Chronic Methylglyoxal Infusion by Minipump Causes Pancreatic β-Cell Dysfunction and Induces Type 2 Diabetes in Sprague-Dawley Rats

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OBJECTIVE—The incidence of high dietary carbohydrate-induced type 2 diabetes is increasing worldwide. Methylglyoxal (MG) is a reactive glucose metabolite and a major precursor of advanced glycation end products (AGEs). MG levels are elevated in diabetic patients. We investigated the effects of chronic administration of MG on glucose tolerance and β-cell insulin secreting mechanism in 12-week-old male Sprague-Dawley rats.

RESEARCH DESIGN AND METHODS—MG (60 mg/kg/day) or 0.9% saline was administered by continuous infusion with a minipump for 28 days. We performed glucose and insulin tolerance tests and measured adipose tissue glucose uptake and insulin secretion from isolated pancreatic islets. We also used cultured INS-1E cells, a pancreatic β-cell line, for molecular studies. Western blotting, quantitative PCR, immunohistochemistry, and transferase-mediated dUTP nick-end labeling (TUNEL) assay were performed.

RESULTS—In rats treated with MG and MG + L-buthionine sulfoximine (BSO), MG levels were significantly elevated in plasma, pancreas, adipose tissue, and skeletal muscle; fasting plasma glucose was elevated, whereas insulin and glutathione were reduced. These two groups also had impaired glucose tolerance, reduced GLUT-4, phosphoinositide-3-kinase activity, and insulin-stimulated glucose uptake in adipose tissue. In the pancreatic β-cells, MG and MG + BSO reduced insulin secretion, pancreatic duodenal homeobox-1, MafA, GLUT-2, and glucokinase expression; increased C/EBPβ, nuclear factor-κB, MG-induced AGE, N^{ε}-carboxymeythyllysine, and receptor for AGEs expression; and caused apoptosis. Alagebrium, an MG scavenger and an AGE-breaking compound, attenuated the effects of MG.

CONCLUSIONS—Chronic MG induces biochemical and molecular abnormalities characteristic of type 2 diabetes and is a possible mediator of high carbohydrate-induced type 2 diabetes. ***Diabetes* 60:899–908, 2011**

Type 2 diabetes is characterized by hyperglycemia, insulin resistance, and progressive decrease in insulin secretion from the pancreas (1). A genetic predisposition has been found in many patients. More recently there has been a staggering increasing in the incidence of type 2 diabetes, many of the cases being reported in children. This explosive increase is attributed to a diet high in carbohydrates, fat, and a sedentary lifestyle (2–6). Oxidative stress is associated with diabetes and has been proposed as one of the causative factors (7,8). An increase in oxidative stress caused the insulin resistance of Zucker obese rats to progress to type 2 diabetes in 1 week (9).

Methylglyoxal (MG) is a reactive dicarbonyl metabolite of mainly glucose metabolism (10). MG reacts with proteins to form advanced glycation end products (AGEs) (10,11), which are implicated in the pathogenesis of vascular complications of diabetes (11,12). Plasma MG levels in healthy humans are 1 μmol/L or less and are elevated two- to fourfold in diabetic patients (13,14). Under physiological conditions, the glyoxalase system degrades MG into D-lactate with the help of reduced glutathione (GSH) (10,14) and keeps plasma MG levels at approximately 1 μmol/L or less (13,14). Incubation of vascular smooth muscle cells with 25 mmol/L glucose or fructose for 3 h significantly increases MG production and oxidative stress (15). In vitro incubation of MG with insulin modifies the insulin molecule and impairs insulin-mediated glucose uptake in adipocytes (16). Incubation of cultured L6 muscle cells with MG (2.5 mmol/L) for 30 min impaired insulin signaling (17). However, the in vitro studies cannot establish whether MG is the cause of diabetes or an effect of diabetes.

The molecular mechanisms of high dietary carbohydrate-induced type 2 diabetes are not clear. It is possible that high carbohydrate-induced chronic elevation of MG causes cumulative pathologic changes that contribute to the development of insulin resistance and type 2 diabetes. We have recently shown that acute MG (50 mg/kg iv) administered to 12-week-old male Sprague-Dawley (SD) rats caused glucose intolerance and reduced adipose tissue insulin-stimulated glucose uptake (18). Here we report the results of a comprehensive study on the effects of chronically administered MG on in vivo glucose tolerance, adipose tissue glucose uptake and insulin secretion from isolated pancreatic islets, and the underlying molecular mechanisms. We administered MG by continuous infusion with minipump for 28 days, a method used for the first time to administer MG.

RESEARCH DESIGN AND METHODS

Animals. All animal protocols were approved by the Animal Care Committee of the University of Saskatchewan. Male SD rats, 11 weeks old, from Charles River Laboratories (Quebec, Canada) were treated according to guidelines of the Canadian Council on Animal Care.

MG (40% solution) was administered to 12-week-old male SD rats for 28 days by means of an osmotically driven infusion minipump (Alzet 2ML4, Durect Corporation, Cupertino, CA) implanted subcutaneously on the back. This pump releases a continuous small amount of MG into the body at a rate of 2.5 μL/h, amounting to 60 mg/kg/day. There was no inflammatory infiltrate or response in the animals with the infusion or the pump implantation. Control rats were

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Received 3 May 2010 and accepted 6 December 2010.
DOI: 10.2337/db10-0627
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0627/-/DC1.
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administered 0.9% saline (2.5 μL/h) by means of subcutaneously implanted pump. GSH plays a key role in degrading MG. We also treated two groups of rats with L-buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (19), which prevents GSH synthesis, decreases MG degradation, and increases MG levels. Alagebrium (AGA) is a MG scavenger and an AGE-breaking compound (18,20).

After one week of acclimatization, the rats were divided into the following treatment groups (n = 6 each): *1*) control – 0.9% saline, *2*) MG (60 mg/kg/day), *3*) MG (60 mg/kg/day) + ALA (30 mg/kg/day in drinking water), *4*) MG (60 mg/kg/day) + BSO (30 mg/kg/day in drinking water), 5) BSO alone (30 mg/kg/day in drinking water), and *6*) ALA alone (ALA, 30 mg/kg/day in drinking water). All of the treatments were for 28 days. ALA alone had no effect.

Biochemical parameters. Blood was collected from anesthetized rats from different treatment groups; the plasma was separated and analyzed for fasting basal levels of glucose, insulin, free fatty acids, total cholesterol, triglycerides, HDL, creatine kinase (CK), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The free fatty acid levels in plasma samples of rat were measured using an ELISA kit (Cusabio Biotech) following the manufacturer's instruction.

Oral glucose tolerance test. After overnight fasting, an oral glucose tolerance test (GTT) was performed. In brief, the trachea, left jugular vein, and right carotid artery were cannulated in anesthetized rats. After a basal blood sample was collected, an oral glucose load (1 g/kg) was given with a stomach tube. Further blood samples were collected at 15, 30, 60, and 120 min from the carotid artery. Plasma glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA), and insulin levels were measured with a rat insulin ELISA assay kit (Mercodia, Winston Salem, NC).

Insulin release from freshly isolated pancreatic islets. The pancreatic islets were freshly isolated from SD rats as described previously (21) and in the Supplementary Data. Insulin release was determined after incubation of the islets with the test compounds.

Glucose uptake. Adipose tissue glucose uptake was performed as described (22) and in the Supplementary Data. [^{3}H]-2-Deoxy glucose (0.1 μCi/500 μL) was used for uptake experiments.

MG assay. MG was measured by a specific and sensitive high-performance liquid chromatography (HPLC) method as described previously and in the Supplementary Data (23).

Measurement of reduced GSH levels. The GSH levels in the plasma and organs were determined by derivation with 5,50-dithio-bis (2-nitrobenzoic acid) and reverse-phase HPLC using ultra-violet detection, as described previously (24).

Isolation of plasma membrane for GLUT4. Adipose tissue plasma membrane was isolated with an isolation kit (BioVision, MountainView, CA) as described in the Supplementary Data.

INS-1E cell culture experiments. INS-1E cells (a pancreatic islet β-cell line, a generous gift from Dr. C. B. Wollheim, Geneva, Switzerland) were cultured as described in the Supplementary Data. These cells were used to determine insulin secretion, peroxynitrite levels, ATP-sensitive K^+ channel (K_{ATP}) channel currents, intracellular calcium levels and phosphoinositide 3-kinase (PI3K) activity as described in the Supplementary Data.

Western immunoblotting. Isolated pancreas and adipose tissue were subjected to Western blotting as described previously (21,22) and in the Supplementary Data.

Real-time quantitative PCR. RNA was isolated from the pancreas using RNA isolation kit (Qiagen Sciences, Germantown, MD). The total RNA was reverse-transcribed in triplicate using RevertAid H Minus M-MuLV reverse transcriptase (MBI, Fermentas Burlington, ON, Canada) in the presence of 5× RT buffer (MBI, Fermentas), random primer (Invitrogen, Carlsbad, CA), and dNTP mixture (Amersham, Pittsburgh, PA) at 42°C for 50 min, followed by 72°C for 10 min. The predesigned primers for pancreatic duodenal homeobox-1 (PDX-1) and MafA were from Qiagen Sciences (Germantown, MD). The real-time PCR was carried out in an iCycler iQ apparatus (Bio-Rad, Life Science Research, Hercules, CA). All PCRs were triplicated and run for 45 cycles at 95°C for 20 s, 62°C for 1 min, and 72°C for 30 s. After cycling, melting curves of the PCR products were acquired by stepwise increase of the temperature from 62° to 95°C.

Determination of cell apoptosis. The transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche Diagnostics, Indianapolis, IN) was used as described in the Supplementary Data. DNA was isolated from pancreas using DNA extraction kit (R & D Systems, Minneapolis, MN). Equal amount and concentration (1 μg) of DNA was loaded on 1% agarose gel and run at 100 V for 2 h. The gel was visualized on Syngene bio-imaging system (Syngene, Frederick, MD) (25).

Chemicals and statistical analysis. All chemicals were of analytical grade. MG and *o*-phenylenediamine (*o*-PD) were purchased from Sigma-Aldrich, Oakville, ON, Canada. Alagebrium (formerly known as ALT-711) was a generous gift from Synvista Therapeutics (Montvale, NJ). Data are expressed as mean ± SEM and analyzed using one-way ANOVA and post hoc Bonferroni's test. P value less than 0.05 was considered significant.

RESULTS

Chronic MG treatment significantly alters metabolic characteristics of SD rats. MG, MG + BSO, and BSO treatment for four weeks significantly increased fasting plasma glucose, free fatty acids, total cholesterol, and triglycerides (Table 1) and decreased fasting plasma insulin and HDL (Table 1) levels compared with the control group. There was no significant difference in the body weight between the treatment groups (Table 1) and markers indicating tissue or organ damage such as serum CK for muscle damage, creatinine for kidney function, and ALT and AST for liver damage (Table 1). Pretreatment with ALA (30 mg/kg/day) significantly attenuated MG-induced changes in the metabolic parameters (Table 1).

MG and GSH levels are different among treatment groups. MG-, MG + BSO-, and BSO-treated groups had significantly elevated plasma, pancreas, adipose tissue, and skeletal muscle MG levels compared with control (Fig. 1*A–D*)

TABLE 1
Plasma levels of several parameters in SD rats treated with MG

Parameter	Control	MG	MG + BSO	BSO	MG + ALA	ALA
Body weight (g)	558 ± 18	556 ± 9	582 ± 13	536 ± 33	588 ± 7	543 ± 21
Fasting plasma glucose (mg/dL)	89 ± 6	121 ± 6*	139 ± 7***	116 ± 10*	95 ± 9	89 ± 8
Fasting plasma insulin (μg/L)	1.2 ± 0.1	0.5 ± 0.1**	0.3 ± 0.02***	0.7 ± 0.1*	1 ± 0.2†	1.25 ± 0.06
Plasma free fatty acids (μg/L)	393 ± 41	619 ± 21***	890 ± 21***†††	601 ± 32***	405 ± 20†††	389 ± 22†††
Total cholesterol (mmol/L)	1.8 ± 0.1	2.4 ± 0.05***	2.8 ± 0.05***††	2.1 ± 0.1**‡‡‡	1.9 ± 0.03††	—
HDL (mmol/L)	1.2 ± 0.05	0.7 ± 0.06**	0.46 ± 0.05***†	0.78 ± 0.1**‡	1.0 ± 0.07†	—
Triglycerides (mmol/L)	0.2 ± 0.03	0.5 ± 0.04***	0.7 ± 0.02***††	0.4 ± 0.05**‡‡‡	0.3 ± 0.02††	—
Creatinine (μmol/L)	55 ± 5	66 ± 6	75 ± 8	58 ± 5	52 ± 2	—
CK (U/L)	315 ± 20	365 ± 25	378 ± 23	355 ± 21	332 ± 15	—
ALT (U/L)	60 ± 10	65 ± 9	71 ± 10	63 ± 7	59 ± 8	—
AST (U/L)	46 ± 5	50 ± 8	56 ± 9	48 ± 4	45 ± 6	—

Values are means ± SE of n = 6 experiments. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic pump for 28 days to all groups of rats (n = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA and ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, basal fasting plasma levels of substances listed in the table were measured. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. respective control group; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ vs. respective MG group; ‡$P < 0.05$, ‡‡‡$P < 0.001$ vs. respective MG + BSO group.

that were attenuated by pretreatment with ALA in the MG + ALA group (Fig. 1*A*–*D*). MG levels were higher in plasma and tissues in the MG + BSO group compared with MG and BSO alone groups (Fig. 1*A*–*D*). MG, MG + BSO, and BSO treatment significantly reduced GSH levels in the plasma, pancreas, and skeletal muscle compared with control (Fig. 1*E*, *F*, and *H*). ALA pretreatment attenuated the decrease in GSH induced by MG (Fig. 1*E*, *F*, and *H*). Adipose tissue

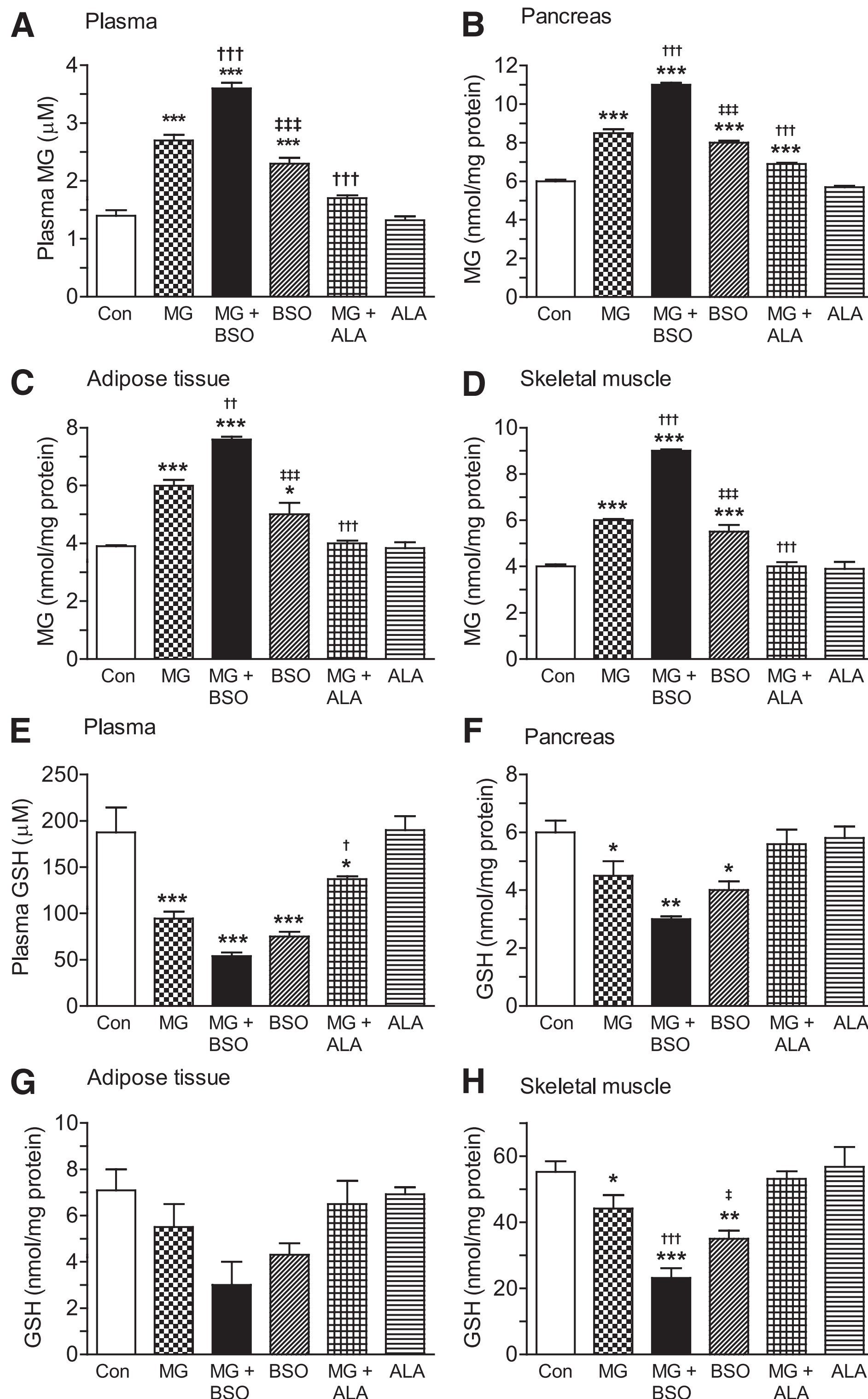

FIG. 1. MG levels are elevated, and reduced GSH levels are decreased in SD rats chronically treated with MG. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n* = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water) (MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water) (MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, MG and GSH levels were determined by HPLC in plasma (*A* and *E*) and organs (*B*–*D* and *F*–*H*). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective control; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. respective MG group; ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 vs. respective MG + BSO group.

and pancreas had much lower GSH levels compared with skeletal muscle (Fig. 1*F–H*).

Chronic MG impairs glucose tolerance in SD rats. MG and MG + BSO treatment significantly impaired in vivo glucose tolerance determined after an oral glucose load in SD rats (Fig. 2*A*). Plasma glucose levels were significantly higher in the MG and MG + BSO group compared with control even 2 h after the glucose load. ALA pretreatment attenuated the impaired glucose tolerance induced by MG (Fig. 2*A*).

MG- and MG + BSO-treated groups had significantly lower plasma insulin levels compared with the control group in the oral GTT (Fig. 2*B*). The plasma insulin levels were lower in the MG + BSO group compared with the MG alone group. The plasma insulin levels were significantly lower than control even 2 h after the glucose load in the MG and MG + BSO groups. ALA pretreatment attenuated the reduced insulin levels induced by chronic MG treatment (Fig. 2*B*). Chronic MG and MG + BSO also impaired the insulin tolerance test in rats, which was attenuated by ALA cotreatment with MG (Supplementary Fig. 1*S*).

Chronic MG treatment reduces glucose uptake, plasma membrane GLUT4 expression, and PI3K activity in adipose tissue. Insulin-stimulated glucose uptake was significantly decreased in adipose tissue freshly isolated from rats treated with MG or MG + BSO compared with those from control rats, attenuated by ALA cotreatment with MG (Fig. 3*A*). To understand the mechanism of MG-induced impaired glucose tolerance and reduced glucose

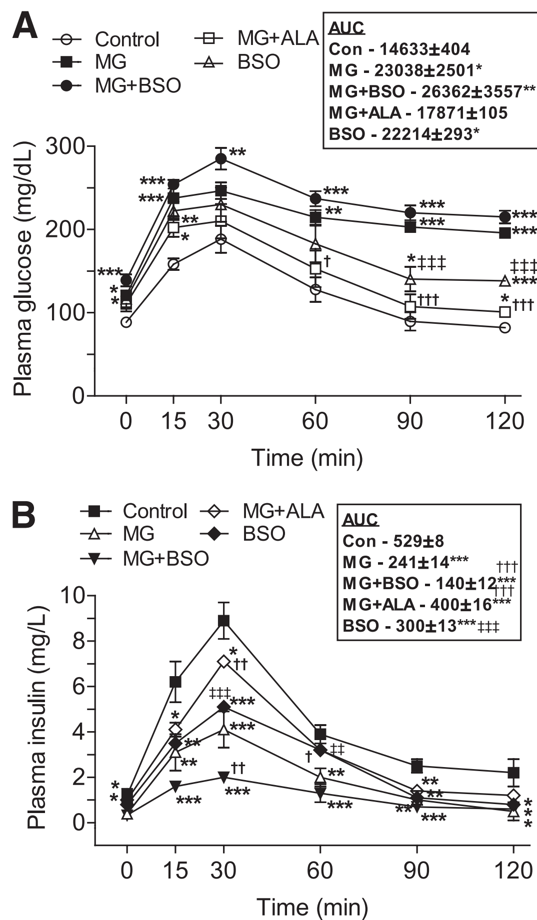

FIG. 2. The oral glucose tolerance test is impaired in SD rats chronically treated with MG. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, the rats were fasted overnight, anesthetized, and cannulated. A basal 0-min blood sample was taken from the carotid artery. After that an oral glucose load (1 g/kg body weight) was administered and blood samples were collected from the carotid artery at different times up to 120 min. Plasma was separated and analyzed for glucose (*A*) and insulin levels (*B*) following the glucose load. The inset box shows the area under curve (AUC) values for different groups. *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective control at the same time point; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. respective MG group at the same time point; ‡‡P < 0.01, ‡‡‡P < 0.001 vs. respective MG + BSO group at the same time point.

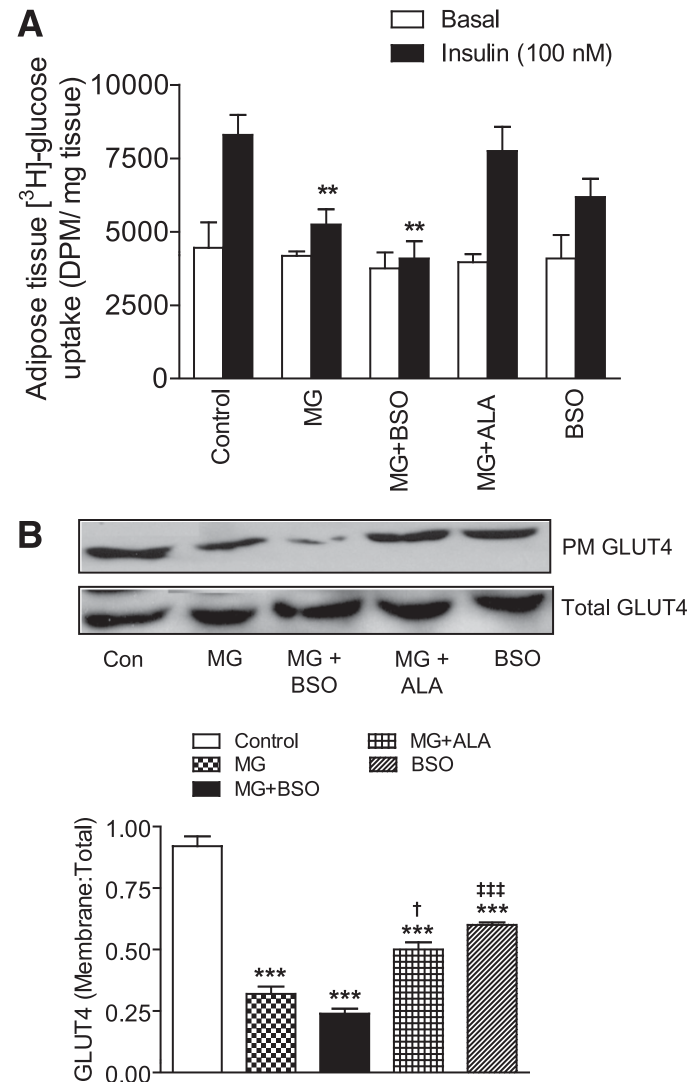

FIG. 3. Adipose tissue glucose uptake and plasma membrane GLUT4 protein are reduced in chronic MG-treated SD rats. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, the rats were fasted overnight, and abdominal visceral adipose tissue (*A*) was removed and tested for insulin-stimulated glucose uptake in vitro. *B*: The adipose tissue was also subjected to Western blotting to determine the plasma membrane (PM) and total GLUT4 protein as described in RESEARCH DESIGN AND METHODS. The plasma membrane was isolated as described in the Supplementary Data. **P < 0.01, ***P < 0.001 vs. control; †P < 0.05 vs. MG group, ‡‡‡P < 0.001 vs. MG + BSO group.

uptake, the adipose tissue GLUT4 translocation to the plasma membrane was determined. There was a significant decrease in plasma membrane GLUT4 in MG- and MG + BSO-treated rats (Fig. 3*B*), attenuated by ALA cotreatment with MG. BSO alone also reduced plasma membrane GLUT4 (Fig. 3*B*). There was no change in insulin receptor (IR) and insulin receptor substrate (IRS-1) protein expression, but phosphorylation of IRS-1 was reduced by MG and MG + BSO (Supplementary Fig. 2*S*). MG and MG + BSO also reduced activity of PI3K in adipose tissue (Supplementary Fig. 2*S*). ALA attenuated the effects of MG in adipose tissue.

Chronic MG treatment reduces total insulin content and glucose-stimulated insulin release from pancreas. The pancreatic insulin content was significantly reduced in MG- and MG + BSO-treated groups compared with control (Fig. 4). ALA attenuated the reduction in insulin content by MG (Fig. 4). Moreover, basal and glucose-stimulated insulin release from isolated pancreatic islets was significantly reduced in MG and MG + BSO groups compared with control (Fig. 5*A*). BSO alone also significantly reduced glucose-stimulated insulin secretion compared with control but less than MG + BSO group (Fig. 5*A*). ALA significantly attenuated MG-induced decrease in glucose-stimulated insulin release from the pancreatic islets (Fig. 5*A*).

Effects of chronic MG on insulin synthesis/secretion pathway in pancreas. To determine the cause of MG-induced reduction of basal plasma insulin level and glucose–stimulated insulin release from pancreatic islets, we looked at the molecular mechanisms of insulin synthesis and secreting pathways. GLUT2 is the transporter for glucose entry into islet cells. PDX-1 (also known as insulin promoter factor 1) and MafA are positive regulators, whereas C/EBPβ is a negative regulator of insulin gene transcription. There was a significant decrease in GLUT2 (Fig. 5*B*), PDX-1, and MafA protein expression in pancreas from MG- and MG + BSO-treated rats (Fig. 6*A*). There was also a significant decrease in mRNA expression of PDX-1 and MafA in MG- and MG + BSO-treated rats as compared with control (Fig. 6*B*). ALA attenuated the decrease in GLUT2 (Fig. 5*B*), PDX-1, and MafA protein and mRNA induced by MG (Fig. 6). At the same time, there was a significant increase in C/EBPβ protein expression and mRNA in MG- and MG + BSO-treated rats, which was attenuated by ALA cotreatment with MG (Fig. 6*A* and *B*). In the isolated islets, MG and MG + BSO also decreased the protein expression of GLUT2, glucokinase, PDX-1, and MafA and increased the protein expression of C/EBPβ, nuclear factor κB (Supplementary Fig. 3*S*), and the receptor for AGE (RAGE) (Supplementary Fig. 4*S*).

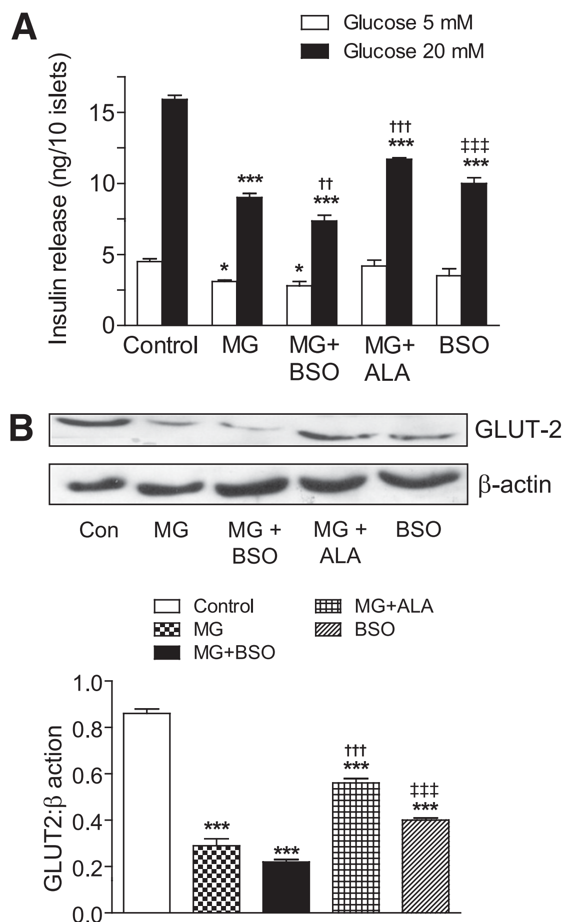

FIG. 5. Pancreatic GLUT2 and insulin release from isolated pancreatic islets are reduced in SD rats chronically treated with MG. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n* = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, the rats were fasted overnight, and the pancreatic islets (*A*) were isolated as described in RESEARCH DESIGN AND METHODS. Glucose-stimulated insulin secretion was evaluated in the isolated islets in vitro. *B*: The pancreatic tissue was removed and processed for determination of GLUT2 protein expression by Western blotting. **P* < 0.05, ****P* < 0.001 vs. respective control; ††*P* < 0.01, †††*P* < 0.001 vs. respective MG group, ‡‡‡*P* < 0.001 vs. respective MG + BSO group.

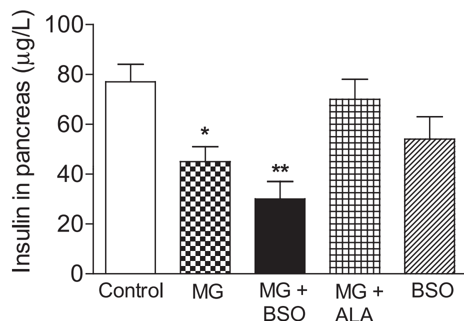

FIG. 4. Pancreatic insulin content is reduced in SD rats chronically treated with MG. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n* = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, the rats were fasted overnight, and the pancreas was removed and evaluated for insulin content. **P* < 0.05, ***P* < 0.01 vs. control.

MG reduces insulin secretion and increases reactive oxygen species in cultured INS-1E cells, a pancreatic β-cell line. In INS-1E-cultured cells, there was no change in glucose-induced intracellular calcium levels between different treatments (Supplementary Fig. 5*S*, *B*). MG, MG + BSO, and BSO increased reactive oxygen species production (Supplementary Fig. 5*S*, *A*), which were attenuated by cotreatment of MG with the superoxide dismutase-mimetic, tempol, or ALA. However, tempol could not attenuate the reduced insulin secretion induced by MG, whereas

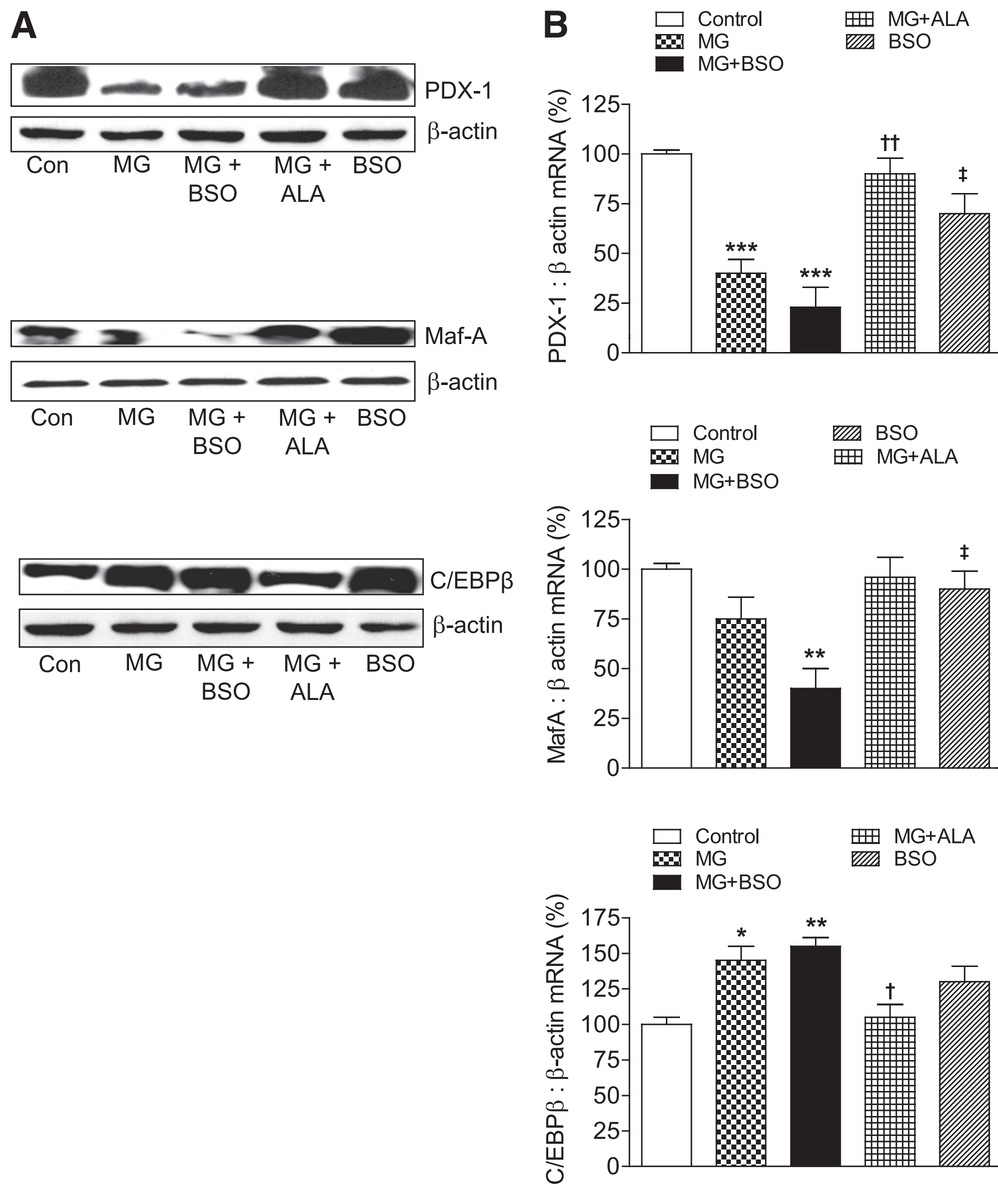

FIG. 6. Insulin gene transcription factors PDX-1 and MafA protein expression and mRNA are reduced; and CCAAT/Enhancer Binding Protein β (C/EBPβ) protein expression and mRNA are enhanced in Sprague-Dawley rats chronically treated with MG. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n* = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, the pancreatic tissue was removed and processed for determination of PDX-1, MafA, and C/EBPβ protein expression by Western blotting (*A*). *B*: PDX-1, MafA, and C/EBPβ mRNA were also determined by RT-PCR in pancreatic tissue isolated from groups of rats described above. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. respective control; †$P < 0.05$, ††$P < 0.01$ vs. respective MG group; ‡$P < 0.05$ vs. respective MG + BSO group.

ALA could (Supplementary Fig. 5*S*, *C*). MG (30 μmol/L) did not affect K_{ATP} channel activity in INS-1E cells (Supplementary Fig. 6*S*).

Chronic MG treatment induces apoptosis of pancreatic β-cells and increases AGE formation. After chronic MG and MG + BSO administration, there was significant DNA fragmentation (Fig. 7*A*) and significant positive BrdUTP staining, indicating apoptosis (Fig. 7*B*) of pancreatic β-cells compared with control, which was attenuated by ALA cotreatment with MG (Fig. 7*A* and *B*). BSO alone also partially induced apoptosis (Fig. 7*B*) but had no effect on DNA fragmentation (Fig. 7*A*). Chronic MG and MG + BSO also increased MG-induced AGE, N^{ε}-carboxymethyllysine (CML) formation in pancreas (Supplementary Fig. 7*S*).

DISCUSSION

In the current study, we report for the first time that MG administered by continuous infusion with a minipump for 28 days to SD rats induces metabolic, biochemical, and molecular abnormalities characteristic of type 2 diabetes. The most notable change induced by MG was an elevation of fasting plasma glucose and reduction of insulin level accompanied by a significantly impaired oral GTT (Figs. 1 and 2). The adipose tissue from MG-treated rats showed significantly reduced insulin–stimulated glucose uptake, reduced plasma membrane GLUT4 protein, unchanged IR and IRS-1 protein, and reduced phosphorylation of IRS-1 and PI3K activity. There was significant pancreatic islet β-cell dysfunction in MG- and MG + BSO-treated rats. Thus

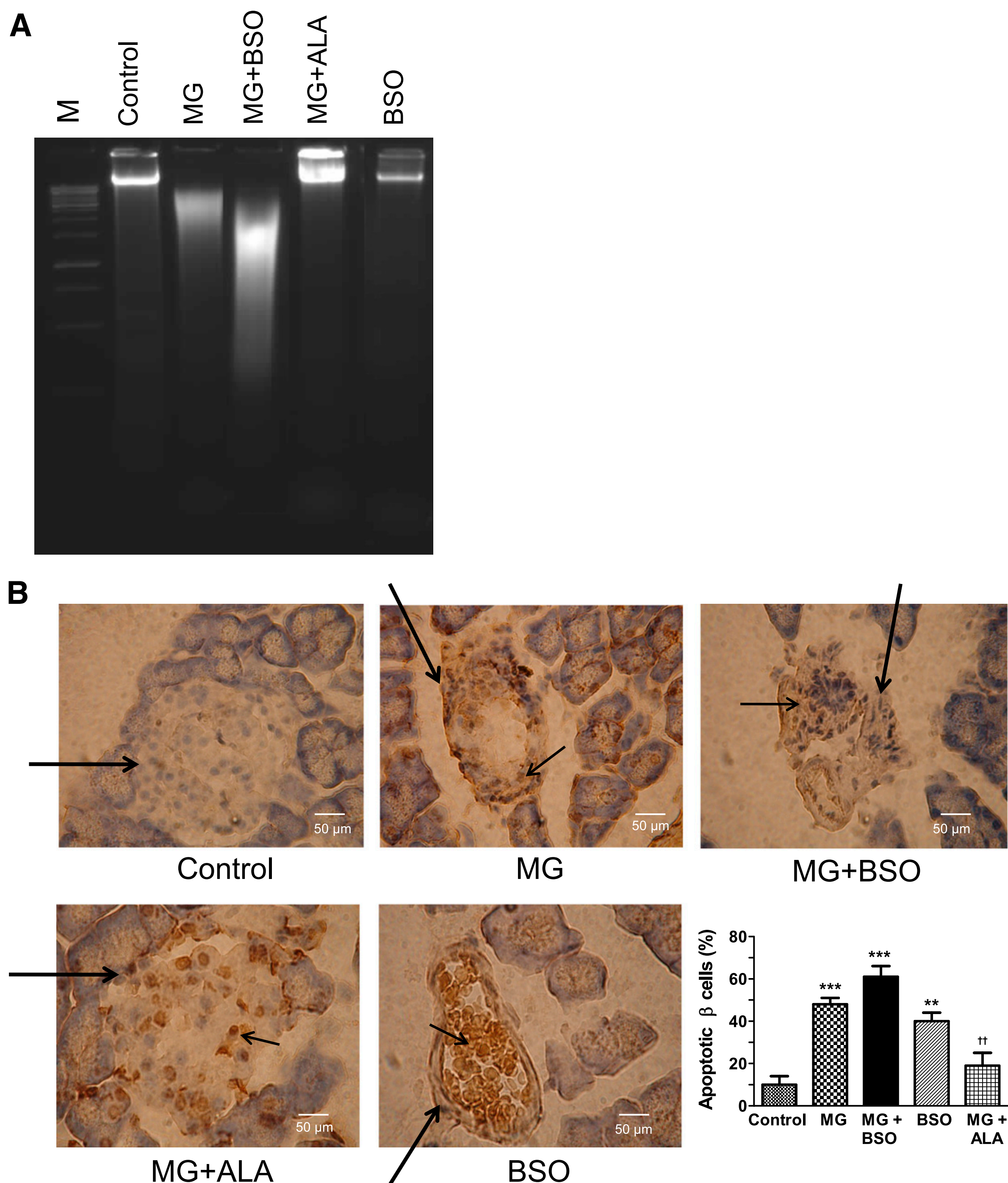

FIG. 7. Chronic MG induces apoptosis in pancreatic islets in SD rats. Either 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n = 6 each). The MG scavenger ALA (30 mg/kg/day in drinking water; MG + ALA) or GSH synthesis inhibitor BSO (30 mg/kg/day in drinking water; MG + BSO and BSO) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days, DNA (*A*) was isolated and run on agarose gel to evaluate DNA integrity. *B*: A part of the pancreas was removed and fixed in 4% paraformaldehyde. The tissue was processed for sectioning and stained for the TUNEL assay. Big black arrows show the pancreatic islet; small black arrows show brown staining of apoptotic cells. (A high-quality digital representation of this figure is available in the online issue.)

glucose–stimulated insulin secretion was reduced in freshly isolated pancreatic islets, GLUT2 protein level was decreased, pancreatic insulin content was lower, and protein as well as mRNA expression of insulin gene transcription factors PDX-1 and MafA were significantly reduced, whereas for C/EBPβ they were increased in MG- and MG + BSO-treated rats. In the pancreatic islets, RAGE protein and the MG-induced AGE, CML, were increased by MG, MG + BSO, and BSO. The deleterious effects of MG were attenuated by the MG scavenger ALA (18,20).

To the best of our knowledge, MG has not been previously administered chronically by continuous infusion, which would mimic the supposedly continuous production of MG in the body and avoid the excessive peaks in plasma associated with repeated intraperitoneal or subcutaneous injections. The plasma levels of MG were significantly

elevated in the MG and MG + BSO groups compared with control (Fig. 1*A*), similar to a range reported in diabetic animals and patients (13,14,22). MG levels were also significantly higher in the pancreas, adipose tissue, and skeletal muscle, which can explain the metabolic changes observed in these organs/tissues. GSH levels were reduced in the plasma, pancreas, adipose tissue, and skeletal muscle. GSH plays a central role in the degradation of MG by binding MG and making it available to the glyoxalase enzymes (10,19,26). A reduction in GSH would decrease MG degradation, increase its levels, and set up a vicious cycle. This was observed in groups of rats treated with the GSH synthesis inhibitor BSO (19). The inhibition of GSH synthesis with BSO and the consequent further elevation of MG levels could be either because of an increase in endogenous MG levels, which add to the exogenously administered MG, or it can be because of inhibition of degradation of exogenously administered MG, or both effects. Thus organ/tissue specific elevation of endogenous MG levels may have different effects in some instances, compared with an increase in MG levels induced by exogenous administration. MG itself has been shown to reduce GSH levels (Fig. 1, *E–H*) (23,26). Pancreatic β-cells have low levels of GSH and antioxidant capacity compared with other tissues, making it more susceptible to oxidative damage (27). GSH levels in pancreas were lower compared with skeletal muscle (Fig. 1*F* and *H*).

The fasting elevated plasma glucose and low insulin levels (Table 1) are features of type 2 diabetes (1), which is characterized by insulin resistance, impaired β-cell function, and glucose regulation, leading to β-cell failure. In the case of type 2 diabetes, functional β-cells vary in numbers, with gradual progressive apoptosis in the advanced stages. In the early type 2 diabetes, the insulin resistance, characterized by defects in the insulin signaling pathway (28), elicits a compensatory increase in pancreatic insulin secretion and higher than normal plasma insulin levels. As the disease progresses, insulin release from the pancreas becomes reduced, because of apoptosis and decrease in β-cell numbers in the later stages, resulting in lower than normal plasma insulin levels and hyperglycemia (1,29). The apoptosis of pancreatic islet β-cells in type 2 diabetes differentiates it from the necrosis of β-cells seen in type 1 diabetes. Thus insulin in the circulation can be either high or low depending on the stage of diabetes. High fructose typically induces insulin resistance with higher plasma insulin levels (22). Thus fructose probably induces milder changes after nine weeks of treatment and the pancreas still can produce more insulin in response to resistance, whereas exogenous MG probably induces more severe changes, which include progressive apoptosis that characterizes the later stages of type 2 diabetes, and reduces the insulin secreting capacity of the pancreas and lower plasma insulin levels.

The oral GTT revealed significantly impaired glucose tolerance and reduced insulin-response to the glucose load. An impaired GTT is a feature of the prediabetic insulin resistance as well as type 2 diabetes. One reason for the elevated plasma glucose levels could be reduced response of insulin sensitive tissues to insulin because of defects in one or more steps of the insulin signaling pathway. We have shown that there is reduced GLUT4 in plasma membrane (Fig. 3), reduced phosphorylation of IRS-1, and reduced activity of PI3K (Supplementary Fig. 2*S*). In vitro studies have shown that incubation of cultured 3T3-L1 adipocytes with MG reduced glucose uptake, decreased insulin-induced IRS-1 tyrosine phosphorylation, and decreased the activity of PI3K (17,22). The Zucker diabetic rat, a genetic model, has a defect of glucose transport in muscle (30). We have recently shown that a single dose of MG (50 mg/kg iv) given to SD rats causes reduced membrane GLUT4 and IRS-1 phosphorylation in adipose tissue (18).

The reduced fasting plasma insulin and the reduced insulin response to the glucose load could be the result of pancreatic islet dysfunction induced by MG. The total pancreatic insulin content was reduced in rats treated with MG and MG + BSO (Fig. 4). This is likely because of reduced transcription of insulin synthesis as indicated by reduced mRNA and protein levels of the insulin gene promoters PDX-1 and MafA induced by MG in the pancreas (Fig. 6) and isolated islets (Supplementary Fig. 3*S*). PDX-1 is an essential regulator of both pancreatic exocrine and endocrine cell development. Inactivation of PDX-1 in the β-cells impairs β-cell function and causes diabetes (31–33). Similarly, MafA activates insulin as well as glucagon gene expression in the pancreatic islet β− and α-cells, respectively (34). The expression of PDX-1 and MafA is reduced in diabetes, resulting in decreased insulin synthesis and secretion (35).

MG and MG + BSO upregulated C/EBPβ in pancreas (Fig. 6) and in isolated islets (Supplementary Fig. 3*S*), which can also be responsible for the reduced insulin levels in these rats because C/EBPβ is a repressor of insulin gene transcription in β-cells and in diabetic animal models (36). MG- and MG + BSO-treated groups also had reduced basal and glucose-stimulated insulin secretion from isolated pancreatic islets and also from cultured INS-1E cells. These results support our previous study (18). GLUT2 acts as a glucose sensor and sends a signal inside the cell to cause insulin secretion when the extracellular glucose concentration increases (37). GLUT2 is reduced in animal models of type 2 diabetes (38). The reduced secretion of insulin in the MG and MG + BSO groups of rats can be partly explained by significantly reduced membrane GLUT2 and glucokinase in the pancreas (Fig. 5*B*, Supplementary Fig. 3*S*).

In type 1 diabetes, there is β-cell death, whereas type 2 diabetes is characterized by gradual apoptosis of islet β-cells, which ultimately results in reduced insulin secretion (29,33,39,40). MG- and MG + BSO-induced DNA fragmentation and caused apoptosis in pancreatic cells (Fig. 7*A* and *B*). Reduced islet number would also contribute to reduced pancreatic insulin content and insulin secretion (Figs. 4 and 5*A*).

To further elucidate the role of MG in the metabolic and biochemical abnormalities described above, one group of rats was treated with MG + ALA. We have recently shown that ALA attenuates acute MG-induced glucose intolerance in SD rats (18). Thus ALA has acute MG scavenging action besides being a known AGEs breaker (20,41,42). The attenuation of chronic MG-induced deleterious effects by ALA strongly supports the role of MG as an inducer of insulin resistance and β-cell dysfunction.

The deleterious effects of MG were not random because there were no significant changes in the levels of biomarkers such as CK, an indicator of muscle damage, creatinine, an indicator of kidney function, and ALT and AST, which are enzyme markers for liver damage (Table 1). The reason for increased plasma levels of free fatty acids, total cholesterol and triglycerides, and reduced levels of HDL induced by MG and MG + BSO treatment (Table 1) is not known and requires further investigations.

The results of this study suggest that chronic high dietary carbohydrate may cause daily abnormal elevations of MG levels, which can cause cumulative pathologic changes in the islet β-cell insulin secreting machinery and in the insulin signaling pathway in tissues, such as the skeletal muscle and adipose tissue. The severity and the time course of progression of these pathologic changes to type 2 diabetes may depend on the overall health of the individual, any underlying oxidative stress, GSH levels, and antioxidant defenses.

In summary, we have shown for the first time that chronic administration of MG to SD rats induces abnormalities in glucose homeostasis and insulin secretion. The direct involvement of MG in these deleterious effects is supported by the GSH synthesis inhibitor BSO, which caused a further increase in MG levels when combined with MG treatment and attenuation of the deleterious effects of MG by the MG scavenger ALA. Our results suggest that MG is a possible mediator of high glucose-induced insulin resistance and type 2 diabetes that is becoming a serious health problem in the Western world (3–6). Further studies may show that specific and safe MG scavengers could be useful in preventing high glucose-induced insulin resistance and type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by the Heart and Stroke Foundation of Saskatchewan (to K.M.D. and L.W.), Canadian Institutes of Health Research (CIHR) (to L.W.), and by the Saskatchewan Health Research Foundation (SHRF) (to K.M.D.). A.D. is supported by a scholarship from the Gasotransmitter Research And Training (GREAT) Program (funded by CIHR and Heart Stroke Foundation of Canada).

No potential conflicts of interest relevant to this article were reported.

A.D. performed most of the experiments, developed some research ideas, contributed to the discussions, wrote the first draft of the article, and reviewed the article. I.D. performed experiments such as INS-1E cell culture, Western blotting, data analysis, and creating graphs and figures. B.J. performed the patch–clamp experiments on cultured INS-1E cells and created the figure for the same. K.M.D. jointly developed the original idea, performed some of the experiments, contributed to the discussions, and reviewed and edited the article. L.W. jointly developed the original idea, contributed to the discussions, and reviewed and edited the article.

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