Methylglyoxal mediates vascular inflammation via JNK and p38 in human endothelial cells

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Yamawaki H, Saito K, Okada M, Hara Y. Methylglyoxal mediates vascular inflammation via JNK and p38 in human endothelial cells. Am J Physiol Cell Physiol 295: C1510–C1517, 2008. First published October 8, 2008; doi:10.1152/ajpcell.00252.2008.—Methylglyoxal (MGO) is a reactive metabolite of glucose. Since the plasma concentration of MGO is increased in diabetic patients, MGO is implicated in diabetes-associated vascular endothelial cells (ECs) injury, which might be responsible for atherosclerosis. In the present study, we examined effects of treatment of human umbilical vein ECs with MGO on EC morphology and inflammatory responses. MGO (24 h) induced cytotoxic morphological changes in a concentration-dependent manner (0–420 μM). MGO induced mRNA and protein expression of cyclooxygenase (COX)-2 in a concentration (0–420 μM)- and time (6–24 h)-dependent manner. COX-2 induction was associated with increased PGE2 release. Acute treatment with MGO (20 min) induced concentration-dependent (0–420 μM) activation of JNK and p38 MAP kinase but not ERK or NF-κB. Both the JNK inhibitor SP600125 and the p38 inhibitor SB203580 prevented the MGO induction of COX-2. However, inhibiting JNK and p38 or COX-2 was ineffective to the morphological damage by MGO (420 μM, 24 h). EUK134, a synthetic combined superoxide dismutase catalyst, mimetic, had no effect on MGO-induced COX-2. Present results indicated that MGO mediates JNK- and p38-dependent EC inflammatory responses, which might be independent of oxidative stress. On the other hand, MGO-induced morphological cell damage seems unlikely to be associated with COX-2-PGE2.

It is well known that diabetes mellitus (DM) is one of the major risk factors for atherosclerosis (7, 18). Vascular endothelial cells (ECs) inflammatory injury represents an important early pathogenic feature of atherosclerosis (17, 28). Although DM is often associated with ECs inflammatory injury (13), the underlying mechanisms are not fully clarified.

Since the plasma concentration of advanced glycation end-products (AGEs) is reported to be significantly elevated in diabetic patients (4, 15, 23), previous investigators have mainly explored the effects of AGEs as well as the high concentration of glucose on vascular endothelium. However, controversial results have been obtained (8, 14, 34, 42). On the other hand, we recently provided evidence that one of the glucose metabolites, glyoxal, is a more powerful inducer for EC inflammatory injury rather than glucose itself or AGEs (38).

Methylglyoxal (MGO) is a reactive metabolite of glucose and serves as an intermediate between glucose and AGEs (30). It is known that MGO can stimulate the cellular signal transduction (2, 3). MGO is produced from triose phosphates under hyperglycemic conditions and eventually metabolized into AGEs mainly by nonenzymatic glycation of protein (1, 25). A recent study (16) revealed that the plasma MGO level is significantly increased in diabetic patients. Furthermore, an increased MGO-derived AGEs level in diabetic patients seems to be associated with diabetic complications such as nephropathy (22) and retinopathy (10). In addition, recent studies (35, 37) indicated that MGO is also implicated in diabetes-related vascular disorders like hypertension. Therefore, we hypothesized that MGO could affect vascular ECs to induce inflammatory injury, which could be responsible for the DM-associated atherosclerosis. To prove this, we examined the effects of treatment with MGO on human vascular ECs by especially focusing on the inflammatory signal events.

Materials and Methods

Materials. Reagent sources were as follows: MGO solution (Sigma-Aldrich, St. Louis, MO); SB203580 (Calbiochem, San Diego, CA); EUK134 and NS398 (Cayman, Ann Arbor, MI); SP600125 (Jena Bioscience, Jena, Germany); and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Antibody sources were as follows: phospho-p38, phospho-ERK, phospho-NF-κB p65 (Ser536), and total JNK2 (Cell Signaling, Beverly, MA); endothelial nitric oxide (NO) synthase (eNOS), cyclooxygenase (COX)-2, IkB-α, total p38, ERK 1, NF-κB p65, and actin (Santa Cruz Biotech, Santa Cruz, CA); and phospho-JNK (Promega, Madison, WI).

Cell culture. Human umbilical vein ECs (HUVECs) were purchased from Kurabo (Osaka, Japan) and cultured in Medium 200 supplemented with low serum growth supplement (Cascade Biologics, Portland, OR) as described previously (38–40). Cells at passage 4 to 7 were used for experiments.

Morphological examination. HUVEC morphological changes were observed under light microscope (CX31, Olympus, Tokyo, Japan) equipped with digital camera (SP-350, Olympus).

Cell counting. The living cell number was counted using a Cell Counting-8 kit (Dojindo Laboratories, Kumamoto, Japan) as described previously (38). After HUVECs were treated at ~90% confluence with MGO (0–420 μM) for 24 h, water-soluble tetrazolium salt (WST-8) was added for 1 h and the culture medium was collected. Conversion of WST-8 into formazan by living cells (active mitochondria) was measured using a standard 96-well plate reader at absorbance 490 nm. The total number of living cells was expressed as relative to untreated control samples (0 μM MGO).

Western blotting. Western blotting was performed as described previously (38–40). Protein lysates were obtained by homogenizing HUVECs with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μM leupeptin, and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan). Protein concentration was determined using...
the bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of proteins (10–15 µg) were separated by SDS-PAGE (7.5–10%) and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI). After being blocked with 3% BSA or 0.5% skim milk, membranes were incubated with primary antibody (1:500–1,000 dilution) at 4°C overnight, and membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the ECL-plus system (Amersham Biosciences, Buckinghamshire, UK). Equal loading of protein was confirmed by measuring total actin or eNOS expression. The resulting autoradiograms were analyzed using National Institutes of Health Image 1.63 software.

Quantitative determination of PGE2 release. The production of PGE2 from HUVECs was measured using an enzyme immunoassay kit (Cayman) in accordance with the instructions of the manufacturer. Briefly, after HUVECs were treated at ~90% confluence with MGO (420 µM) for 24 h, the culture medium was collected. PGE2 released into the culture medium was measured at absorbance 410 nm using a standard 96-well plate reader.

Immunofluorescence staining. Immunofluorescence staining was performed as described previously (5). Cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at 4°C and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After being blocked with 5% normal goat serum for 60 min, cells were incubated with NF-κB p65 antibody (1:500 dilution) at 4°C over night followed by fluorescent-conjugated secondary antibody (Alexa Fluor 488; 1:500 dilution, 1 h, Invitrogen). Images were obtained using fluorescence microscope (BX-51, Olympus) equipped with cooled CCD camera (MicroPublisher 5.0 RTV, Roper Japan, Tokyo, Japan).

RT-PCR. Total RNA was extracted from HUVECs by using QuickGene SP kit (Fuji Film, Tokyo, Japan) according to the instructions of the manufacturer. The first strand of cDNA was synthesized using random hexamers and Superscript II RT at 65°C for 5 min, 25°C for 2 min, 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. PCR amplification was performed using HotStarTaq MasterMix kit (Qiagen, Valencia, CA). The oligonucleotide primers for COX-2 were GAG CTG TAT CCT GCC CTT CTG GTA (sense) and GGA AGA ACT TGC ATT GAT GGT GAC (antisense). The oligonucleotide primers for GAPDH were GCT GAT GCC CCC ATG TTT G (sense) and GGG TGG TGG ACC TCA TGG C (antisense). After initial activation at 95°C for 15 min, 28 cycles of amplifications at 94°C for

Fig. 1. A: representative photomicrographs of human umbilical vein endothelial cells (HUVECs) treated for 24 h with methylglyoxal (MGO); a, f: 0 µM MGO; b: 42 µM MGO; c: 140 µM MGO; d: 280 µM MGO; and e, g: 420 µM MGO. Magnification: ×100 (a–e) and ×200 (f, g). B: total number of living cells was counted using water-soluble tetrazolium salt. Results are shown as relative to 0 µM MGO (n = 6). **P < 0.01 vs. 0 µM MGO.
0.5 min, 52°C for 0.5 min, and 72°C for 1 min followed by final extension at 72°C for 10 min were done with a thermal cycler (PC707, ASTEC, Fukuoka, Japan). PCR products were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. Detectable fluorescent bands were visualized by an ultraviolet transilluminator and analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

**Statistical analysis.** Data are means ± SE. Statistical evaluations were performed using one-way ANOVA followed by Tukey’s test. Values of *P* < 0.05 were considered statistically significant.

**RESULTS**

**Effects of chronic treatment of HUVECs with MGO.** We first examined morphological changes after chronic treatment of HUVECs with MGO. Treatment for 24 h with MGO induced a cytotoxic morphological change in a concentration-dependent manner (0–420 μM; Fig. 1A, a–e). Especially in cells treated with high concentration of MGO (420 μM), the cell density seemed decreased and spindle typed cells were often observed (Fig. 1A, f, g). Figure 1B showed that morphological damage by MGO was associated with decreased living cell number (at 420 μM MGO, 20.9 ± 5.3% decrease from 0 μM MGO; *n* = 6; *P* < 0.01).

To examine whether the morphological changes are associated with vascular inflammatory states, expressions of inflammation-associated proteins were measured by Western blotting. Treatment of HUVECs with MGO (24 h) induced COX-2 protein expression in a concentration-dependent manner (0–420 μM; *n* = 3). On the other hand, induction of VCAM-1 protein was not detectable by the treatment with MGO.

**Acute effects of MGO on inflammatory signaling in HUVECs.** After HUVECs were treated with MGO (0–420 μM) for 20 min, total cell lysates were harvested. A: activation of JNK, p38, and ERK was determined by Western blotting using phospho-specific antibodies (*n* = 3). Equal protein loading was confirmed using eNOS antibody. B, C: phosphorylation of JNK (B) or p38 (C) is shown as fold increase relative to 0 μM MGO. *P* < 0.05, **P** < 0.01 vs. 0 μM MGO. D: total amount of MAP kinases (JNK, p38, and ERK) and eNOS after MGO treatment (0–420 μM, 24 h) was shown. Equal protein loading was confirmed using total actin antibody.
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MGO (0–420 μM; n = 3; data not shown). COX-2 induction by MGO (420 μM) was observed after 6 h treatment (1.3 ± 0.8-fold relative to 0 μM MGO; n = 3), became maximum after 12 h (2.9 ± 0.7-fold; n = 3), and remained clearly detectable after 24 h (1.9 ± 0.4-fold; n = 3; Fig. 2B). Figure 2C showed that increased COX-2 expression by MGO (420 μM, 24 h) was associated with increased PGE₂ release from HUVECs [2,037.0 ± 284.1 pg/mg protein in 420 μM MGO vs. 1,178.1 ± 141.4 pg/mg in 0 μM MGO (control); n = 15; P < 0.05].

Signaling transduction mechanisms relating to MGO-mediated COX-2 induction. To examine the signal mechanisms responsible for COX-2 induction by MGO, the activation of MAP kinases was measured by Western blotting using phosphorylation-specific antibodies. Acute treatment of HUVECs with MGO (20 min) induced activation of JNK and p38 but not ERK in a concentration-dependent manner (0–420 μM; Fig. 3A). Maximal phosphorylation of JNK or p38 was obtained by the treatment with 420 μM MGO (p-JNK1/2, 2.8 ± 0.5-fold relative to 0 μM MGO; n = 3; P < 0.05; Fig. 3B; and p-p38, 6.2 ± 0.8-fold; n = 3; P < 0.01; Fig. 3C). We confirmed that the total amount of these kinases was not changed by the treatment with MGO (0–420 μM, 24 h; Fig. 3D).

The activation of NF-κB pathway was next examined. Phosphorylation of NF-κB p65 at Ser536 was not induced by the treatment of HUVECs with MGO (0–420 μM, 20 min; Fig. 4A; n = 4) in contrast to TNF-α treatment (10 ng/ml, 5 min). Consistently, degradation of IkB-α protein was not caused by the MGO treatment (Fig. 4A; n = 4). We also observed that nuclear translocation of NF-κB p65 was not induced by MGO (420 μM, 15–60 min; n = 3; Fig. 4B), suggesting that the NF-κB pathway is not involved in the COX-2 induction by MGO.

To clarify whether activation of JNK and/or p38 is responsible for the COX-2 induction by MGO, HUVECs were pretreated with a specific inhibitor of JNK or p38 (30 min) before MGO stimulation (420 μM, 24 h). Pretreatment with the specific JNK inhibitor SP600125 (3 μM) significantly suppressed the COX-2 induction by MGO (COX-2 expression in MGO + SP600125, 1.1 ± 0.1-fold relative to 0 μM MGO, n = 8, P < 0.01 vs. 420 μM MGO; Fig. 5A). We confirmed that SP600125 significantly inhibited the MGO (420 μM, 20 min)-mediated JNK phosphorylation (n = 6; P < 0.01; Fig. 5B). Figure 6A showed that pretreatment of HUVECs with the specific p38 inhibitor SB203580 (100 μM, 30 min) significantly inhibited the MGO (420 μM, 20 min)-mediated p38 phosphorylation (n = 5; P < 0.05). We observed that SB203580 at <100 μM was ineffective to MGO-induced p38 phosphorylation (n = 4; data not shown). However, this concentration of SB203580 (100 μM) was toxic to HUVECs if treated chronically (24 h). Therefore, SB203580 was washed before MGO (420 μM, 24 h) stimulation according to the methods previously described (11). Figure 6B showed that pretreatment of HUVECs with SB203580 (100 μM, 30 min) significantly suppressed the COX-2 induction by MGO (n = 5; P < 0.01).

It was also confirmed that COX-2 mRNA was significantly increased by the treatment of HUVECs with MGO (420 μM, 6 h, P < 0.01 vs. 0 μM MGO; n = 3) and that the COX-2 induction was significantly prevented by the pharmacological inhibition of JNK (3 μM SP600125, 30 min, P < 0.01 vs. 420 μM MGO; n = 3) or p38 (100 μM SB203580, 30 min, P < 0.05 vs. 420 μM MGO; n = 3; Fig. 7).

MGO-induced COX-2-PGE₂ is dissociating from EC morphological damage. We next examined whether MGO-induced COX-2 is associated with EC morphological damage. Treatment of HUVECs with SP600125 (3 μM), SB203580 (100 μM), or the specific COX-2 inhibitor NS398 (1–10 μM) did not prevent MGO (420 μM, 24 h)-induced morphological damage (Fig. 8), suggesting that MGO-induced COX-2-PGE₂ is dissociating from morphological damage.

Effects of anti-oxidant on MGO-mediated EC inflammation. To finally examine whether EC inflammation by MGO is associated with increased oxidative stress, HUVECs were pretreated with EUK134 (3 μM, 30 min) before MGO stimulation (420 μM, 24 h). EUK134 is a synthetic superoxide dismutase/catalase mimetic (27, 29). Treatment of HUVECs with EUK134 had no effect on both EC morphology (data not shown) and MGO-mediated COX-2 induction (Fig. 9; n = 3), suggesting that the effect of MGO is independent of reactive oxygen species.

DISCUSSION

The major findings of the present study are that MGO, a metabolite of glucose induced inflammatory responses as determined by COX-2 induction and PGE₂ production in human vascular ECs (Fig. 10). It was clarified that induction of COX-2 was mediated via activating the cellular signal transduction of JNK and p38 MAP kinase but not reactive oxygen species.
We also found that MGO induced cytotoxic morphological damage in ECs, which is independent of COX-2-PGE2. Since ECs inflammatory response and morphologic damage are early pathogenic steps of atherosclerosis, our results suggest that MGO might play a significant role in diabetes-related atherosclerosis.

The present study used 56–420 μM of MGO for in vitro experiments, and this concentration of MGO caused ECs inflammatory injury in a concentration-dependent manner. There is a report (16) showing that plasma MGO concentration in poorly controlled human diabetic patients is ~400 μM, whereas others (20, 33) demonstrated that it was much less. It has been suggested that the local MGO concentration in tissues is much higher than the plasma level (26). It was also reported that cultured cells may produce larger amounts of MGO (as much as 310 μM; Ref. 9). Nevertheless, determination of the chronic effects of a lower concentration of MGO especially in blood vessels and whole animals is necessary to validate the present in vitro results.

In the present study, we determined that COX-2 induction by MGO was mediated via activating JNK and p38 MAP kinase. It is well known that COX-2 induction by various stimuli is mainly regulated by the MAP kinase family (ERK, JNK, and p38; Refs. 11, 24, 36) and/or NF-κB (31), supporting the findings presented in this study.

**Fig. 5.** Effect of a JNK inhibitor on MGO-mediated COX-2 induction in HUVECs. After HUVECs were treated with 420 μM MGO for 24 h (A) or 20 min (B) in the absence or presence of SP600125 (3 μM, pretreatment for 30 min), total cell lysates were harvested. Expression of COX-2 (A; n = 6–8) and phosphorylation of JNK (B; n = 6–7) were determined by Western blotting. Equal protein loading was confirmed using actin antibody. COX-2 expression (A) or JNK phosphorylation (B) is shown as fold increase relative to 0 μM MGO. **P < 0.01 vs. 0 μM MGO; ##P < 0.01 vs. 420 μM MGO.

**Fig. 6.** Effect of a p38 MAP kinase inhibitor on MGO-mediated COX-2 induction in HUVECs. After HUVECs were treated with 420 μM MGO for 20 min (A) or 24 h (B) in the absence or presence of SB203580 (100 μM, pretreatment for 30 min), total cell lysates were harvested. Phosphorylation of p38 (A; n = 5) and expression of COX-2 (B; n = 5) were determined by Western blotting. Equal protein loading was confirmed using eNOS antibody. Phosphorylation of p38 (A) or COX-2 (B) expression is shown as fold increase relative to 0 μM MGO. *P < 0.05, **P < 0.01 vs. 0 μM MGO; #P < 0.05, ##P < 0.01 vs. 420 μM MGO.
our results. In contrast, our recent results (38) showed that glyoxal, one of the metabolites of glucose, induced COX-2 protein but failed to activate JNK and p38. MGO is different from glyoxal only at the methyl group, and we could not explain why such differences occurred in the cellular signal transduction. It is speculated that another target rather than protein kinases might also exist for the effects of glucose metabolites. The recent report (41) that MGO modifies nuclear transcriptional regulatory proteins might support the concept.

Fig. 7. MGO induced mRNA for COX-2 in HUVECs, which is due to JNK and p38 activation. After HUVECs were treated for 6 h with MGO (420 μM), total RNA was harvested. A: expression of COX-2 mRNA was determined by RT-PCR analysis using the gene-specific primers to human COX-2. Equal loading of template cDNA was confirmed by ensuring the GAPDH expression (n = 3). B: expression is shown as fold increase relative to 0 μM MGO. **P < 0.01 vs. 0 μM MGO; #P < 0.05, ##P < 0.01 vs. 420 μM MGO.

Fig. 8. Representative photomicrographs of HUVECs treated for 24 h with MGO (A: 0 μM; B–E: 420 μM) in the presence of SP600125 (C: 3 μM, pretreatment for 30 min), SB203580 (D: 100 μM), SP600125 + SB203580 (E), or NS398 (F: 10 μM) are shown. Magnification: ×100.

Fig. 9. Effect of antioxidant on MGO-induced COX-2. HUVECs were treated with MGO (420 μM, 24 h) in the absence or presence of EUK134 (3 μM, pretreatment for 30 min). Effect of EUK134 on COX-2 expression was determined by Western blotting. COX-2 expression is shown as fold change relative to 0 μM MGO (n = 3).
On the other hand, we observed that MGO-induced ECs morphological damage was not associated with increased COX-2-PGE2. There are two possible explanations. MGO-induced COX-2-PGE2 was related to other ECs inflammatory states such as hyperpermeability (6, 19) and/or increased leukocyte-ECs interaction (12, 21) rather than direct ECs morphological damage. Alternatively, MGO might directly interact with DNA and damage the cell, since it is suggested that MGO mediates DNA glycation, which may be associated with mutation, DNA strand breaks, and cytotoxicity (32).

Whether the effects of MGO are due to extracellular effects or intracellular effects seems an important issue, since MGO is produced in the cytosol under hyperglycemic condition (30). Furthermore, whether the present results are caused directly via MGO or indirectly via MGO-derived AGEs is also an important issue. We postulate that the results obtained here might be mediated mainly via extracellular MGO itself but not AGEs by the following reasons. 1) Our preliminary data showed that MGO-derived AGEs was not detectable even 6 h after treatment of HUVECs with MGO (420 μM) as measured by anti-Nε-(carboxethyl) lysine (CEL), a specific MGO-derived AGEs Western blotting. 2) In our former study (38), we provided evidence that the pathological concentration of AGEs (100–500 μM, 24 h) had no effect on both COX-2 induction and morphology in HUVECs. However, it could not be ruled out that exogenously applied MGO might move inside the cells and cause cytotoxic injury by unknown mechanisms. Our preliminary results that a higher concentration of MGO (560 μM, 6–12 h), which is able to induce apoptosis in HUVECs (5), produced AGEs (CEL) specifically in the nuclei (as determined by immunofluorescence) might support the concept.

In summary, we demonstrated in cultured human vascular ECs that MGO, a reactive metabolite of glucose, could be a stimulant for vascular inflammatory responses. EC inflammation by MGO was mediated via the activation of JNK and p38 MAP kinase. Further investigations in blood vessels and whole animals might contribute to obtain further mechanistic insights into the diabetes-associated atherosclerosis.

**Fig. 10.** Proposed pathways leading to MGO-mediated endothelial cell inflammation and morphological injury, which might be responsible for the diabetes-associated atherosclerosis.

**REFERENCES**

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