Long-Term Methylglyoxal Treatment Causes Endothelial Dysfunction of Rat Isolated Mesenteric Artery

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Abstract. Methylglyoxal (MGO) is a metabolite of glucose and likely related to pathogenesis of diabetes-related vascular complications including hypertension. In this study, long-term effects of MGO on endothelial function were examined. Rat isolated mesenteric artery was treated for 3 days with MGO using an organ culture method. The contractility, morphology and protein expression of organ-cultured artery were examined. MGO (42 μM, 3 days) impaired acetylcholine (ACh: 1 nM–300 μM)-induced endothelium-dependent relaxation, while it had no effect on sodium nitroprusside (0.1 nM–10 μM)-induced endothelium-independent relaxation. MGO decreased ACh (3 μM)-induced nitric oxide (NO) production as measured by a fluorescence NO indicator, diaminofluorescein-2. Consistently, MGO inhibited ACh (3 μM)-induced phosphorylation of vasodilator stimulated phosphoprotein (an indicator of cyclic GMP production). MGO induced apoptosis in endothelium as detected by TdT-mediated dUTP-biotin nick-end labeling staining. MGO induced accumulation of superoxide in endothelium as detected by dihydroethidium staining. MGO decreased protein expression of endothelial NO synthase (eNOS). Gp91ds-tat (0.1 μM), an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), prevented the impairment of endothelium-dependent relaxation and the decrease in eNOS protein caused by MGO. The present results demonstrated that long-term MGO treatment impairs endothelium-dependent relaxation through NOX-derived increased superoxide-mediated endothelial apoptosis.

Key words: apoptosis, endothelium-dependent relaxation, methylglyoxal, organ culture, reactive oxygen species.


Methylglyoxal (MGO) is an alpha-dicarbonyl compound that is spontaneously formed in a process of glycolysis from dihydroxyacetone phosphate as a by-product during a formation of glyceraldehyde 3-phosphates in most mammalian cells including vascular endothelial cells [20] and smooth muscle cells [25]. In addition, MGO is formed in some enzymatic processes by the enzymes including MGO synthase, cytochrome P450 2E1 and semicarbazide-sensitive amine oxidase [4]. MGO binds to arginine, cysteine and lysine residues in proteins, which causes a non-enzymatic formation of a number of advanced glycation end-products (AGEs) [28] including argpyrimidine [19] and Nε-(carboxyethyl)lysine [1].

MGO concentration significantly increased in diabetic patients [8, 9]. Increased levels of blood MGO-derived AGEs seem to be associated with diabetic microvascular complications including diabetic nephropathy [12] and retinopathy [6]. In addition, we have recently demonstrated that glyoxal and MGO are more powerful inducers for large vascular complications including atherosclerosis and hypertension. In fact, MGO accumulated in aorta from spontaneously hypertensive rats (SHR) with aging, and the increased MGO accumulation in aorta correlated with increased blood pressure [22]. Furthermore, there are reports demonstrating that treatment with MGO by drinking water not only increased blood pressure in Wistar-Kyoto rats (WKY) [21] but also caused salt-sensitive hypertension and insulin resistance in Sprague-Dawley rats [7]. Although it is logical to hypothesize that MGO could directly affect vascular reactivity in addition to inflammation, it remains to be fully clarified.

We have previously shown that short-term (30 min) treatment of endothelium-denuded rat aorta and mesenteric artery with MGO inhibited noradrenaline (NA)-induced contraction via activating smooth muscle large conductance Ca2+-activated K+ (BKCa)-channel [14]. Moreover, we have shown that short-term treatment of rat aorta with MGO enhanced sodium nitroprusside (SNP)-induced endothelium-independent relaxation through activation of BKCa-channel, while short-term MGO had no effect on acetylcholine (ACh)-induced endothelium-dependent relaxation [16]. Furthermore, we have demonstrated that short-term treatment of rat carotid artery with MGO augmented angiotensin II-induced contraction via increasing endothelium-produced reactive oxygen species (ROS) [15]. In addition, we have recently shown that long-term (3 days) treatment of endothelium-denuded rat mesenteric artery with MGO inhibited NA-induced contraction, which is mediated via increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)1-derived superoxide production and subsequent apoptosis of smooth muscle [13]. However, it remains to be fully clarified how long-term treatment with MGO affects vascular endothelial function. Therefore, in
the present study, we sought to clarify effects of long-term treatment with MGO on endothelium-dependent relaxation of rat isolated mesenteric artery using a recently established organ culture method [10, 11, 13].

MATERIALS AND METHODS

Tissue preparation and organ culture method: Male Wistar rats (0.2–0.4 kg, 7–10–week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The main branch of superior mesenteric artery was isolated under sterile conditions. After removal of fat and adventitia, the mesenteric artery was cut into rings (1 mm in diameter) for organ culture [10, 11, 13]. Arterial rings were then placed in serum-free Dulbecco’s Modified Eagle Medium supplemented with 1% penicillin-streptomycin in the absence (control) or presence of MGO (42 µM). We chose the MGO concentration, because previous literatures demonstrated that blood MGO concentration in human diabetic patients and hypertensive rats is around 2 µM [9, 18] and 30 µM [23], respectively. They were maintained at 37°C in an atmosphere of 95% air and 5% CO2 for 3 days. Animal care and treatment were conducted in conformity with the institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental work was approved by ethical committee of School of Veterinary Medicine, The Kitasato University.

Measurement of isometric contraction: After organ culture, the arterial rings were placed in normal physiological salt solution (PSS), which contained (mM): NaCl 136.9, KCl 5.4, CaCl2 1.5, MgCl2 1.0, NaHCO3 23.8, glucose 5.5, and EDTA 0.001. The high KCl solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O2-5% CO2 mixture at 37°C and pH 7.4. Smooth muscle contractility was recorded isometrically with a force-displacement transducer (Nihon Kohden, Tokyo, Japan) as described previously [10, 11, 13–17]. The arterial preparations were equilibrated for 30 min under a resting tension of 0.5 g. They were then repeatedly exposed to 72 mM KCl solution, until the responses became stable (45 min). Concentration–response curves were obtained by the cumulative application of ACh (1 nM–300 µM) or SNP (0.1 nM–10 µM) to the artery pre-contracted equally with submaximal concentrations of KCl. Long-term (3 days) treatment of endothelium-intact mesenteric artery with MGO (42 µM) inhibited KCl-induced contraction [13]. In the present study, we thus chose the different concentrations of KCl to produce similar pre-contraction between control and MGO-treated artery (30 mM for control and 40 mM for MGO-treated).

Nitric oxide (NO) assay by 4,5-diaminofluorescein (DAF)-2: Levels of NO production were measured by using a specific NO-sensitive dye, DAF-2 (Sekisui Medical, Tokyo, Japan). After equilibration for 30 min in a 3 ml organ bath, the organ-cultured tissue samples were treated with KCl and DAF-2 (100 nM) as described previously [10]. After 10 min, 200 µl of incubation solution (PSS) in each bath was collected and transferred to a 96-well plate as basal NO release control (before ACh stimulation). ACh (3 µM) was then applied for 5 min, and 200 µl of PSS was also collected. Fluorescence at excitation 485 nm and emission 535 nm was measured using a Tristar LB 941 fluorometer (Berthold Technologies, Bad Wildbad, Germany).

Western blotting: Western blotting was done as described previously [13, 16, 17, 26, 27]. Protein lysates were obtained by homogenizing organ-cultured mesenteric artery with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM β-glycerol phosphate 1 mM Na3VO4, 1 µg/ml leupeptin and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan). Protein concentration in lysate was measured using the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). Equal amounts of proteins (10–15 µg) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, U.S.A.). After being blocked with 3% bovine serum albumin or 0.5% skim milk, membranes were incubated with primary antibodies (1:200–500 dilution) at 4°C overnight and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 hr) and the EZ-ECL system (Biological Industries, Kibbutz Beit Hesmek, Israel). Equal protein loading was confirmed by measuring total actin expression.

Morphological examinations: The organ-cultured tissue samples were fixed in 4% paraformaldehyde at 4°C overnight and incubated in the sucrose gradients (10–20%) at 4°C for 8 hr. After they were embedded in OCT compound (Sakura Finetek, Tokyo, Japan), frozen sections (10 µm-thick) were made using a cryostat (Leica, Nussloch, Germany) [13]. After the hematoxylin and eosin (HE) staining was performed, they were observed under a light microscope (BX-51; Olympus, Tokyo, Japan).

Assessment of apoptosis: To identify apoptotic cells, TdT-mediated dUTP nick end labeling (TUNEL) staining was utilized [13]. The frozen sections (10 µm-thick) were made, and TUNEL staining was performed by using the commercial kit according to the manufacturer’s instructions (Roche; Indianapolis, IN, U.S.A.). The ratio of apoptotic cells was expressed as TUNEL-positive cells per 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml, 5 min, Dojindo; Kumamoto, Japan)-positive nuclei.

Fluorometric measurement of superoxide: Levels of superoxide production were measured by using a superoxide-sensitive dye, dihydroethidium (DHE; Invitrogen, Carlsbad, CA, U.S.A.). The frozen sections (10 µm-thick) were treated for 30 min with DHE (100 nM). Images were obtained with a fluorescence microscope (BX-51, Olympus) equipped with CCD camera (MicroPublisher 5.0 RTV, Roper Industries, FL, U.S.A.). ImageJ software was used for the quantitative analysis.

Statistical analysis: Results are expressed as mean ± SEM. Statistical evaluation of the data was performed by Student’s t-test for comparisons between two groups and by ANOVA followed by Bonferroni’s test for comparisons in three groups. Results were considered significant when P value was less than 0.05.
Chemicals: The chemicals used were as follows: MGO solution and SNP (Sigma-Aldrich, St. Louis, MO, U.S.A.); gp91ds-tat (Anaspec, Fremont, CA, U.S.A.); and ACh (Daiichi Pharmaceutical, Tokyo, Japan). All drugs were dissolved in distilled water. The antibody sources were as follows: total endothelial NO synthase (NOS) (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.); total actin (Sigma-Aldrich); and phospho-vasodilator stimulated phosphoprotein (VASP) (Alexis, San Diego, CA, U.S.A.).

RESULTS

Effects of long-term MGO treatment on ACh-induced relaxation, SNP-induced relaxation and ACh-induced NO production in rat mesenteric artery: We first examined effects of long-term MGO treatment on ACh (1 nM–300 µM)-induced endothelium-dependent relaxation in mesenteric artery. In the control artery, ACh induced concentration-dependent relaxation. The relaxation was observed after 10 nM ACh and reached maximum at 100 µM ACh. MGO treatment (42 µM, 3 days) significantly impaired ACh-induced endothelium-dependent relaxation (Fig. 1A, n=6). Next, we examined effects of long-term MGO treatment on SNP (0.1 nM–10 µM)-induced endothelium-independent relaxation. The relaxation was observed after 3 nM SNP and reached maximum at 10 µM SNP. MGO treatment (42 µM, 3 days) had no effect on SNP-induced relaxation (Fig. 1B, n=6–8). We next examined whether long-term MGO treatment decreases ACh-induced NO production by using DAF-2. MGO had no influence on the basal NO production (n=8, data not shown). MGO (42 µM, 3 days) significantly decreased ACh
(3 µM)-induced NO production (Fig. 1C, n=6–7). Consistent with this result, MGO significantly reduced ACh (3 µM)-induced phosphorylation of VASP, a known substrate for cyclic GMP-dependent protein kinase [2] (Fig. 1D, n=5).

Morphological changes of endothelium after long-term MGO treatment: In order to clarify whether long-term MGO treatment alters morphology of endothelium, we observed the HE stained section of mesenteric artery after organ-culture with MGO (42 µM) for 3 days. In endothelium of the control arteries, flat-shaped endothelial cells were laid along the internal elastic lamina (Fig. 2A-a). In endothelium of the MGO-treated arteries, the condensed nuclei were often observed (Fig. 2A-b). We further assessed whether MGO induces apoptosis in endothelium by performing a TUNEL-staining. MGO treatment significantly increased the TUNEL-positive cells (Fig. 2B–D, n=6).

**DISCUSSION**

The major finding of the present study is that long-term MGO treatment impaired ACh-induced endothelium-dependent relaxation in organ-cultured rat mesenteric artery, while MGO did not affect SNP-induced endothelium-independent relaxation. Consistent with the results, MGO decreased ACh-induced NO production. In the arteries treated with MGO for 3 days, endothelial apoptotic morphological changes and superoxide accumulation were observed. Moreover, MGO reduced eNOS protein expression. Finally, it was revealed that a specific NOX inhibitor prevented the impairment of endothelium-dependent relaxation and the decrease in eNOS expression caused by MGO. The results collectively indicate that long-term MGO treatment impairs ACh-induced endothelium-dependent relaxation through NOX-derived...
Fig. 3. Effects of long-term MGO (42 µM, 3 days) treatment on superoxide production. (A) Representative photomicrographs of dihydroethidium (DHE) stained sections of rat mesenteric arteries cultured in the absence (Control; A-a) or presence of MGO (A-b). Scale bar: 10 µm. (B) Fluorescent intensity of DHE in endothelium was measured using ImageJ software and shown as fold increase relative to control (n=4). **P<0.05 vs. control.

Fig. 4. Effects of an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), gp91ds-tat (0.1 µM). (A) Concentration-contraction relationships to ACh in mesenteric arteries cultured in the absence (Control: open circle, n=9) or presence of MGO (42 µM, 3 days) co-treated without (MGO: closed circle, n=10) or with gp91ds-tat (open triangle, n=10). ACh (1 nM–300 µM) was cumulatively added after the contraction induced by KCl had reached a steady state. Results were expressed as the mean ± SEM. 100% represents KCl-induced pre-contraction. (B) Effects of a NOX inhibitor on the inhibitory effect of MGO on eNOS expression. After rat mesenteric arteries were cultured in the absence (Control) or presence of MGO (42 µM, 3 days) co-treated without (MGO) or with gp91ds-tat (gp91ds-tat+MGO), total cell lysates were harvested. eNOS expression was determined by Western blotting (n=9). Results were shown as fold increase relative to control. Equal protein loading was confirmed using total actin antibody. *P<0.05, **P<0.01; control vs. MGO, ##P<0.01; MGO vs. gp91ds-tat+MGO.
increased superoxide-mediated endothelial apoptosis. Here, we for the first time clarified the mechanisms of the long-term effects of MGO on endothelial function in organ-cultured rat mesenteric artery.

Recently, Brouwers et al. [3] reported that acute treatment (2 hr) of rat mesenteric artery with high concentration of MGO (1 mM) impaired ACh-induced relaxation in a pathway that is dependent on oxidative stress. In addition, Dhar et al. [5] demonstrated that acute treatment (2 hr) of rat aorta with MGO (30 µM) impaired ACh-induced relaxation through impairment of eNOS phosphorylation and subsequent NO production via increasing ROS. These previous reports determining acute MGO effects support our present results that MGO mediates impairment of ACh-induced relaxation via increasing ROS production. To advance the previous studies, we here especially focused on the long-term (3 days) effects of MGO on endothelial function using an organ culture technique, and for the first time revealed that long-term MGO induced impairment of ACh-induced relaxation through the decrease in eNOS protein via NOX-derived increased superoxide-mediated endothelial apoptosis.

In the present study, we showed that long-term treatment (3 days) of rat mesenteric artery with MGO impaired ACh-induced endothelium-dependent relaxation. In contrast, our previously data showed that short-term treatment (30 min) of rat aorta and carotid artery with MGO had no influence on ACh-induced endothelium-dependent relaxation [15, 16]. The discrepancy may be due to the differences in the duration of treatment (short-time vs. long-term). Because hypertension and diabetes are chronically developed diseases and MGO accumulates chronically in vascular walls [22], the present study might contribute to provide the mechanistic insights into roles of MGO on the pathogenesis of diabetic-related hypertension. Our recent in vivo data that MGO accumulation in mesenteric artery may mediate development of hypertension in SHR at least in part via increased ROS-mediated impairment of endothelium-dependent relaxation [17] might support the concept.

In the present study, we showed that gp91ds-tat, a specific inhibitor of NOX, prevented the decrease in eNOS expression and the impairment of endothelium-dependent relaxation caused by MGO. It was reported that gp91ds-tat inhibited activity of both NOX1 and NOX2 [24]. In addition, we have previously shown that long-term treatment (3 days) of endothelium-denuded rat mesenteric artery with MGO increased smooth muscle expression of NOX1 but not NOX2 [13]. Therefore, it might be possible that the effects of MGO on endothelium are mediated through increasing expression and/or activity of NOX1. Further biochemical studies might help to clarify the mechanisms through which MGO activates endothelial NOX.

In conclusion, we demonstrated that long-term MGO treatment impairs ACh-induced endothelial-dependent relaxation in organ-cultured rat mesenteric artery. The inhibitory effect is likely mediated through the decrease in eNOS expression via NOX-derived increased superoxide production and subsequent endothelial apoptosis. Further investigations on MGO may contribute to develop new pharmaceutical approaches against the diabetes-related hypertensive vascular disorders.

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REFERENCES


carboxyethyl cysteine, are elevated and related to nephropathy in patients with diabetes. Mol. Cell. Biochem. 302: 35–42. [Medline] [CrossRef]


