EUROPEAN SOCIETY OF CARDIOLOGY®
Cardiovascular Research (2011) **92**, 494–503
doi:10.1093/cvr/cvr239

Upregulation of aldolase B and overproduction of methylglyoxal in vascular tissues from rats with metabolic syndrome

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Received 1 April 2011; revised 8 July 2011; accepted 29 August 2011; online publish-ahead-of-print 2 September 2011

Time for primary review: 23 days

Aims Methylglyoxal (MG) overproduction has been reported in metabolic syndrome with hyperglycaemia (diabetes) or without hyperglycaemia (hypertension), and the underlying mechanism was investigated.

Methods and results Contributions of different pathways or enzymes to MG formation were evaluated in aorta or cultured vascular smooth muscle cells (VSMCs). In all four animal models of metabolic syndrome, i.e. chronically fructose-fed hypertensive Sprague–Dawley rats, spontaneously hypertensive rats, obese non-diabetic Zucker rats, and diabetic Zucker rats, serum and aortic MG and fructose levels were increased, and the expression of GLUT5 (transporting fructose) and aldolase B (converting fructose to MG) in aorta were up-regulated. Aortic expressions of aldolase A, semicarbazide-sensitive amine oxidase (SSAO), and cytochrome P450 2E1 (CYP 2E1), accounting for MG formation during glycolysis, protein, and lipid metabolism, respectively, was unchanged/reduced. Fructose (25 mM) treatment of VSMCs up-regulated the expression of GLUT5 and aldolase B and accelerated MG formation. Insulin (100 nM) increased GLUT5 expression and augmented fructose-increased cellular fructose accumulation and MG formation. Glucose (25 mM) treatment activated the polyol pathway and enhanced fructose formation, leading to aldolase B upregulation and MG overproduction. Inhibition of the polyol pathway reduced the glucose-increased aldolase B expression and MG generation. The excess formation of MG in under these conditions was eliminated by knock-down of aldolase B, but not by knock-down of aldolase A or inhibition of SSAO or CYP 2E1.

Conclusion Upregulation of aldolase B by accumulated fructose is a common mechanism for MG overproduction in VSMCs and aorta in different models of metabolic syndrome.

Keywords Aldolase B • Fructose • Methylglyoxal • Metabolic syndrome

1. Introduction

Increased levels of methylglyoxal (MG) and MG-glycated proteins or advanced glycation end-products is the key pathogenic event in the vascular dysfunction in diabetes and hypertension.[1–3] MG induces mitochondrial dysfunction and increases production of superoxide and peroxynitrite in vascular smooth muscle cells (VSMCs).[4,5] The interaction between MG and hydrogen sulfide, a natural vasorelaxant, in VSMCs has also been reported.[6] However, the underlying mechanism for MG overproduction was unknown.

MG is generated through several metabolic pathways. Spontaneous non-enzymatic fragmentation of triosephosphates, glyceraldehyde 3-phosphate (GA3P), and dihydroxyacetone phosphate (DHAP) is a primary source for endogenous MG generation.[7,8] Increased GA3P and DHAP generation, in turn, may occur due to increased availability of glucose and fructose. In the cytosol, glucose is metabolized enzymatically through the glycolytic pathway into fructose-1,6-diphosphate, which subsequently forms GA3P and DHAP catalysed by aldolase A.[9] In contrast, fructose, either derived from the diet (e.g. table sugar) or converted from glucose by aldose reductase and sorbitol dehydrogenase via the polyol pathway, can be phosphorylated by fructokinase to fructose 1-phosphate, which is cleaved by aldolase B to generate GA3P and DHAP.[9] Other potential sources of MG include the oxidation of aminoacetone (generated during protein catabolism) by semicarbazide-sensitive amine oxidase (SSAO) and the oxidation

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of acetone (generated from lipolysis) by cytochrome P450 2E1 (CYP 2E1).[10]

Most glucose is physiologically metabolized through the glycolytic pathway to GA3P and DHAP, and, therefore, MG is traditionally considered an intrinsic metabolite of glycolysis.[11] High levels of glucose with accumulation of endogenous MG have received much attention in diabetes research owing to the potentially pathogenic roles of MG and advanced glycation end-products in the development of diabetes and diabetic complications.[1] However, the importance of glycolysis in MG overproduction during hyperglycaemia is challenged because high levels of glucose did not change or even impaired glycolysis, but activated the polyol pathway and enhanced fructose production in diabetic states.[12–14] Moreover, elevated MG levels are also present in the metabolic syndrome without hyperglycaemia, such as in hypertension.[2] Our recent studies show that as spontaneously hypertensive rats (SHRs) develop hypertension, MG levels in plasma and aorta increase in an age-dependent fashion,[2] although no difference in blood glucose levels between SHRs and control rats is apparent.[15] When Sprague–Dawley (SD) rats were fed with fructose for 16 weeks, elevated MG levels in serum and aorta, with development of vascular remodelling and high blood pressure were evident,[16] but plasma glucose levels were within the physiological range.[17] Indeed, fructose treatment directly increased levels of MG in cultured VSMCs.[18] Aldolase B is a rate-limiting enzyme in fructose metabolism, and a deficiency of aldolase B resulted in fructose intolerance in humans.[19–21] These observations raise an important question regarding whether fructose and aldolase B are commonly and predominantly responsible for vascular MG overproduction in metabolic syndrome with normal blood glucose, such as in hypertension and obesity, and with hyperglycaemia, such as in diabetes.

In this study, different rat models, including fructose-fed SD rats, SHRs, obese and diabetic Zucker rats, and their respective controls, were used to investigate the involvement of fructose and aldolase B in MG formation *in vivo*. The gene expression of enzymes (aldose reductase, fructokinase, aldolase A, aldolase B, SSAO, and CYP 2E1) and glucose transporter 5 (GLUT5), a specific fructose transporter,[22] was examined in aorta from these rats and in cultured VSMCs (the major cellular constituent of aorta) after treatment with fructose or glucose. The inhibitors for aldose reductase, sorbitol dehydrogenase, SSAO, and CYP 2E1, as well as short hairpin RNA (shRNA) targeting aldolase A and B, were employed in VSMCs to compare the relative contributions of these enzymes to fructose- or glucose-induced MG overproduction.

2. Methods

2.1 Animal studies

All animal care and experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), as well as Directive 2010/63/EU of the European Parliament, and were approved by the Animal Care Committee at The University of Saskatchewan, following guidelines of the Canadian Council on Animal Care. Male 4-week-old SD rats, 20-week-old Wistar–Kyoto (WKY) rats and SHRs, and 16-week-old lean, obese, and diabetic Zucker rats were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). Four-week-old SD rats were treated with or without 60% fructose (in chow) for another 16 weeks. Rats were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) or isoflurane (5%, by inhalation). The depth of anaesthesia was evaluated by limb withdrawal reflexes. A supplemental dose of pentobarbital (30 mg/kg, intraperitoneally) or a longer duration of isoflurane inhalation was applied until rats' reflexes disappeared. Then rats were killed by exsanguination, involving collection of blood from the heart, under deep anaesthesia, and their tissues were quickly collected. Blood glucose and insulin were measured using a glucose assay kit (Bioassay Systems, Hayward, CA, USA) and a rat-specific insulin ELISA kit (Mercodia AB, Sylveniusgatan, Uppsala, Sweden), respectively. Blood fructose was detected with a method based on fructose dehydrogenase,[23] after deproteinization with 1/4 volume of 1 N perchloric acid and neutralization with 2.5 M K_2CO_3.

2.2 Cell culture

A-10 cells, a rat aortic smooth muscle cell line, were cultured in Dulbecco's modified Eagle's medium containing 10% bovine serum. Sub-confluent (80%) VSMCs were starved for 24 h in bovine serum-free Dulbecco's modified Eagle's medium before further treatments in Dulbecco's modified Eagle's medium containing 10% bovine serum.

2.3 Plasmid shRNA knock-down of aldolase A or aldolase B

Knock-down of aldolase A or B was established by transfection of A-10 cells with SureSilencing plasmid shRNAs (aldolase A, CACTGCCAA TAAACAGCTATT; aldolase B, CTAGAGCACTGCCAGTATGTT; and control, GGAATCTCATTCGATGCATAC). The transfected cells were selected in medium containing 600 μg/mL G418 for 2 weeks, and the surviving cells were maintained in 200 μg/mL G418. Gene knock-down was verified with real-time PCR and western blotting according to the manufacturer's instructions (Superarray, Frederick, MD, USA).

2.4 MG measurement

MG levels in aorta and VSMCs were determined with an *o*-phenylenediamine-based assay.[24] Briefly, samples were sonicated and centrifuged at 10 000 g (10 min, 4°C). A portion of the supernatant (240 μL) was mixed with 60 μL of perchloric acid (1 N), kept on ice for 10 min, and deproteinized by centrifugation at 10 000 g (10 min, 4°C). Then 180 μL of supernatant were incubated with 90 μL *o*-phenylenediamine (100 mM) for 3 h at room temperature in the dark. The mixture was centrifuged at 10 000 g (5 min, 4°C). A portion of the supernatant (180 μL) was mixed with 20 μL of 5-methylquinoxaline (internal standard) and analysed by high-performance liquid chromatography with mobile phase buffer containing 17% acetonitrile, 8% 50 mM NaH_2PO_4 (pH 4.5), and 75% water.

MG levels in serum were determined using our recently modified method.[25] Briefly, 135 μL serum were incubated with 135 μL perchloric acid (1 N) and 30 μL *o*-phenylenediamine (100 mM) for 24 h at room temperature in the dark. The mixture was centrifuged at 10 000 g (10 min, 4°C). A portion of the supernatant (180 μL) was mixed with 20 μL 5-methylquinoxaline and analysed by high-performance liquid chromatography.

2.5 Biochemical assays

Cell or tissue samples were sonicated and centrifuged at 10 000 g (10 min, 4°C). Total protein levels were determined with a bicinchoninic acid protein assay kit (Sigma, Oakville, ON, Canada). Intracellular fructose levels were measured by a fructose assay kit (BioVision, Mountain View, CA, USA). Aliquots of supernatants were deproteinized by 1/4 volume of perchloric acid (1 N) and neutralized by 2.5 M K_2CO_3 to measure the levels of glucose 6-phosphate, sorbitol, GA3P, and DHAP using the enzymatic fluorometric methods.[26,27] Glyoxalase 1 or 2 activity was assayed by using a spectrophotometric method to monitor the increase and decrease of S-D-lactoylglutathione, respectively, as described.[28]

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2.6 Analysis of gene expression

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) and converted to cDNA with an iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada). Real-time PCR was performed using SYBR Green PCR Master Mix (Bio-Rad, Mississauga, Ontario, Canada) with the flowing primers: rat aldolase A forward 5′-CCAACTGCTGCTGACTG-3′, reverse 5′-GGGCACTACACCCT-TATC-3′; aldolase B forward 5′-ACAGCCTCCTACACCTACT-3′, reverse 5′-GCTCATACTCGCACTTCA-3′; CYP 2E1 forward 5-GGGAAACAGGGTAA-3, reverse 5-GCTCAGCAGGTAGAA-3; and β-actin forward 5′-CGTTGACATCCGTAAAGAC-3′, reverse 5′-TAGGAGCCAGGGCAGTA-3′. Primers of aldolase A and B to verify shRNA knock-down were provided by Superarray (Frederick, MD, USA). Primers of SSAO, GLUT5, aldose reductase, and fructokinase were purchased from Qiagen (Mississauga, ON, Canada). Protein levels were analysed by western blotting using antibodies as follows: aldolase A (1:5000 dilution; Sigma), aldolase B (1:500 dilution; Epitomics, Burlingame, CA, USA), and β-actin (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.7 Materials

Sorbinil and CP-470711 were generous gifts from Pfizer Inc. (Groton, CT, USA). Diallyl disulfide was purchased from Sigma. (E)-2-(4-fluorophenethyl)-3-fluoroallylamine (MDL-72974) was a generous gift from Dr Peter Yu (Department of Pharmacology, University of Saskatchewan, SK, Canada).

2.8 Statistics

Data are expressed as means ± SEM from at least four rats ($n \geq 4$) or from at least five independent experiments in the cell study ($n \geq 5$ per group). Statistical analyses were performed using parametric Student's *t*-test (two-tailed) or one-way ANOVA.

3. Results

3.1 Upregulation of aldolase B and increased MG levels in fructose-fed hypertensive SD rats

Our previous study indicated that SD rats chronically fed with fructose displayed a normal fasting blood glucose but increased serum and aortic MG levels, with the development of hypertension and insulin resistance.[16,17] To reveal why fructose resulted in MG overproduction, in the present study we focused on the fructose-related in the present study transport and metabolic pathway. We observed that in addition to increased MG and fructose levels in aorta (*Figure 1A*), fructose feeding of SD rats for 16 weeks increased mRNA levels of GLUT5, but did not alter mRNA levels of aldose reductase (*Figure 1B*). As our *in vitro* studies found that GA3P (95%) or DHAP (26%) converted to MG non-enzymatically after 12 h incubation (see Supplementary material online, *Figure S1*), we investigated whether fructose feeding affects gene expression of aldolase B, which cleaves fructose 1-phosphate to generate GA3P and DHAP. As shown in *Figure 1C*, fructose feeding elevated mRNA levels of aldolase B in aorta, but had no effect on the mRNA levels of aldolase A, SSAO, and CYP 2E1. In addition, fructose feeding up-regulated aortic mRNA levels of fructokinase, an enzyme that phosphorylates fructose into fructose 1-phosphate (see Supplementary material online, *Table S1*).

3.2 Increased fructose, aldolase B mRNA, and MG levels in SHRs

Twenty-week-old SHRs and WKY rats displayed similar levels of serum glucose; however, serum MG, fructose, and insulin levels were markedly increased in SHRs (*Table 1*). Increased levels of MG and fructose, up-regulated mRNA expression of GLUT5, and unaltered mRNA expression of aldose reductase were observed in aorta of SHRs (*Figure 1D* and *E*). SHRs had higher levels of fructokinase and aldolase B mRNA in aorta than WKY rats (*Figure 1F*; also see Supplementary material online, *Table S1*). Both SHRs and WKY rats displayed similar mRNA levels of aldolase A and CYP 2E1 in aorta, but SHRs had lower levels of SSAO mRNA than WKY rats (*Figure 1F*).

3.3 Increased fructose, aldolase B mRNA, and MG levels in obese, non-diabetic Zucker rats

When compared with lean rats, obese rats had similar levels of serum glucose, but much higher levels of serum MG and insulin (*Table 1*). Serum fructose levels in obese rats were increased (*Table 1*), although not significantly. Obese rats displayed higher levels of MG and fructose, as well as up-regulated mRNA expression of GLUT5, fructokinase, and aldolase B in aorta (*Figure 2A*, *B* and *D*); however, aortic mRNA levels of aldose reductase, aldolase A, SSAO, and CYP 2E1 were not significantly different in obese and lean rats (*Figure 2C* and *D*).

3.4 Activation of polyol pathway and increased fructose and MG levels in diabetic Zucker rats

In comparison with obese rats, diabetic rats had similar but exacerbated alterations. Diabetic rats exhibited higher levels of fructose and MG in serum and aorta, as well as higher levels of aldolase B mRNA in aorta than non-diabetic lean and obese rats (*Table 1*, *Figure 2A* and *D*). Aortic mRNA levels of GLUT5 and fructokinase in diabetic rats were higher than those in lean rats, but not significantly different from those in obese rats (*Figure 2B*). Unlike obese, hypertensive, and fructose-fed rats, diabetic rats displayed higher glucose and lower insulin levels in serum (*Table 1*). We also investigated whether the polyol pathway is involved in aortic fructose and MG overproduction in diabetic Zucker rats. As shown in *Figure 2C*, aortic levels of aldose reductase mRNA and sorbitol were higher in diabetic rats than in non-diabetic lean and obese rats. In contrast, mRNA levels of aldolase A were decreased in diabetic rats (*Figure 2D*).

3.5 MG accumulation in VSMCs induced by fructose, fructose plus insulin, and glucose

To investigate whether fructose and aldolase B are primarily responsible for increased MG levels in aorta in the different rat models, cultured A-10 cells were used. We chose glucose at 25 mM to mimic the hyperglycaemia of diabetics and chose fructose at 25 mM for comparative purposes. We observed that fructose (25 mM) induced time-dependent increases of cellular MG and fructose (*Figure 3A* and *C*). In the presence of insulin (100 nM), the cellular MG and fructose levels induced by fructose (25 mM; 6 and 12 h) were further augmented (*Figure 3B* and *C*).

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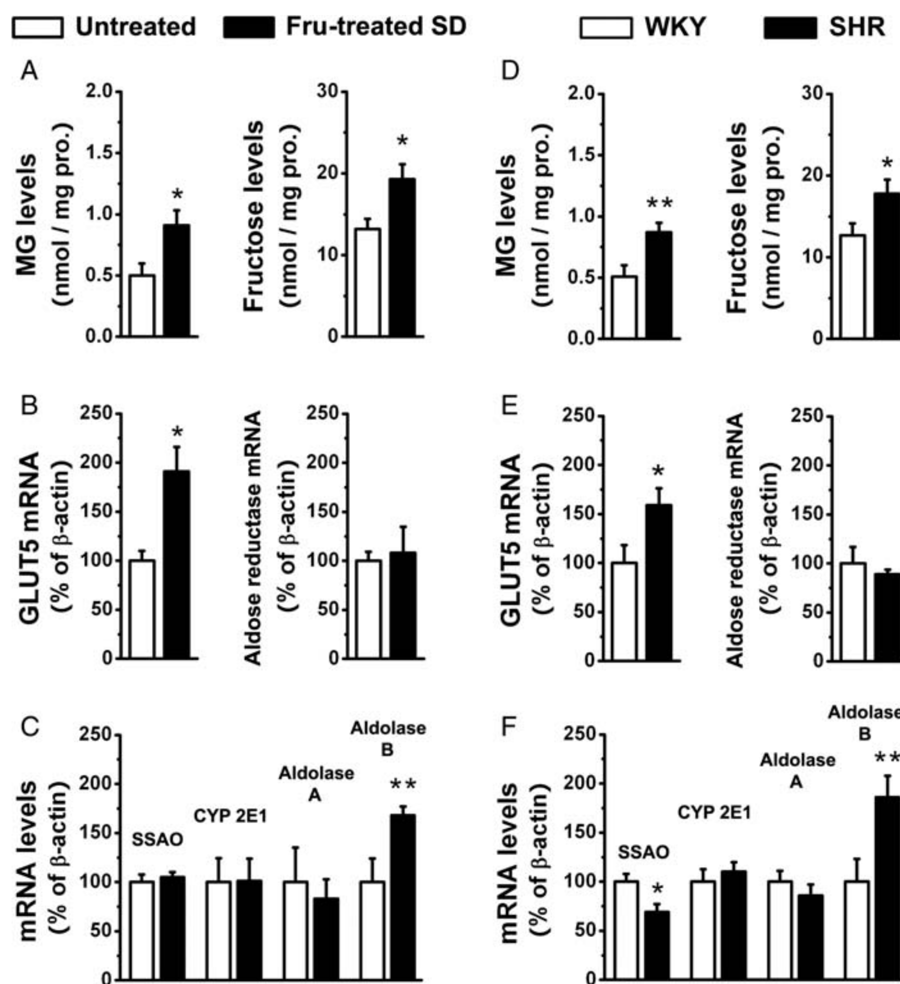

Figure 1 Fructose, aldolase B, and MG in aorta of fructose-induced or genetically hypertensive rats. (*A*) Levels of MG and fructose. Levels of mRNA of glucose transporter 5 (GLUT5) and aldose reductase (*B*), semicarbazide-sensitive amine oxidase (SSAO), cytochrome P450 2E1 (CYP 2E1), aldolase A, and aldolase B (*C*) in aorta of 20-week-old Sprague–Dawley (SD) rats treated with 60% fructose for 16 weeks or untreated. (*D*) Levels of MG and fructose. (*E* and *F*) Levels of mRNA of different genes in aorta of 20-week-old spontaneously hypertensive rats (SHRs) and control Wistar–Kyoto (WKY) rats. $*P < 0.05$, $**P < 0.01$ vs. untreated SD or WKY rats. $n = 4$ for each group in *A–F*.

Insulin alone (100 nM) had no effect on cellular levels of MG and fructose (*Figure 3B* and *C*). Glucose (25 mM) also increased cellular MG and fructose levels in a time-dependent manner (*Figure 3A* and *C*). Co-application of insulin (100 nM) had no effect on cellular fructose levels induced by glucose (25 mM; *Figure 3C*). In comparison with glucose (25 mM), fructose at the same concentration raised MG and fructose levels in a similar pattern, but which started earlier (<3 h), reached the peak quicker, and stayed at elevated levels longer (~18 h).

We explored whether or not glucose or fructose induces a change of glyoxalase 1 and glyoxalase 2 activities, because most of the cellular MG is degraded by these two enzymes into D-lactic acid.[28] Glucose (25 mM), fructose (25 mM), or fructose plus insulin (100 nM) incubations of cultured A-10 cells increased the activity of glyoxalase 1 but not glyoxalase 2 after 12 h treatment, and increased the activity of both glyoxalase 1 and glyoxalase 2 after 24 h treatment (*Figure 3D*).

3.6 Fructose- or fructose plus insulin-enhanced MG formation was totally prevented by aldolase B knock-down in VSMCs

After the cells were treated with fructose (25 mM) and insulin (100 nM), respectively, we observed a similar rise in mRNA levels of GLUT5; with co-treatment of fructose and insulin, the GLUT5 mRNA was further elevated (*Figure 4A*). Fructose (25 mM) up-regulated gene expression of fructokinase (mRNA) and aldolase B (mRNA and protein), but had no effect on aldolase A mRNA (*Figure 4A–C*). In the presence of insulin (100 nM), fructose-elevated fructokinase and aldolase B gene expressions were further raised (*Figure 4A–C*). Insulin alone had no effect on fructokinase mRNA, aldolase A mRNA, and aldolase B mRNA and protein levels (*Figure 4A–C*). Transfection with shRNA targeting aldolase B successfully knocked down aldolase B in A-10 cells, which reduced aldolase B

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Table 1 Basal parameters in serum of rat models

Rat models	Age (weeks)	*n*	MG (μM)	Fructose (mM)	Glucose (mM)	Insulin (ng/mL)
WKY	20	4	1.2 ± 0.04	0.27 ± 0.06	5.3 ± 0.1	3.5 ± 0.2
SHR	20	4	1.7 ± 0.05**	0.46 ± 0.04*	5.6 ± 0.3	10.1 ± 1.7**
Lean Zucker	16	5	1.1 ± 0.05	0.25 ± 0.05	4.9 ± 0.06	2.4 ± 0.2
Obese Zucker	16	5	1.4 ± 0.04**	0.33 ± 0.06	5.3 ± 0.3	5.8 ± 0.4**
Diabetic Zucker	16	5	2.3 ± 0.1**##	0.53 ± 0.05**#	19.2 ± 0.6**##	1.1 ± 0.1**##

*$P < 0.05$, **$P < 0.01$ vs. WKY or lean Zucker rats; #$P < 0.05$, ##$P < 0.01$ vs. obese Zucker rats.

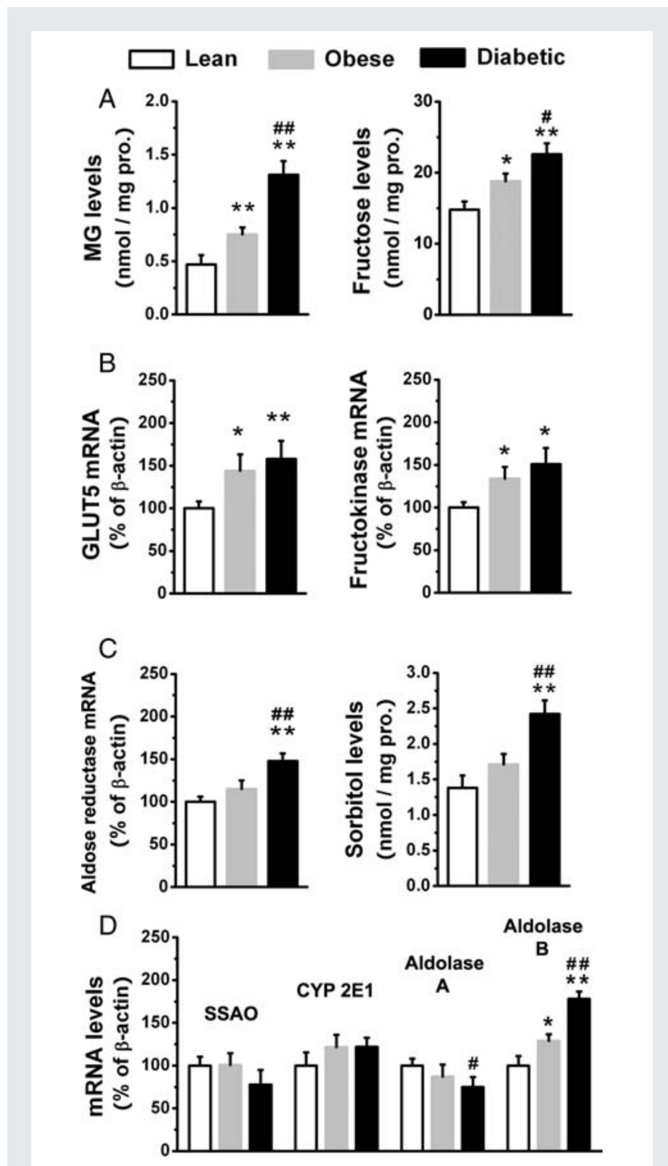

Figure 2 Fructose, aldolase B, and MG in aorta of Zucker rats. Levels of MG and fructose (*A*). levels of GLUT5 and fructokinase mRNA (*B*). levels of aldose reductase mRNA and sorbitol (*C*). and levels of SSAO, CYP 2E1, aldolase A, and aldolase B mRNA (*D*) in aorta of 16-week-old lean, obese, and diabetic Zucker rats. *$P < 0.05$, **$P < 0.01$ vs. lean rats and #$P < 0.05$, ##$P < 0.01$ vs. obese rats. $n = 5$ for each group in *A*–*D*.

by 75% in mRNA levels and by 84% in protein levels compared with the transfection with control shRNA (*Figure 4D*). In these cells transfected with aldolase B shRNA, the increases in cellular MG production induced by fructose (25 mM) or fructose plus insulin (100 nM) were completely inhibited (*Figure 4D*).

3.7 Glucose-increased MG formation was reduced by inhibition of the polyol pathway and totally prevented by knock-down of aldolase B in VSMCs

Treatment with glucose (25 mM; 12 h) elevated levels of the polyol pathway metabolites sorbitol and fructose, as well as GA3P, DHAP, and MG in A-10 cells, but did not change the levels of glucose 6-phosphate (Figures 3*C* and 5*A*). The glucose-increased MG levels were reduced in the presence of sorbinil (a specific aldose reductase inhibitor) and CP-470711 (a specific sorbitol dehydrogenase inhibitor; *Figure 5B*). Glucose (25 mM; 12 h) up-regulated aldolase B expression, but down-regulated aldolase A expression in mRNA and protein levels (*Figure 5C* and *D*). The down-regulation of aldolase A and upregulation of aldolase B evoked by glucose (25 mM) were reduced in the presence of sorbinil, CP-470711, or their combination (*Figure 5C* and *D*). MG levels were raised in control shRNA cells treated with glucose (25 mM) vs. glucose (5 mM; 12 h). However, high-glucose-increased MG levels were totally abolished by transfection with aldolase B shRNA (*Figure 5E*). Transfection with aldolase A shRNA reduced aldolase A mRNA (by 69%) and protein levels (by 86%) in A-10 cells (*Figure 5E*), but this aldolase A knock-down only partly inhibited glucose-induced MG overproduction (*Figure 5E*). Basal cellular MG levels in VSMCs treated with normal levels of glucose (5 mM) were unchanged by co-application of sorbinil or CP-470711, or by knock-down of aldolase B, but were reduced by knock-down of aldolase A (*Figure 5A* and *E*).

3.8 CYP 2E1 and SSAO are not implicated in MG formation in VSMCs

Treatment with acetone, glycine, or threonine (glycine and threonine are precursors of aminoacetone) at 25 mM for 3, 12, or 24 h did not alter MG levels in A-10 cells (see Supplementary material online, *Figure S2*). Co-application of diallyl disulfide or MDL-72974, the specific inhibitor of CYP 2E1 and SSAO, respectively, for 12 h had no effect on basal MG formation induced by normal glucose (5 mM) and excess MG production induced by high glucose (25 mM), fructose (25 mM), or high fructose plus insulin (100 nM; see Supplementary material online, *Figure S2*).

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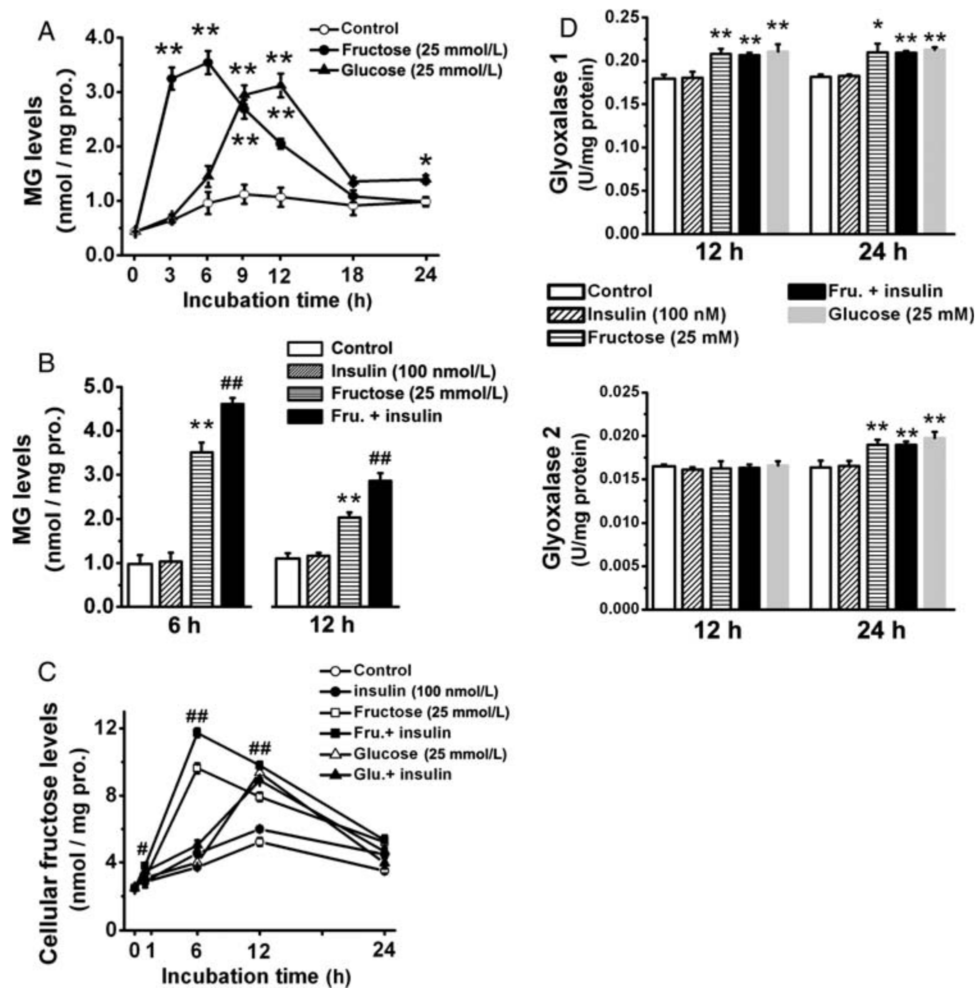

Figure 3 MG and fructose formation in A-10 cells. (*A*) MG formation in A-10 cells induced by fructose (25 mM) or glucose (25 mM). (*B*) MG levels in cells co-treated with fructose (25 mM) and insulin (100 nM) for 6 and 12 h. (*C*) Time-dependent increases in fructose levels in cells treated with fructose, glucose, and/or insulin. (*D*) Activity of glyoxalase 1 or glyoxalase 2 in cells after 12 or 24 h treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (5 mM glucose) and $\#P < 0.05$, $\#\#P < 0.01$ vs. without insulin. $n = 5$ for each group in *A–C* and $n = 6$ for each group in *D*.

4. Discussion

The functional alterations induced by increased methylglyoxal in the aorta *in vivo* or VSMCs *in vitro* have been shown in numerous studies from our laboratory. For example, we observed mitochondrial dysfunction, increased oxidative stress, and enhanced proliferation in cultured VSMCs treated with MG.[4,5,29] We also found that MG injection (intravenous or intraperitoneal) induced endothelial dysfunction, such as decreases in eNOS activity, NO production, and endothelium-dependent relaxation, in aorta of SD rats.[30] Using MG scavengers, we found that the over-produced MG contributed to the vascular remodelling in hypertensive rats.[16,31] With all these functional studies having been done, the focus and the novelty of the present study are to illustrate the molecular mechanisms for vascular MG overproduction, and the present study demonstrated that upregulation of aldolase B by increased fructose is a common and primary mechanism for the increased MG production in vascular tissues, including both smooth muscle cells and endothelial cells, in different forms of the metabolic syndrome (obesity, hypertension, and diabetes), despite their varying blood glucose and insulin levels. Evidence supporting our hypothesis includes the following observations: (1) in all four rat models of metabolic syndrome, levels of fructose and MG in serum and aorta were increased, and gene expression of aldolase B, but not aldolase A, SSAO, and CYP 2E1, in aorta were up-regulated; and (2) in cultured VSMCs, glucose (25 mM), fructose (25 mM), or fructose plus insulin (100 nM) elevated cellular fructose accumulation, up-regulated aldolase B gene expression, and increased MG formation, and the excess MG formation in these conditions was completely inhibited by knock-down of aldolase B, but not by knock-down of aldolase A or inhibition of SSAO or CYP 2E1.

As high-fructose diets impair vascular relaxation and increase blood pressure in arteries,[32,33] the role of fructose in cardiovascular diseases is gradually receiving more attention. We observed that a rat model of hypertension or obesity showed normoglycaemia but higher levels of fructose and MG in serum than their respective controls (*Table 1*). Increased mRNA levels of fructose transporter GLUT5 were found in VSMCs treated with fructose (25 mM) and in aorta of fructose-fed rats, SHR, and obese Zucker rats (*Figures 1* and *2*), which may, in turn, have facilitated more fructose entering VSMCs. Fructose is well

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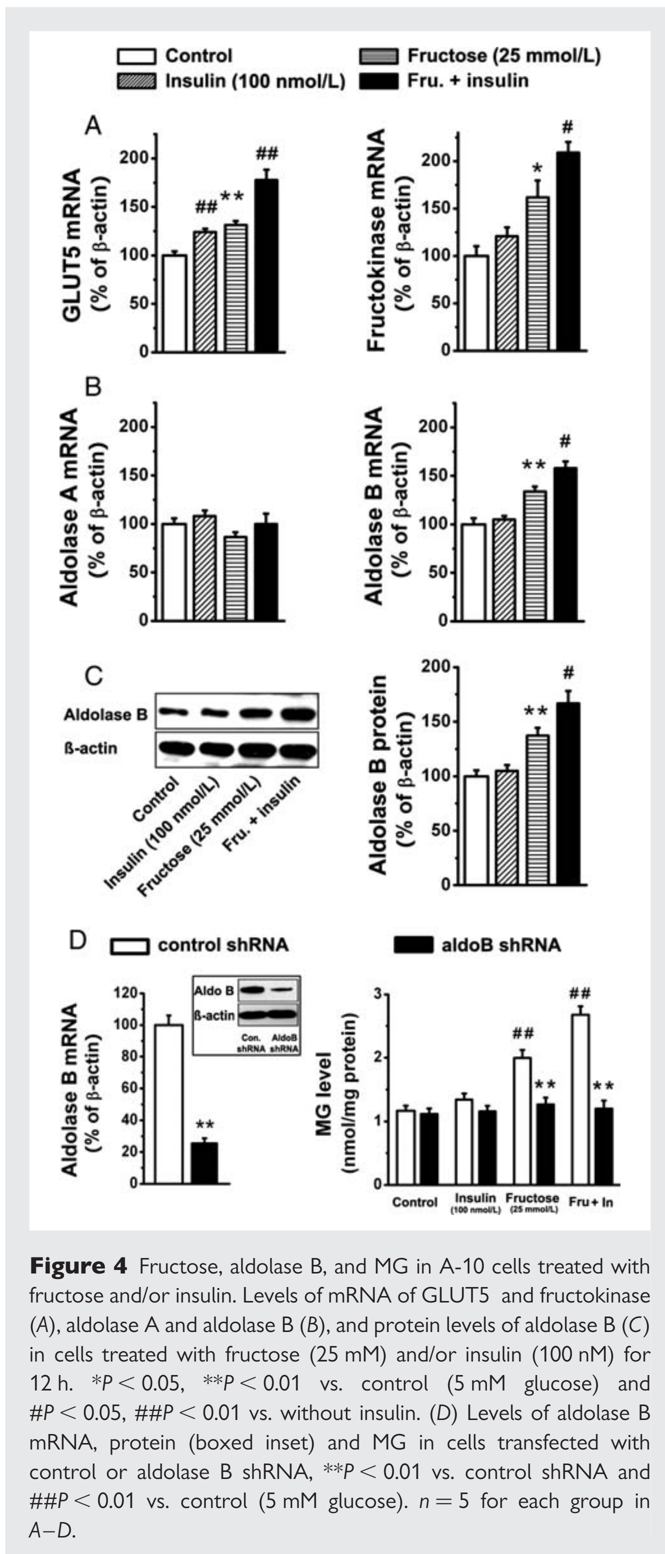

Figure 4 Fructose, aldolase B, and MG in A-10 cells treated with fructose and/or insulin. Levels of mRNA of GLUT5 and fructokinase (*A*), aldolase A and aldolase B (*B*), and protein levels of aldolase B (*C*) in cells treated with fructose (25 mM) and/or insulin (100 nM) for 12 h. $*P < 0.05$, $**P < 0.01$ vs. control (5 mM glucose) and $\#P < 0.05$, $\#\#P < 0.01$ vs. without insulin. (*D*) Levels of aldolase B mRNA, protein (boxed inset) and MG in cells transfected with control or aldolase B shRNA, $**P < 0.01$ vs. control shRNA and $\#\#P < 0.01$ vs. control (5 mM glucose). $n = 5$ for each group in *A–D*.

known as a precursor of MG in VSMCs and aorta.[16,18,34] Our data indicate that the increased circulating fructose levels in serum and subsequent fructose accumulation in aorta could be the cause for aortic MG overproduction in rats with metabolic syndrome without hyperglycaemia.

Insulin is likely to promote fructose-induced fructose and MG accumulation in hypertension and obesity. High levels of insulin and fructose but normal glucose in serum were observed in obese (obese Zucker) and hypertensive rats (SHRs; *Table 1*), and, as previously reported, in rats fed with fructose.[33,35] Indeed, many clinical studies report a similar phenomenon in obese or hypertensive patients who have normal glucose (normoglycaemia) but high insulin (hyperinsulinaemia).[36–38] In VSMCs, we observed that fructose-elevated levels of cellular MG and gene expression of fructokinase (mRNA) and aldolase B (mRNA and protein) were further augmented in the presence of insulin (100 nM), although insulin alone had no effect on the expression of these enzymes, nor on cellular fructose and MG formation (*Figures 3* and *4*). One possible reason for this increase in fructose-induced MG production induced by insulin is that insulin may up-regulate GLUT5 expression and by doing so, when extracellular fructose is increased, it may enhance fructose transport and accumulation in cells. Our data showed that insulin alone up-regulated GLUT5 expression, and co-application of insulin augmented the fructose-increased GLUT5 mRNA levels and cellular fructose accumulation (*Figures 3* and *4*). Thus, hyperinsulinaemia may augment fructose-enhanced MG formation in obese and hypertensive rats through its upregulation of GLUT5 rather than its effect on fructose catabolism.

A role for aldolase B in fructose accumulation-induced MG overproduction seems paramount. Aldolase B splits fructose 1-phosphate, the phosphorylated product of fructose, into MG precursors GA3P and DHAP, and plays a rate-limiting role in fructose metabolism.[19,20] Our data displayed elevated MG levels associated with an up-regulated fructose metabolism pathway, especially the key enzyme aldolase B in aorta of fructose-induced or genetically hypertensive, or obese non-diabetic rats (*Figures 1* and *2*). As GA3P and DHAP are considered direct sources for endogenous MG formation[7,8] and showed high efficiencies of non-enzymatic conversion to MG (see Supplementary material online, *Figure S1*), our study suggests that upregulation of aldolase B expression in metabolic syndrome is the triggering event for fructose accumulation-induced MG overproduction. However, aortic gene expression of aldolase A, SSAO, and CYP 2E1, which are responsible for MG formation during glycolysis, protein, and lipid metabolism, respectively, were unchanged or even reduced in these hypertensive or obese rats (*Figures 1* and *2*). Our *in vitro* studies with cultured VSMCs treated with fructose or fructose plus insulin, conditions mimicking hypertension and obesity, also identify a major role of aldolase B in the fructose-induced excess MG formation. A high fructose concentration (25 mM) significantly increases mRNA and protein levels of aldolase B in VSMCs (*Figure 4*). As mentioned earlier, co-application of insulin (100 nM) can increase fructose transport and augment fructose-enhanced aldolase B expression, although insulin alone has no effect on aldolase B expression (*Figure 4*). In the VSMCs transfected with shRNA targeting aldolase B, the excess MG production induced by fructose or fructose plus insulin was totally prevented (*Figure 4*). These observations *in vivo* and *in vitro* indicate that fructose accumulation and up-regulated aldolase B expression are mainly responsible for the aortic over production of MG in obesity and hypertension without hyperglycaemia.

Fructose and MG formation in diabetic rats seem more complicated. Diabetic Zucker rats had higher fructose and glucose (hyperglycaemia), but lower insulin levels (hypoinsulinaemia) in serum than both lean and obese rats (*Table 1*), similar to clinical observations in diabetic patients.[39,40] Glucose can be metabolized to fructose via the polyol pathway.[1] Diabetic rats displayed an upregulation of GLUT5 and an activation of the polyol pathway (evident by the elevated levels of aldose reductase mRNA and sorbitol) in aorta when

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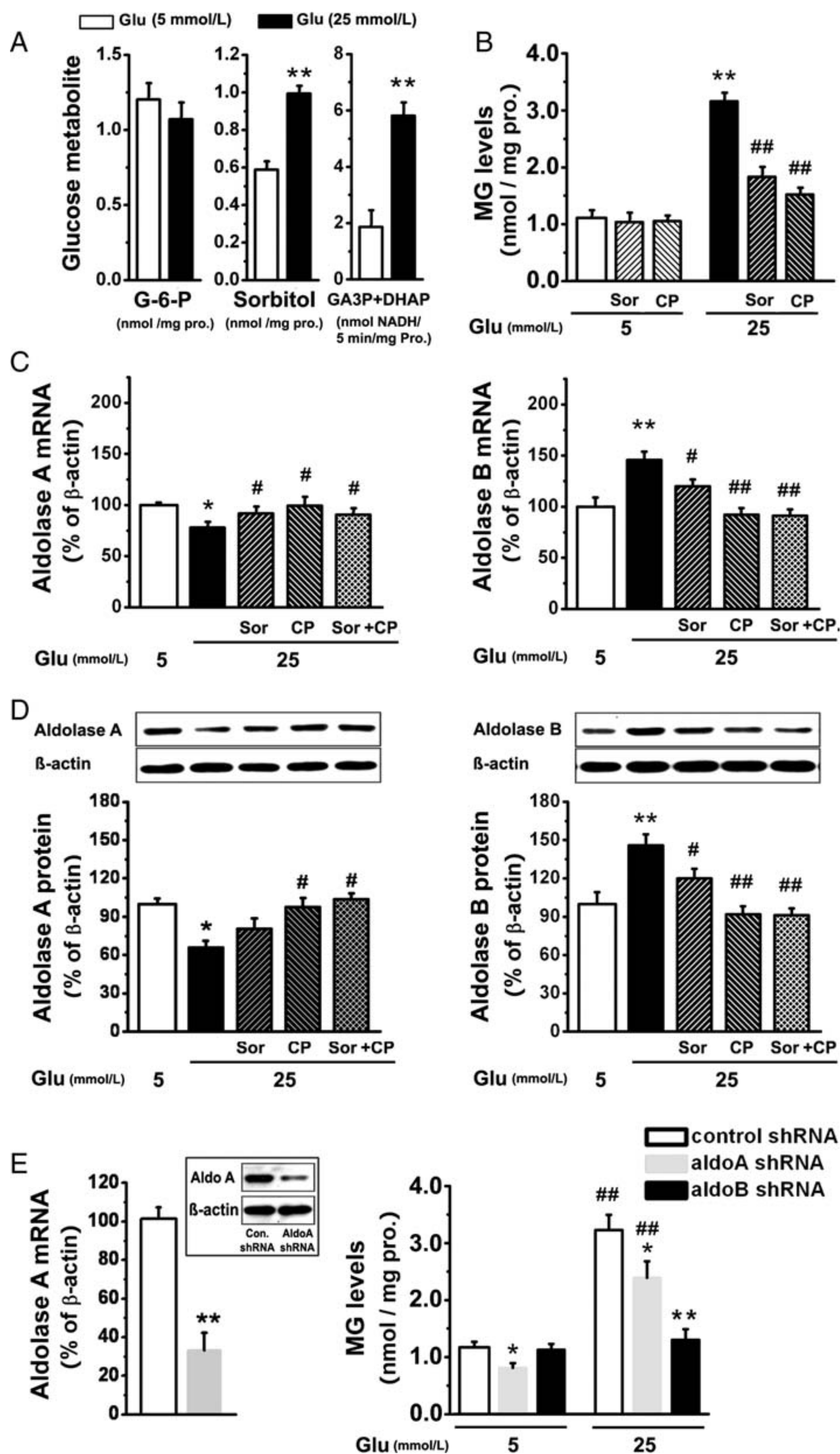

Figure 5 Fructose, aldolase B, and MG in A-10 cells treated with glucose. (*A*) Levels of glucose 6-phosphate (G-6-P), sorbitol, and GA3P + DHAP in cells treated with glucose for 12 h. Levels of MG (*B*), and levels of mRNA of aldolase B (*C*), and protein of aldolase A or aldolase B (*D*) in cells treated with glucose in the presence or absence of sorbinil (Sor; 10 μM) and CP-470711 (CP; 1 μM) for 12 h. *$P < 0.05$, **$P < 0.01$ vs. 5 mM glucose, #$P < 0.05$, ##$P < 0.01$ vs. 25 mM glucose. (*E*) Levels of aldolase A mRNA, protein (boxed inset), and MG in cells transfected with different shRNAs. *$P < 0.05$, **$P < 0.01$ vs. control shRNA and ##$P < 0.01$ vs. control (5 mM glucose). $n = 5$ for each group in *A*–*E*.

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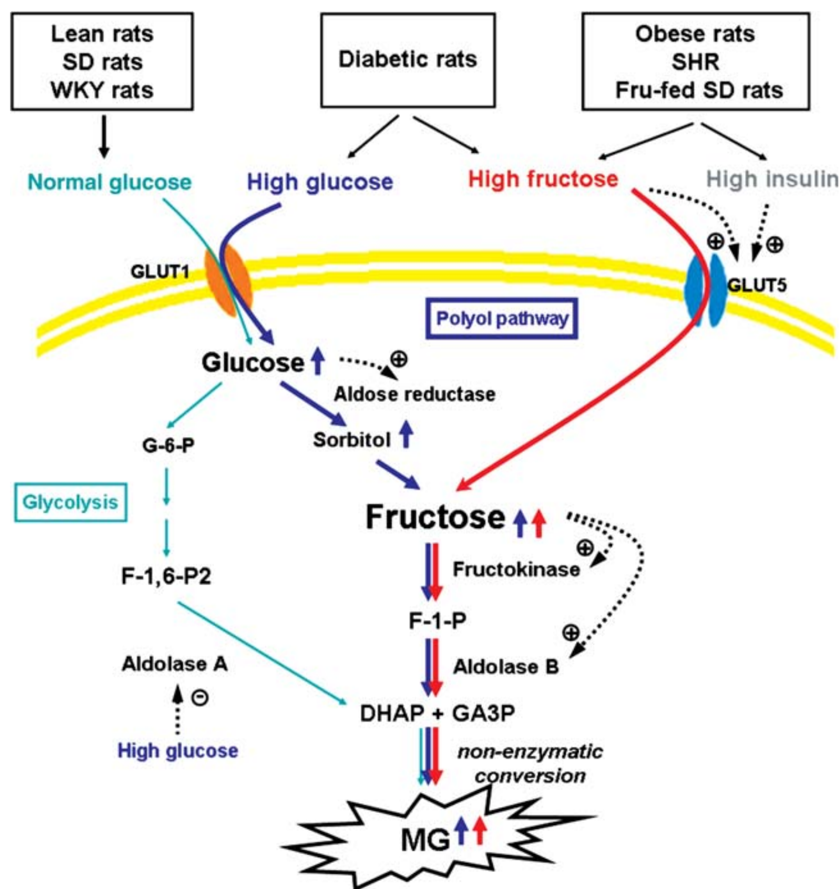

Figure 6 Summary of the mechanism for vascular MG overproduction in rat models of metabolic syndrome. Abbreviaitons: SD, Sprague–Dawley; WKY, Wistar–Kyoto; SHR, spontaneously hypertensive rat; MG, methylglyoxal; G-6-P, glucose 6-phosphate; F-1-P, fructose 1-phosphate; F-1,6-P_2, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; GLUT1 and GLUT5, glucose transporter 1 and 5, respectively.

compared with non-diabetic lean and obese rats (*Figure 2*), indicating that both increased serum fructose and glucose contribute to aortic fructose formation. These diabetic rats showed exacerbated alterations in fructose accumulation, aldolase B expression, and MG formation compared with non-diabetic obese and hypertensive rats (*Figures 1* and *2*). However, mRNA levels of aldolase A, SSAO, and CYP 2E1 were unchanged or even reduced in aorta of diabetic rats. Our data suggest that fructose and aldolase B also predominately contribute to MG overproduction in metabolic syndrome with hyperglycaemia.

Most glucose is physiologically metabolized through the glycolytic pathway, and the traditional view is that glucose-induced MG formation is a result of glycolysis.[11] We indeed observed that the basal cellular MG levels in cells treated with glucose (5 mM) were reduced by shRNA knock-down of aldolase A, but not affected by knock-down of aldolase B or inhibition of the polyol pathway, SSAO, or CYP 2E1 (*Figure 5*; see Supplementary material online, *Figure S2*), which indicates a critical role for aldolase A and glycolysis in the maintenance of basal MG levels in normoglycaemic conditions, as shown in aorta of untreated SD, WKY, and lean Zucker rats. However, hyperglycaemia developing in the diabetes is unlikely to increase metabolism through glycolysis in vascular cells.[12,13] In the present study, we found that levels of glucose 6-phosphate, the first metabolite of glycolysis, in VSMCs treated with glucose at concentrations of 5 and 25 mM were not significantly affected (*Figure 5*). The gene expression of aldolase A was decreased in VSMCs treated with high glucose (*Figure 5*). In contrast, high glucose (25 mM) elevated cellular levels of the polyol pathway metabolites sorbitol and fructose, up-regulated aldolase B expression, and increased GA3P, DHAP, and MG formation (*Figures 3* and *5*). High glucose induced a time-dependent increase of cellular MG and fructose, but in a considerably delayed pattern compared with fructose treatment (*Figure 3*). Inhibition of aldose reductase and/or sorbitol dehydrogenase reduced the glucose-elevated aldolase B expression and MG levels in VSMCs (*Figure 5*). More importantly, glucose-induced excess MG generation was completely prevented by shRNA knock-down of aldolase B in VMSCs, but only partly reduced by shRNA of aldolase A, and not affected by inhibitors of SSAO or CYP 2E1 (*Figure 5*; see Supplementary material online, *Figure S2*). These findings provide direct evidence that, in hyperglycaemic conditions, the polyol pathway but not the glycolytic pathway was activated, and subsequently fructose accumulated and aldolase B was up-regulated, resulting in excess MG formation in VSMCs.

In conclusion, and as shown in *Figure 6*, with normoglycaemia and normal levels of fructose and insulin, aortic glucose is metabolized through the glycolytic pathway, which results in basal levels of MG

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formation in lean Zucker, SD, and WKY rats. However, the situation changes with metabolic syndrome. With normoglycaemia but increased serum fructose and hyperinsulinaemia, aortic GLUT5 mRNA levels are up-regulated by both fructose and insulin, and, as a consequence, fructose levels in aorta are elevated. Increased fructose up-regulates gene expression of fructokinase and aldolase B, leading to increased fructose metabolism and excess MG generation in aorta of obese Zucker, SHR, and fructose-fed SD rats. When hyperglycaemia with high levels of fructose but low levels of insulin in serum is established in diabetes, much higher increases in fructose metabolism and MG formation are observed in aorta of diabetic rats than in SHRs or obese Zucker rats. The increased transport of serum fructose (resulting from raised GLUT5 mRNA levels in aorta) and the high glucose-activated polyol pathway metabolism (indicated by the elevated levels of aldose reductase mRNA and sorbitol in aorta) both account for the higher levels of cellular fructose, fructose-induced fructokinase, and aldolase B expression and MG in aorta of these diabetic rats. Unchanged levels of glucose 6-phosphate and down-regulated aldolase A expression by high glucose suggests a reduced contribution of glycolysis to aortic MG overproduction in diabetic rats.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We are grateful to Mrs Arlene Drimmie (Department of Pharmacology, University of Saskatchewan) for her excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by operating grants from Canadian Institutes of Health Research and the Heart and Stroke Foundation of Saskatchewan to L. Wu. J. Liu was supported by College of Medicine Graduate Scholarships, University of Saskatchewan.

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