} supplementation. We prepared a stock solution of 16 mg/ml $MnCl_2$ and gavaged the rats according to weights, administrating 0.1 ml/100 g of body weight. Mn^{2+} content of the diet is 71 ppm, which equals to 71 mg/kg of diet and 3 mg/50 g of diet. Therefore, total supplementation in our experiment was 8 mg/rat, which is 0.016% Mn^{2+} supplementation in the diet.

The rats were maintained under standard housing conditions at 22 ± 2 °C with 12:12-h light/dark cycles and fed Purina 5008 lab chow diet (Charles River Laboratories). Food intake was monitored on two separate occasions during the 7-week period to assess consumption. At the end of 7 weeks, the rats were fasted overnight and then euthanized for analysis by exposure to isoflurane. Blood was collected into EDTA BD Vacutainer tubes via cardiac puncture with a 19.5-gauge needle. Plasma was isolated after centrifuging blood at 3000 rpm for 15 min at 4 °C. Immediately after blood collection, livers were perfused with cold saline, extracted, and stored in −70 °C freezers. A portion of blood from rats in each group was sent to the clinical laboratory of LSU Health Shreveport (located in the same building) for clinical tests to determine glucose, HbA_1c, liver, and renal function and red blood cell counts.

MCP-1 and ICAM-1 Assay—Cytokine levels were determined using the sandwich ELISA kits from R&D Systems, Inc. (Minneapolis, MN) for MCP-1 and from Fisher Thermo Scientific for ICAM-1. All appropriate controls and standards as specified by the manufacturer's kit were used. Control samples were analyzed each time to check the variation from plate to plate on different days of analysis.

All chemicals were purchased from Sigma unless otherwise mentioned. Data were analyzed statistically using one-way analysis of variance between different groups using Sigma Plot 11 software (Systat Software Inc., San Jose, CA). A p value of less than 0.05 was considered significant.

RESULTS

Effects of Mn^{2+} Supplementation in Cell Culture—Fig. 1 shows the effects of Mn^{2+} pretreatment on MCP-1 secretion in endothelial cells and adipocytes exposed to high glucose. There was a significant reduction in MCP-1 secretion in both cell types in the presence of 10 μM Mn^{2+}, indicating that the effect of Mn^{2+} on MCP-1 secretion inhibition is not specific to one cell type.

Fig. 2 illustrates the effects of Mn^{2+} supplementation on monocyte adhesion to endothelial cells. Adhesion assays between HUVECs and THP-1 monocytes were performed after treating both cell types with and without Mn^{2+} (see "Experimental Procedures" for details). Mn^{2+} supplementation significantly inhibited monocyte adhesion to endothelial cells in both high glucose-treated and MCP-1-treated cells. These results suggest a potential beneficial effect of Mn^{2+} in reducing mono-

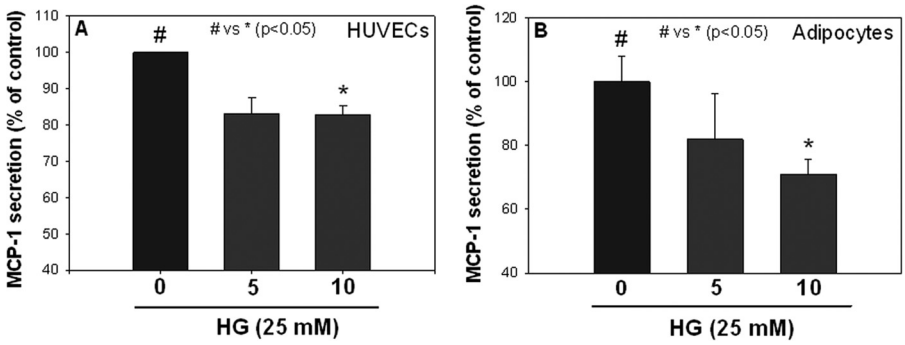

FIGURE 1. **Effects of manganese (Mn^{2+}) supplementation on MCP-1 secretion in HUVECs (*A*) and 3T3L1 adipocytes (*B*).** Values are ± S.E., $n = 3$, and expressed as a percentage of HG-treated cells.

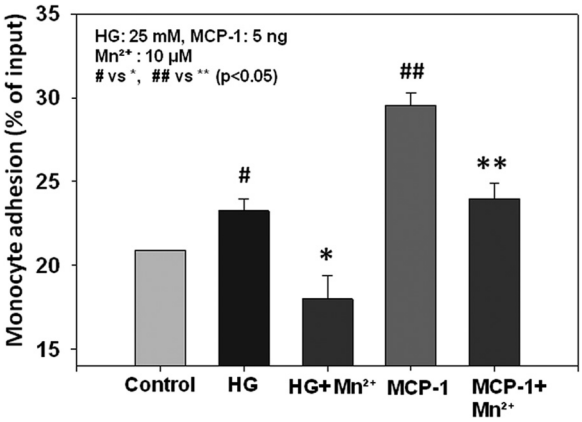

FIGURE 2. **Effects of Mn^{2+} supplementation on monocyte (THP-1) adhesion to endothelial cells (HUVECs).** Values are ± S.E., $n = 3$, and expressed as a percentage of input fluorescence.

cyte adherence to endothelial cells, a key event in the initiation of endothelial dysfunction.

To determine what adhesion molecules mediate the reduction of monocyte adhesion to endothelial cells caused by Mn^{2+} supplementation, we measured total and surface ICAM-1 expression in HUVECs cultured with high glucose. Mn^{2+} supplementation caused a significant down-regulation of both total and surface ICAM-1 expression in endothelial cells (Fig. 3, *A* and *B*). The effect on ICAM-1 down-regulation was Mn^{2+}-dose dependent. Magnesium (Mg^{2+}) supplementation did not have any effect on total ICAM-1 regulation. Because Mn^{2+} caused a decrease in MCP-1 secretion and ICAM-1 expression, we then examined whether ICAM-1 regulation is associated with MCP-1 secretion in endothelial cells. Exogenous addition of MCP-1 *per se* also caused activation of ICAM-1 expression, and Mn^{2+} supplementation inhibited the MCP-1-induced up-regulation of ICAM-1 in endothelial cells. This suggests that inhibition of MCP-1 secretion caused by Mn^{2+} supplementation may play a role in down-regulation of ICAM-1 expression.

To further investigate the mechanisms by which Mn^{2+} supplementation reduces monocyte adhesion to endothelial cells and MCP-1 secretion, we measured ROS levels in HUVECs treated with or without Mn^{2+}. Fig. 3C shows that Mn^{2+} supplementation inhibits the high glucose increase in ROS levels in a dose-dependent manner.

To evaluate the role of MnSOD in the regulation of ICAM-1 and ROS levels, we first measured MnSOD expression and activity. MnSOD expression was not different in endothelial cells treated with HG or MCP-1 with and without Mn^{2+} supplementation (data not shown). MnSOD activity was decreased in cells exposed to high glucose and increased in cells supplemented with Mn^{2+} and exposed to HG (Fig. 4).

To further investigate the role of MnSOD, we knocked down its expression using siRNA before treating the cells with Mn^{2+}. Fig. 5 shows the efficiency of our knockdown system, where MnSOD was knocked down about 70%, but the expression of the other SOD enzyme, Cu/ZnSOD, was unchanged in HUVECs. As shown in Fig. 6, monocyte adhesion to endothelial cells was still inhibited by Mn^{2+} supplementation in cells where MnSOD was knocked down. In addition, we still observed a down-regulation of ICAM-1 total and surface expression (Fig. 7, *A* and *B*), as well as a decrease in ROS levels (Fig. 7*C*), suggesting that the effects of Mn^{2+} supplementation on monocyte adhesion to endothelial cells are mediated by inhibition of ICAM-1 expression and ROS production, and not MnSOD. MnSOD activity was not detectable in HUVECs treated with MnSOD siRNA (data not shown). This is the first time that a role of Mn^{2+} is reported to have a beneficial effect on ICAM-1 and ROS levels in endothelial cells, without MnSOD.

Effects of Mn^{2+} Supplementation in ZDF Rats—Elevated levels of ICAM-1 and lipids are a hallmark of endothelial cell dysfunction and are risk factors in the development of atherosclerosis. Fig. 8 shows that blood levels of ICAM-1 (*A*) and cholesterol (*B*) were significantly lower in Mn^{2+}-supplemented when compared with placebo-supplemented ZDF rats. Fig. 8 also shows that blood levels of MCP-1 (*C*) and triglycerides (*D*) were lower but not statistically significant in Mn^{2+}-supplemented rats when compared with placebo-supplemented rats. Further studies are needed on the effect of dose and duration of Mn^{2+} supplementation to determine its optimal efficacy *in vivo*. Nevertheless, these results demonstrate that our novel *in vitro* findings, that Mn^{2+} supplementation down-regulates ICAM-1 expression and progression of endothelial dysfunction, are validated *in vivo*.

There were no differences in body weight (345.6 g ± 7.6 *versus* 345 g ± 7.7), food consumption (42.4 g/day ± 2.6 *versus* 40.9 g/day ± 3.9), insulin (1.94 ± 0.46 *versus* 2.06 ± 0.36 ng/ml), blood glucose (278.4 ± 18 *versus* 323.3 ± 35.3 mg/dl), or glycated hemoglobin (HbA1, 17.6 ± 0.34 *versus* 16.5 ± 0.39%) in the Mn^{2+}-supplemented group in comparison with the control diabetic group ZDF rats. Mn^{2+} supplementation

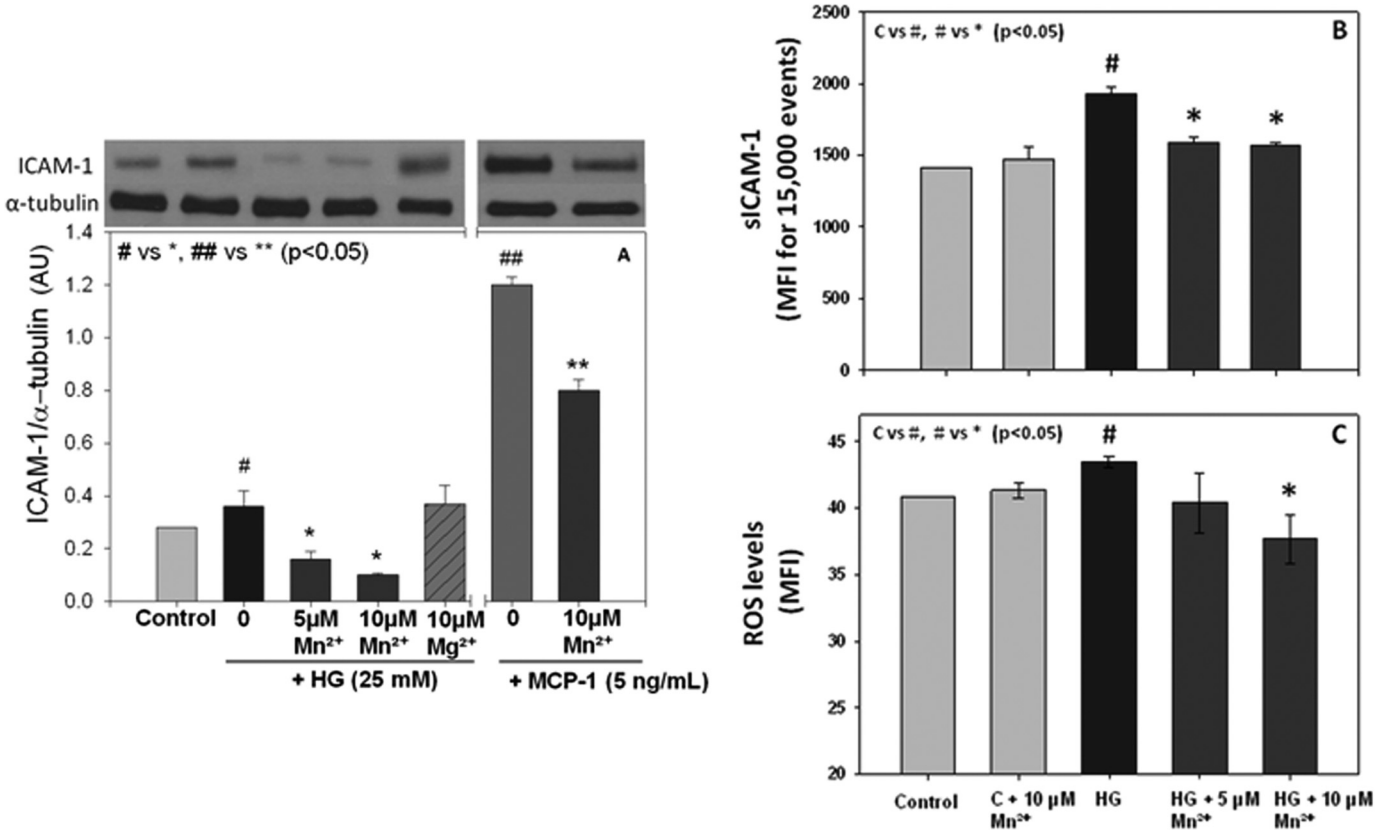

FIGURE 3. **Effects of Mn^{2+} supplementation on ICAM-1 total expression (*A*), surface expression (*B*), and ROS (*C*) in HUVECs.** Quantification of band intensity is shown in arbitrary units (*AU*). Values are ± S.E., $n = 3$, expressed as mean fluorescence intensity (*MFI*) for surface ICAM-1. Values are ± S.E., $n = 4$ for ROS. *C* indicates control.

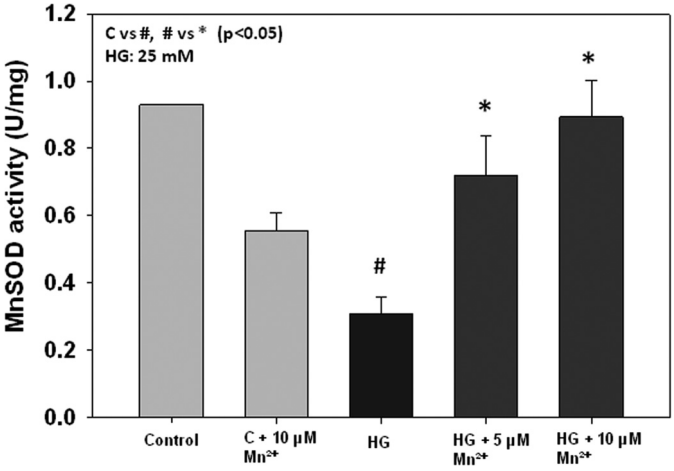

FIGURE 4. **Effects of Mn^{2+} supplementation on MnSOD activity in HUVECs.** MnSOD activity is expressed as units/mg of protein. Values are ± S.E., $n = 3$. *C* indicates control.

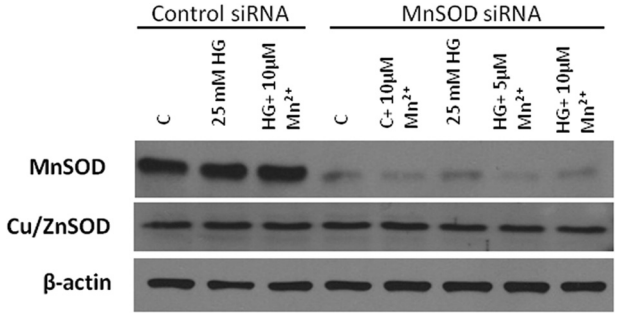

FIGURE 5. **Effects of Mn^{2+} supplementation on MnSOD siRNA-transfected HUVECs on MnSOD and Cu/ZnSOD expression.** *Lane C* indicates control.

did not affect hemoglobin, hematocrit, or RBC count, or markers of liver or kidney functions in diabetic rats, as assessed by alanine aminotransferase and aspartate aminotransferase, or creatinine levels.

DISCUSSION

Complications of atherosclerosis cause most morbidity and mortality in patients with diabetes (24). More than 25 million persons in the United States have at least one clinical manifestation of atherosclerosis (25). The key early event in the development of atherosclerosis is dysfunction of the endothelium, which is characterized by increased expression of cellular adhesion molecules, such as ICAM-1, and secretion of chemokines such as MCP-1. These events lead to the recruitment of monocytes to the arterial wall where they become macrophages and initiate chronic inflammation, leading to hyperlipidemia and atherosclerotic lesion development.

Various studies report blood concentrations of manganese between 0.15 and 7 μM (7, 8, 26, 27). Some studies have reported lower Mn^{2+} levels in lymphocytes (7), plasma, and hair samples (8) of type 2 diabetic patients and in the heart and aorta tissue of patients with atherosclerosis when compared with those of healthy controls (9). Manganese supplementation has been

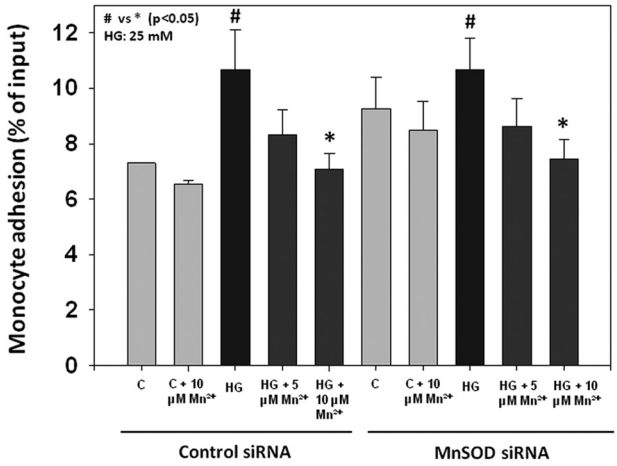

FIGURE 6. **Effects of Mn^{2+} supplementation on monocyte (THP-1) adhesion to endothelial cells (HUVECs).** Values are ± S.E., $n = 3$, and expressed as a percentage of input fluorescence. *C* indicates control.

shown to cause a decrease in total serum cholesterol and aorta cholesterol and regression of atherosclerosis in cholesterol-fed rabbits (10). However, there is no evidence showing a direct beneficial role of Mn^{2+} supplementation on endothelial function and vascular inflammation, and the mechanisms by which Mn^{2+} can reduce cholesterol levels are unknown. MCP-1 is a chemokine that promotes the recruitment of monocytes and macrophages to the subendothelial cell layer. Deposition of lipids within these monocytes and macrophages then leads to development of atherosclerotic lesions. MCP-1 is also produced after induction of oxidative stress or growth factors by a variety of cell types, including monocytes, smooth muscle cells, and endothelial cells, and plays an important role in vascular inflammation and atherosclerotic lesion formation (28–31). Mn^{2+} is a potent antioxidant; it is the cofactor of the enzyme MnSOD, the main antioxidant enzyme in the mitochondria, and can also scavenge oxygen radicals itself. Various studies using Mn^{2+} link its effects with the function and role of MnSOD (4, 6).

This study demonstrates for the first time that Mn^{2+} supplementation down-regulates ICAM-1 expression, reduces ROS production, MCP-1 secretion, and monocyte adhesion in endothelial cells exposed to high glucose, and lowers blood levels of ICAM-1 and cholesterol in ZDF rats. In addition, our study provides a molecular mechanism for the beneficial effects of Mn^{2+} supplementation on lowering vascular inflammation markers in HUVECs and ZDF rats. We found that Mn^{2+} supplementation inhibits secretion of MCP-1 in endothelial cells and adipocyte cells and that the MCP-1-induced up-regulation of ICAM-1 expression and monocyte adhesion to endothelial cells can be inhibited with Mn^{2+} supplementation. Interestingly, Mn^{2+} supplementation also inhibited both the ICAM-1 down-regulation and the monocyte adhesion induced by exogenous MCP-1 treatment in endothelial cells. This demonstrates that the inhibition of MCP-1 secretion caused by Mn^{2+} supplementation may mediate the down-regulation of ICAM-1 expression and monocyte adhesion in endothelial cells.

Hyperglycemia is known to increase oxidative stress and glycation of protein (32–34). In this study, we observed that ROS,

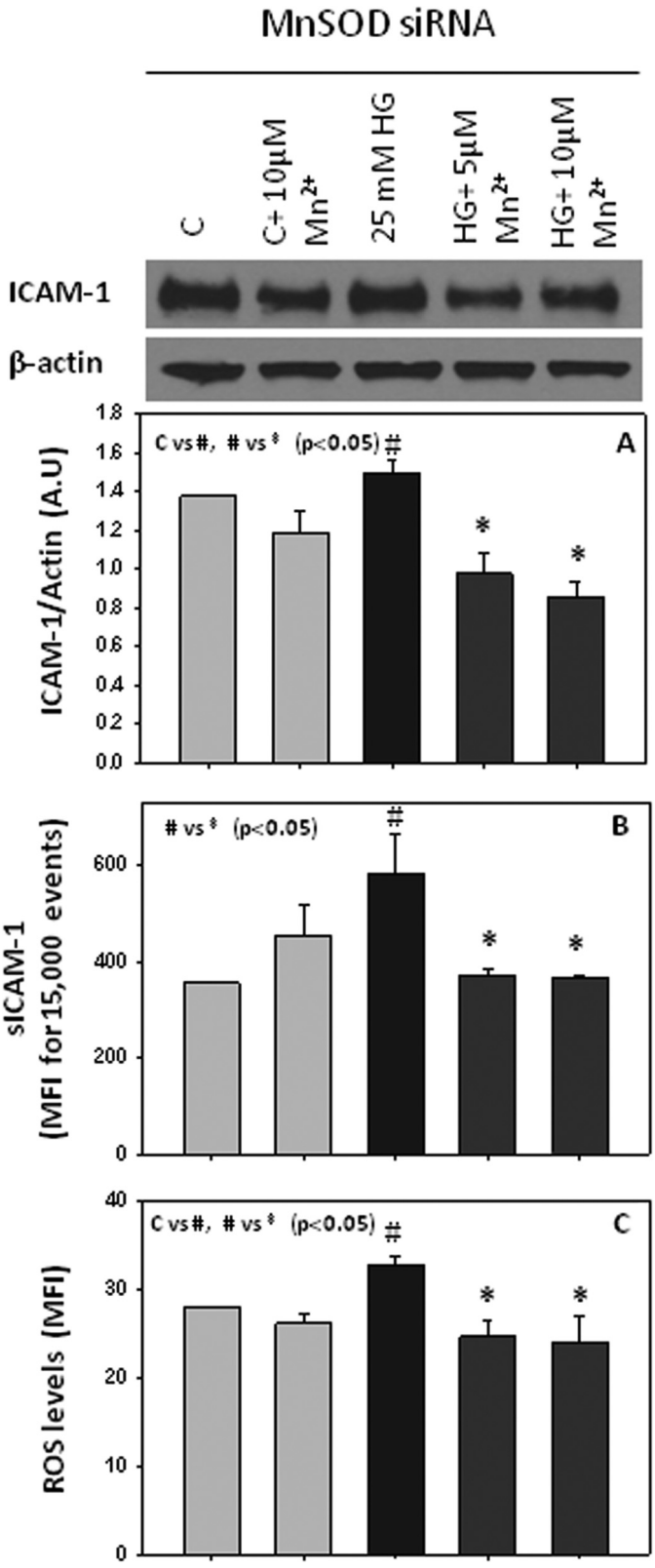

FIGURE 7. **Effects of Mn^{2+} supplementation on ICAM-1 total expression (A), surface expression (B), and ROS (C) in HUVECs transfected with MnSOD siRNA.** Values are ± S.E., $n = 3$. *C* indicates control. *A. U.*, arbitrary units.

which was inhibited by Mn^{2+} supplementation, was increased in HG-treated HUVEC cells. Also, the decrease in MnSOD activity observed could be due to increase in oxidative stress or

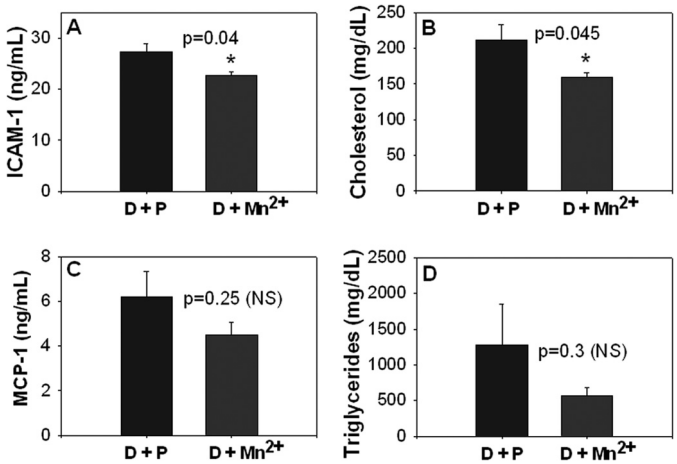

FIGURE 8. **Effects of Mn^{2+} supplementation on plasma levels of ICAM-1 (A), cholesterol (B), MCP-1 (C), and triglycerides (D).** Values are ± S.E. of placebo-supplemented rats ($D + P$, $n = 6$) and manganese-supplemented rats ($D + Mn^{2+}$, $n = 5$).

glycation of MnSOD. This study reports that Mn^{2+} supplementation increases MnSOD activity in HG-treated HUVECs. However, further investigation shows that the effect of Mn^{2+} on monocyte adhesion to endothelial cells is independent of MnSOD. Although activity of MnSOD increases in cells supplemented with Mn^{2+}, similar effects of Mn^{2+} supplementation on monocyte adhesion to endothelial cells, inhibition of ICAM-1 and ROS levels, were observed in MnSOD knockdown cells, suggesting that MnSOD does not play a role in the decreased monocyte-endothelial cell adhesion in Mn^{2+}-supplemented endothelial cells.

This study suggests that the effects of Mn^{2+} supplementation on monocyte adhesion to endothelial cells are mediated by the inhibition of ROS and ICAM-1 expression. This study also demonstrates that Mn^{2+} supplementation significantly lowers blood levels of ICAM-1 and cholesterol in ZDF rats. Thus, this study provides a novel molecular mechanism by which Mn^{2+} supplementation can prevent or delay endothelial dysfunction and atherosclerosis development using both cell culture and *in vivo* studies.

The findings reported in this study could have broader significance. In addition to atherosclerosis, adhesion molecules such as ICAM-1 are also implicated in the progression of infection (35–37). A recent study reported that manganese blocks intracellular trafficking of Shiga toxin and that manganese-supplemented mice were completely resistant to a lethal Shiga toxin challenge (38). Whether manganese inhibition of ICAM-1 expression occurs in other cell types and can play a beneficial role in preventing the progression of infection is not known and warrants investigation.

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