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Methylglyoxal augments intracellular oxidative stress in human aortic endothelial cells

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Abstract

Methylglyoxal (MGO) is a non-enzymatic metabolite in the glycolytic pathway and its concentration in blood and tissues is elevated in diabetes and renal failure. MGO induces tissue injuries via ROS; however, the mechanism remains to be clarified. The present study examined the harmful actions of MGO. Human aortic endothelial cells were assessed under real-time fluorescent microscopy with continuous superfusion. Increases in intracellular ROS were measured with fluorescent indicator, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFH-DA). The addition of MGO rapidly increased the ROS in a dose-dependent manner. The increment of DCF was entirely abolished by pre-treatment with superoxide anion scavenger and membrane-permeable catalase, indicating that MGO induces superoxide production. The increment was completely inhibited by 2-thenoyltrifluoroacetone or carbonyl cyanide 3-chlorophenylhydrazone and partially inhibited by N-methyl-L-arginine. These data suggest that MGO stimulates superoxide production from mitochondria and partially stimulates nitric oxide synthase in human endothelial cells.

Keywords: *Methylglyoxal, oxidative stress, endothelial cell, carbonyl stress*

Introduction

Endothelial dysfunction is the initial step in the development of atherosclerosis. Diabetes mellitus and end-stage renal disease (ESRD) cause atherosclerosis and arteriosclerosis through endothelial damage [1].

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound produced mainly by the degradation of glucose, via fructose in the glycolytic pathway. MGO reacts with the peptide forming MGO-derived lysine dimer, one of the advanced glycation end-products (AGEs) [2]. MGO is also known as a protein-bound uremic toxin [3] and the free form of MGO also exists stably in plasma. The plasma level of MGO is elevated in diabetic patients [4], patients with end-stage renal disease [5] and the corresponding animal models [6–8]. Several studies have suggested that MGO induces peritoneal tissue injury via oxidative stress [9]. MGO stimulates the transcription of angiopoietin-2 expression, which causes endothelial cell death and acellular capillary formation in the retina [10] and kidney [11]. In addition, a substantial portion of glucose-induced tissue injuries are caused by MGO [12]. Based on such findings, increased attention has been focused on the role of MGO in various pathological conditions such as diabetes mellitus, renal failure and hypertension [12–14]. However, the biological actions of MGO have not been well studied.

Reactive oxygen species (ROS), such as superoxide anion ($O^{\cdot}{}_2^-$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are crucial risk factors in the progression of endothelial damage [15–17]. We have recently reported that MGO itself is not an ROS, as

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determined by a chemiluminescence method; however, we detected free radicals by an electron spin resonance spectrum method when MGO was present with H_2O_2 [18]. MGO induces ROS production in neutrophils [19], platelets [20] and vascular smooth muscle cells [21]. However, the pathway of ROS production induced by MGO has not been examined enough in aortic endothelial cells, especially in the acute period after stimulation.

In the present study, we determined if MGO at concentrations detected in physiological or pathological conditions induced ROS in human aortic endothelial cells (HAECs) *in vitro*. We also examined the pathways of MGO-induced ROS production.

Materials and methods

Cell culture and reagents

Human aortic endothelial cells (HAECs) were obtained from Cambrex (Charles City, IA) and cultured according to the supplier's instructions. Methylglyoxal, elastase, polyethylene-glycol binding catalase (PEG-Cat), 2-thenoyltrifluoroacetone (TTFA), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), apocynin and N-methyl-L-arginine (L-NAME) were purchased from Sigma-Aldrich (St. Louis, MO) and 5- (and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). Tiron and H_2O_2 were purchased from Dojindo (Kumamoto, Japan).

Assessment of direct reaction between DCFH-DA and MGO

To confirm that DCFH-DA does not chemically react with MGO in a cell-free condition, 1, 10 and 100 μmol/L MGO or various concentrations of H_2O_2 were added to DCFH-DA in Falcon 96-well plates. Elastase was added to the well for conversion of DCFH-DA to DCFH 3 min before the addition of H_2O_2 or MGO. DCFH reacted with H_2O_2 or $ONOO^-$, but not $O^{\cdot}{}_2^-$, and was converted to the fluorescent form of the probe oxidation product (2',7'-dichlorofluorescein, DCF). The fluorescent signals of DCF were captured with excitation at 480 nm and emission at 535 nm by using a Fluoroscan Ascent plate reader (Thermo Labsystems, Waltham, MA). Data were analysed with Ascent software (Thermo Labsystems, Waltham, MA).

Measurement of DCF under real-time fluorescent microscopy with a continuous superfusion system

HAECs at the 5–7th passage were cultured on 15 mm cover slips and grown to 80–90% confluence under 95% O_2 and 5% CO_2. The cover slip was then placed on the stage of a real-time fluorescent microscope IX71 (Olympus, Tokyo, Japan) and the chamber was set for continuous superfusion.

The temperature of the chamber was maintained at 37°C by warming the superfusate with a TC-344 and TA-29 thermo-warmer (Warner Instruments, Hamden, CT). The superfusion rate of the buffer was maintained at 1 ml/min with a PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) and the chamber volume was kept at 5 mL by continuous aspiration. Signals of DCF were detected through an Ixon cool CCD video camera (Andor Co., Tokyo, Japan) equipped with an emission filter of 535 nm after excitation at 480 nm by a DG-4 and 175-W Xenon-Arc lamp (Sutter Instruments, Navato, CA). The signals were captured every 10 s and quantified with MetaFluor imaging software (Universal Imaging Co., Ypsilanti, MI).

Protocol

Cells were loaded with a membrane-permeable, non-fluorescent probe, DCFH-DA (5 μmol/L) for 15 min at 37°C in Hanks' Balanced Salt Solution (HBSS) under the light-shielding condition [22]. After washing the dye with superfusion buffer for 5 min, the vehicle buffer was exchanged with buffer that contained 0, 1, 10 or 100 μmol/L MGO and cells were observed for 20 min. Then the fluorescence of DCF was recorded. For the inhibition study, cells were pre-treated with inhibitors such as Tiron, TTFA, CCCP, apocynin and L-NAME for 6 h prior to the study. In the case of PEG-catalase, the cells were treated for 15 min before loading DCFH-DA. To compare ROS productivity after each treatment, mean rates of oxidation (units/s) were calculated for 800 s immediately after starting MGO superfusion during every experiment. These values are rough but illustrative estimates of the rate of oxidation of the probe which is not constant in time (especially for higher rates).

Statistical analysis

All data from at least five independent experiments were expressed as means ± SEM. The unpaired Student's *t*-test was used to analyse the data of mean oxidation rates in each independent experimental group.

Results

MGO did not chemically produce DCF in the cell-free assay

DCFH-DA is a fluorescent indicator used extensively to detect intracellular H_2O_2, but it may have potential artifacts. We assessed the direct interaction between MGO and DCFH-DA by using microplate readers to confirm that DCFH-DA is useful as a fluorescent

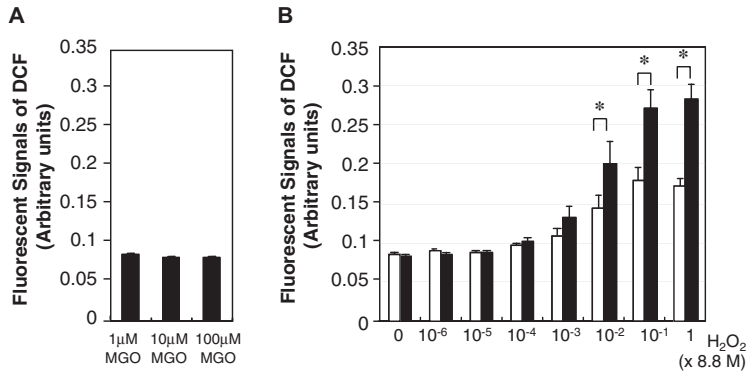

Figure 1. DCFH-DA did not react directly with MGO. (A) MGO did not form DCF from DCF. (B) DCFH-DA formed DCF from DCFH in the presence of H_2O_2 at concentrations higher than 8.8 mmol/L. Elastase was added to form DCFH from DCFH-DA. Open bars, absence of elastase; closed bars, presence of elastase. $^*p < 0.05$ absence of elastase vs the presence of elastase; $\#p < 0.05$ vs H_2O_2 $8.8*10^{-4}$ mol/L.

marker to measure intracellular H_2O_2 and $ONOO^-$. The amount of DCF in the presence of elastase was increased in a H_2O_2-concentration dependent manner and significantly greater than the amount in the absence of elastase at concentrations of H_2O_2 greater than 8.8 mM (Figure 1B). However, MGO did not directly convert DCFH to DCF (Figure 1A). These results suggested that MGO is not a ROS molecule that chemically reacts with DCFH, so DCFH-DA is available in the next protocol with HAECs.

MGO induced DCF formation dose-dependently

MGO increased the amount of intracellular DCF in HAECs. In order to evaluate the rate of increase, the intensities of the DCF were plotted against the elapsed time (Figure 2) and the mean rates of oxidation were calculated (Figure 3).

MGO increased the DCF signals in a dose- and time-dependent manner. Superfusion of MGO at the concentration of 10 and 100 μmol/L significantly increased the production of ROS. The rate for 0 (control), 1, 10 and 100 μmol/L MGO was 0.51 ± 0.18, 0.42 ± 0.09, 1.90 ± 0.12* and 2.64 ± 0.23 units/s*, respectively ($^*p = 0.0029$, 10 μmol/L MGO vs control, $p = 0.0014$, 100 μmol/L MGO vs control).

Inhibition study of MGO-induced ROS formation

To determine the ROS production by MGO in HAECs (Figure 3), the cells were pre-treated with a membrane-permeable type of catalase (50 U/mL of PEG-Cat) or $O^{\cdot}{}_2^-$ scavenger [23] (1 mmol/L of Tiron). Pre-treatment with either PEG-Cat (0.26 ± 0.25 units/s, $p = 0.00\,071$ vs 100 μmol/L MGO) or Tiron (0.29 ± 0.07 units/s, $p = 0.00\,005$ vs 100 μmol/L MGO) completely abolished the MGO-induced DCF to the vehicle level. These data suggest that MGO promotes the production of $O^{\cdot}{}_2^-$ and that the $O^{\cdot}{}_2^-$ is converted to H_2O_2, which increases the intracellular levels of DCF.

Next, we investigated which pathway was involved in the MGO-induced $O^{\cdot}{}_2^-$ production. Cells were pre-treated with an uncoupler of oxidative phosphorylation

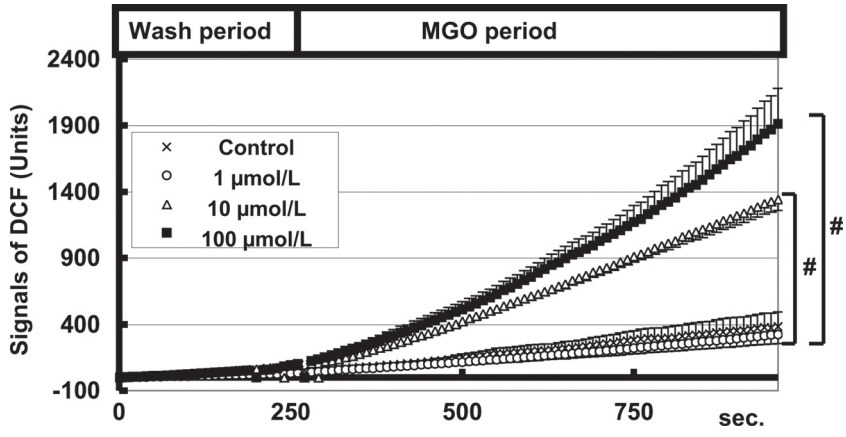

Figure 2. Intracellular ROS production by MGO. Increases in the amounts of DCF indicate the production of MGO-induced ROS. Acute infusion of MGO induced the production of intracellular ROS in a dose- and time-dependent manner in HAECs. The wash period indicates the vehicle in the each experiment. $\#p < 0.05$, control vs 10 μmol/L or 100 μmol/L.

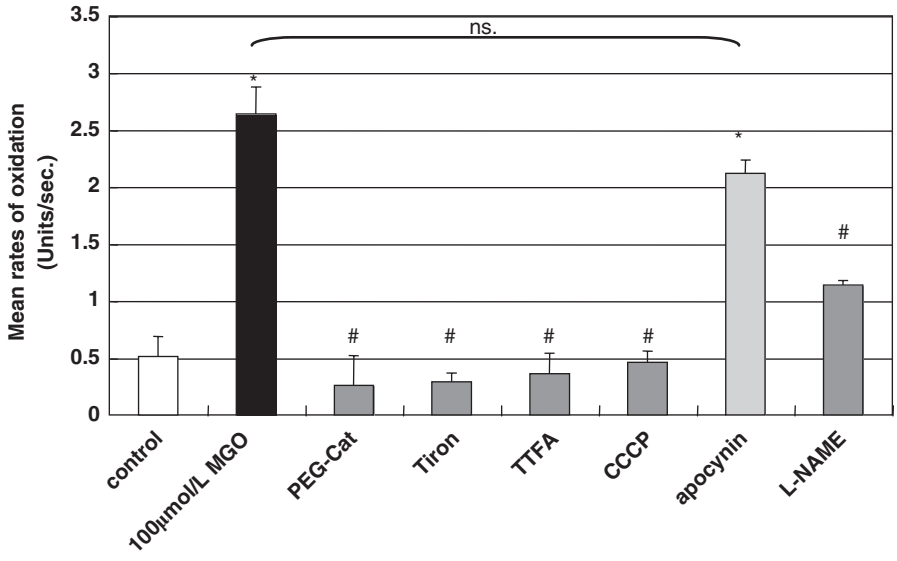

Figure 3. Effects of inhibitors on the MGO-induced ROS production. MGO (100 μmol/L) significantly increased the intracellular ROS production (*$p < 0.05$). Pre-treatment with PEG-Cat, Tiron, TTFA or CCCP completely inhibited the 100 μmol/L MGO-induced ROS (#$p < 0.05$). Pre-treatment with apocynin did not significantly inhibit the ROS formation vs 100 μmol/L MGO (ns), but significantly increased the formation of ROS vs control (*$p < 0.05$). Pre-treatment with L-NAME partially and significantly inhibited the formation of ROS (#$p < 0.05$).

that abolishes the mitochondrial membrane proton gradient (5 μmol/L of CCCP), an inhibitor of the mitochondrial electron transport chain complex II (10 μmol/L of TTFA), nitric oxide synthase (NOS) inhibitor (100 μmol/L of L-NAME) or an inhibitor of NADPH oxidase (NOX) (1 mmol/L of apocynin) for 6 h. Increases in the level of DCF were completely inhibited to the level of vehicle by pre-treatment with CCCP (0.46 ± 0.09 units/s, $p = 0.0001$ vs 100 μmol/L MGO) or TTFA (0.36 ± 0.18 units/s, $p = 0.0002$ vs 100 μmol/L MGO). Pre-treatment with L-NAME (1.14 ± 0.04 units/s, $p = 0.0012$ vs 100 μmol/L MGO) significantly suppressed the response to 54% of the response induced by 100 μmol/L MGO. These results suggest that pathological concentrations of MGO increase the intracellular production of $O^{\cdot}{}_2^-$ in HAECs and that the production is mainly mediated by the mitochondrial electron-transport pathway and partially mediated by the NOS pathway. However, pre-treatment with apocynin also partially suppressed the production of DCF, although not significantly (2.12 ± 0.11 units/s, $p = 0.09$ vs 100 μmol/L MGO, $p = 0.0016$ vs control).

Discussion

MGO is one of the uremic toxins found in patients with diabetic nephropathy and ESRD, but its contribution to tissue toxicity has remained controversial. In the present study, we demonstrate that MGO increased the production of ROS in HAECs by using real-time fluorescent microscopy. For the first time, we demonstrated that the increase occurred immediately after exchanging the vehicle buffer with MGO in the chamber. DCFH is useful as an indicator of $ONOO^-$ as well as H_2O_2 [24]. Superoxide anion is converted into H_2O_2 or $ONOO^-$, so DCF is able to indirectly measure the total amounts of superoxide. Tiron inhibited DCF after MGO stimulation; thus, probably MGO primarily stimulates the production of $O^{\cdot}{}_2^-$. Tiron has been reported to be a Ca^+ chelator in addition to a scavenger of $O^{\cdot}{}_2^-$ like a SOD mimetic [25]. Therefore, it is possible that tiron diminishes ROS production via another unknown pathway.

Certain levels of MGO exist in normal tissues throughout the whole body and the plasma level of MGO is increased in hyperglycaemic conditions (1–4 μmol/L) [26], in end-stage renal disease (9.7–11.5 μmol/L) [5] or by ageing [27,28]. In the present study we used 1, 10 or 100 μmol/L of MGO, which are the concentrations found in the plasma of healthy persons, diabetic patients and ESRD patients, respectively. MGO is a key component in pathological conditions such as DM and ESRD. We measured the plasma concentration of MGO in healthy individuals and found that it was less than 1 μmol/L. Even after healthy individuals consumed beverages, MGO concentrations were not greater than 1 μmol/L (unpublished data). Thus, the daily intake of food does not appear to contribute to endothelial toxicity in healthy individuals, because our results show that a low level of MGO does not increase the production of intracellular ROS. A previous study showed that cigarette smoke contains 0.19–0.83 μmol of MGO per cigarette [29]. Chronic oral administration of MGO at 0.69 mmol per kg of body weight led to

kidney collagen accumulation in an animal study [30]. Thus, the toxicity of MGO may be involved not only in some disease conditions, but also in activates such as smoking and the large intake of foods containing high levels of MGO.

The present study demonstrated that pathological concentrations of MGO rapidly elicit ROS production in human endothelial cells. Previous studies have used rather high concentrations of MGO to demonstrate its biological actions [19–21]. The higher sensitivity of our system may be due to our superfusion system and real time measurements of ROS. When cells are treated with MGO for a prolonged period under culture conditions, secondary changes may occur, and these changes may obscure the detection of subtle changes. Since our system allowed real-time measurements in the absence of the influences of secondary changes, we were able to detect significant effects of MGO at low concentrations.

Davidson and Duchen [31] reported that mitochondria play an important role in production of $O^{\cdot}_2{}^-$ in the endothelial cells. Superoxide is generated from two main sites in the inner mitochondrial membrane, NADH dehydrogenase at complex I and at the interface between ubiquinone and complex III. High glucose loads stimulate mitochondrial $O^{\cdot}_2{}^-$ production from the latter site in aortic endothelial cells [32] and retinal endothelial cells [33]. Rosca et al. [34] showed that the MGO-induced modification of mitochondrial complex III was associated with the excessive formation of $O^{\cdot}_2{}^-$ in streptozotocin-derived diabetic rats. Long-standing hyperglycaemia alters the mitochondrial function and increases the formation of $O^{\cdot}_2{}^-$ due to alterations in mitochondrial metabolism [35]. Shangari et al. [36] reported that a monocarbonyl compound metabolized from glucose, glyoxal markedly increased ROS-induced cytotoxicity in rat hepatocytes. In the present study, two types of mitochondrial inhibitors (TTFA and CCCP) completely suppressed the MGO-induced ROS. TTFA inhibits mitochondrial electron transport chain complex II and CCCP uncouples oxidative phosphorylation, which abolishes the mitochondrial membrane proton gradient. Our results suggest that the mitochondrial pathway is crucial for MGO-induced ROS production, which probably originates from $O^{\cdot}_2{}^-$.

Superoxide reacts with nitric oxide (NO) very quickly and forms $ONOO^-$ [37]. Zou et al. [38] demonstrated that increases in the level of $ONOO^-$ reduced the amount of BH_4 and increased the uncoupling form of eNOS. Cai [39] suggested that H_2O_2 originating from vascular NAD(P)H oxidases propagates its own production via the enhancement of several pathways of ROS production including uncoupling eNOS. The uncoupling eNOS increases the production of $O^{\cdot}_2{}^-$. However, our protocols of measuring DCF could not distinguish the production of H_2O_2 from $ONOO^-$. Thus, we could not distinguish uncoupling eNOS from coupling eNOS to consider their individual contributions to the ROS production. $ONOO^-$ is also a harmful ROS molecule and L-NAME inhibits uncoupling eNOS as well as coupling eNOS. Based on our results, eNOS is associated with the MGO-mediated ROS production. These findings suggest that MGO triggers a vicious cycle involved in formation of uncoupling eNOS (Figure 4). This pathway may augment the MGO toxicity in the cells.

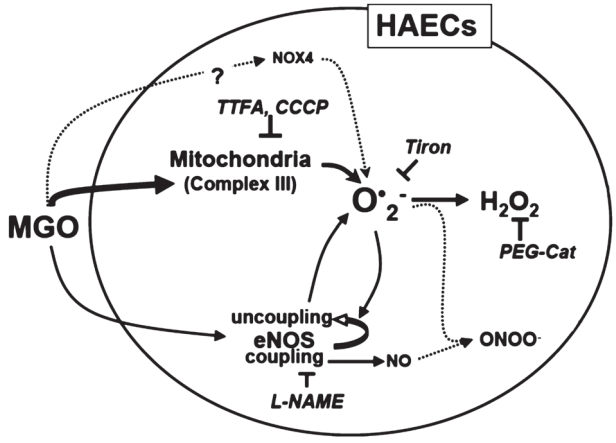

Figure 4. Putative pathways by which MGO induces ROS production in HAECs. To increase $O^{\cdot}_2{}^-$ production, MGO primarily affects the mitochondria and partially affects eNOS. MGO might also potentially affect Nox. The increment of intracellular $O^{\cdot}_2{}^-$ increases the uncoupling of eNOS and probably trigs a vicious cycle on the oxidative stress.

In vascular endothelial cells, the Nox family is an important source of $O^{\cdot}_2{}^-$. Nox2 and Nox4 are abundantly expressed in human endothelial cells [40]. A previous study suggested that Nox was responsible for the MGO-induced $O^{\cdot}_2{}^-$ formation in mesangial cells [41]. However, we did not find a significant contribution of Nox to the acute ROS production induced by MGO in HAECs when we used apocynin to inhibit Nox. Apocynin is activated in the presence of H_2O_2 and myeloperoxidase (MPO) [42] and it inhibits the activation of Nox2 by inhibition of the translocation of p47phox. A recent report found that apocynin is an antioxidant and is not used as an NADPH oxidase inhibitor [43]. HAEC has a rare expression pattern of MPO that is different from neutrophils. Furthermore, the pre-treated apocynin was washed out before the experimental period, so apocynin could not exert antioxidant effects in our protocol. Nox4 requires p22phox for its activity but does not require other regulatory sub-units [44]. It is possible that Nox4 produced superoxide in our study, but we could not address this possibility in the present study.

Recently we reported that MGO reacted with H_2O_2, forming a novel carboxy-methyl radical [18]. The radical was formed higher under MGO with H_2O_2 than under glyoxal (GO) with H_2O_2. This observation is consistent with our supplemental data (online version only) showing that the ROS production rate by MGO

(4.11 ± 0.79 units/s) is greater than that by GO (0.82 ± 0.05 units/s, $p = 0.02$). As MGO reacts with glutathione and forms S-D-lactoylglutathione, it is possible that MGO decreases the intracellular capacity of antioxidants [45], but GO does not [46]. On the other hand, H_2O_2 is known as an endothelial-derived hyperpolarization factor [47]. Thus, MGO may reduce H_2O_2 localized around endothelial cells and damage the artery.

MGO is a radical modulator that forms advanced glycation end-products (AGEs). Aminoguanidine (AG) inhibits AGE formation from glucose and methylglyoxal [48]. AGE is also an inducer of $O^{\cdot}{}_2^-$. We examined the ability of AG and arginine (L-Arg) to inhibit AGE formation from MGO. As shown in the supplemental figure (online version only), both L-Arg and AG entirely inhibited the ROS production (mean oxidation rates (units/s): MGO alone, 4.11 ± 0.79; MGO with L-Arg, 0.014 ± 0.01; MGO with AG, –0.09 ± 0.06; control, 0.31 ± 0.12). MGO reacts with guanidine residue [49] and produces superoxide, which is inhibited by AG [50]. Arginine is a kind of guanidino compound. It is possible that the extracellular $O^{\cdot}{}_2^-$ generated form L-Arg and MGO was continuously drained from the chamber because of our continuous superfusion system. As DCF is an accumulative indicator, it is probable that AG almost completely inhibited ROS by the basal production and that we observed a photo-bleaching phenomenon of DCF. Because the guanidine residue is a constituent of cyclic GMP, which is an intracellular signal of NO to dilate vessels, MGO may interfere with NO functions. It has been reported that MGO activated caspase-3 and induced the cell death of endothelial cells [51]. The expression and activity of caspase-3 was stimulated by superoxide [51]. This study supports our finding that MGO stimulates the production of superoxide in endothelial cells. Here, we show the mechanism by which MGO produces ROS in endothelial cells. Further studies are needed to conclusively determine if the produced ROS is derived from $O^{\cdot}{}_2^-$.

In conclusion, we found that high concentrations of MGO rapidly induce the production of ROS in HAECs. The putative source of the ROS is the mitochondrial pathway and also partially the eNOS pathway (Figure 4). The increased level of MGO in patients with renal dysfunction and/or diabetes probably plays an important role in the endothelial dysfunction and is a putative risk for developing atherosclerosis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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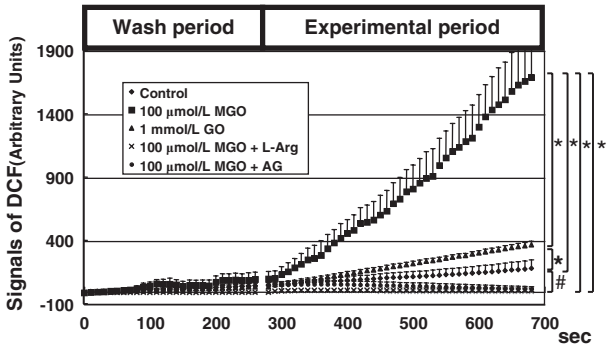
Supplementary Material



Supplementary Figure 1. Vehicle buffer was continuously superfused during the wash period and then switched to buffer that contained MGO (100 μmol/L) or glyoxal (GO, 1 mmol/L) during the experimental period. Signals of DCF were detected and captured by a 535-nm emission filter with 480-nm excitation wavelength every 10 s. The responses by MGO were measured in the presence or absence of 10 mmol/L Aminoguanidine (AG) or 100 μmol/L L-arginine (L-Arg). $^{*}p < 0.05$, $\#p = 0.016$ control vs 100 μM MGO + AG.