Increased Methylglyoxal and Oxidative Stress in Hypertensive Rat Vascular Smooth Muscle Cells

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Abstract—Methylglyoxal can yield advanced glycation end products via nonenzymatic glycation of proteins. Whether methylglyoxal contributes to the pathogenesis of hypertension has not been clear. The aim of the present study was to investigate whether the levels of methylglyoxal and methylglyoxal-induced advanced glycation end products were enhanced and whether methylglyoxal increased oxidative stress, activated nuclear factor–κB (NF-κB), and increased intracellular adhesion molecule-1 (ICAM-1) content in vascular smooth muscle cells from spontaneously hypertensive rats. Basal cellular levels of methylglyoxal and advanced glycation end products were more than 2-fold higher ($P<0.05$) in cells from hypertensive rats than from normotensive rats. This correlated with levels of oxidative stress and oxidized glutathione that were significantly higher in cells from hypertensive rats, whereas levels of glutathione and activities of glutathione reductase and glutathione peroxidase were significantly lower. Basal levels of locally catalyzed NF-κB p65 and ICAM-1 protein expression were higher in cells from hypertensive rats than from normotensive rats. Addition of exogenous methylglyoxal to the cultures induced a greater increase in oxidative stress and advanced glycation end products in cells from hypertensive rats compared with normotensive rats and significantly decreased the activities of glutathione reductase and glutathione peroxidase in cells of both rat strains. Methylglyoxal activated NF-κB p65 and increased ICAM-1 expression in hypertensive cells, which was inhibited by $N$-acetylcyesteine. Our study demonstrates an elevated methylglyoxal level and advanced glycation end products in cells from hypertensive rats, and methylglyoxal increases oxidative stress, activates NF-κB, and enhances ICAM-1 expression. Our findings suggest that elevated methylglyoxal and associated oxidative stress possibly contribute to the pathogenesis of hypertension. (Hypertension. 2002;39:809-814.)

Key Words: glycation ■ methylglyoxal ■ oxidative stress ■ muscle, smooth, vascular

Methylglyoxal (MG) is a highly reactive dicarbonyl compound. It causes nonenzymatic glycation of proteins to yield irreversible advanced glycation end products (AGEs), leading to cross-linking or degradation of proteins. MG is formed from acetone and the glycolytic intermediates, the triose phosphates, in mammalian cells, including vascular smooth muscle cells (VSMCs). The inactivation of MG is catalyzed by the glyoxalase system that uses glutathione (GSH) as a cofactor. Increased MG production, which in turn gives rise to AGEs, has been linked to the development of chronic complications of diabetes such as microvascular damage (retinopathy or nephropathy). AGEs are also found in human end-stage renal disease; of interest is that increase in AGE formation in nephrectomized rats could be inhibited with an angiotensin II inhibitor. Increased AGE formation has been found in the aorta of stroke-prone spontaneously hypertensive rats (SHR). Furthermore, Vasdev et al reported that the systolic blood pressure was significantly increased 1 week after Wistar Kyoto rats (WKY) were treated with MG (0.2% to 0.8%) or fructose (4%), a precursor of MG, in drinking water. In these studies, increased aldehyde conjugates and microvascular damage were also observed in kidney but not in other tissues such as heart, liver, and muscle. These reports suggest that MG may specifically induce the structural and functional changes of blood vessels. It is unclear whether the levels of MG and MG-induced AGE formation are elevated in VSMCs from SHR and what the role of MG is in the development of essential hypertension. MG can increase oxidative stress by inactivating antioxidant enzymes such as glutathione reductase and glutathione peroxidase because of their glycation. Furthermore, AGEs can interact with their receptors (receptor for AGEs [RAGE] and scavenger receptors), which are present in endothelial cells, VSMCs, and mononuclear phagocytes; activation of these receptors results in strong oxidant production. Oxidative stress in turn activates nuclear factor–κB (NF-κB), which has been observed in human endothelial cells. Activation of NF-κB has downstream effects, such as expression of proinflammatory genes. Recently, an increased activation of NF-κB has been reported in macrophages from deoxycorticosterone acetate hypertensive rats. The interaction of
AGEs with RAGEs promotes expression of cell adhesion molecules—such as vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 (ICAM-1)—on endothelial cells and causes general vasculopathy; this is likely mediated by NF-κB activation caused by the oxidative stress associated with RAGE activation.

The aims of the present study were to investigate whether the levels of MG and AGEs are elevated and whether MG increases oxidative stress, activates NF-κB, and upregulates ICAM-1 in VSMCs from SHR. To this end, cellular MG levels were measured, and basal AGE levels or MG-induced AGE formation was determined and compared in VSMCs from SHR and WKY. Cellular status of oxidative stress was measured in the absence or presence of MG, and the effect of MG on the cellular GSH and related enzyme activities were evaluated. Finally, the effects of MG on ICAM-1; the inhibitory κBs, IκBα and IκBβ; and nuclearly localized NF-κB p65 were also examined.

**Methods**

**VSMC Preparation**

Single aortic VSMCs from SHR or WKY (Charles River, Ste-Constant, Quebec, Canada) were isolated and identified as previously described. Rats were treated in accordance with guidelines of the Canadian Council on Animal Care. Cells between passages 2 to 8 were thawed from frozen stocks and used to establish the cultures. A minimum of 3, and up to 5, separate culture batches was examined for each assay. After different incubation treatments, cell suspensions were sonicated for 30 seconds on ice and then centrifuged at 14 000g at 4°C for 10 minutes. The supernatants were used freshly or stored at −80°C for different enzyme assays. There was no significant difference in the MG-induced cellular oxidative stress or GSH levels among cultured VSMCs from passages 2 to 8.

**Measurement of MG and AGEs**

MG level was measured using the most widely accepted α-phenyl-enediamine (α-PD)-based assay as described by Chaplen et al. VSMC suspension was sonicated and then centrifuged. The supernatant was mixed with α-PD and phosphate buffer. Thereafter, the MG levels were determined by measuring derivatives of MG with α-PD, using high-performance liquid chromatography. As described in our previous study, the formation of AGEs in VSMCs was assessed by their characteristic fluorescence with excitation at 370 nm and emission at 440 nm, using a microplate fluorometer.

**Measurement of Oxidative Stress and Reduced and Oxidized Glutathione Levels**

Oxidative stress was determined as described in our previous studies by examining the oxidation of 5-(and-6)-carboxy-2,7′-dichlorodihydrofluorescein (DCFH) to DCF. The reduced-glutathione (GSH) and oxidized-glutathione (GSSG) levels were determined by reverse-phase high-performance liquid chromatography using ultraviolet detection and precolumn derivatization with 5,5′-dithio-bis(2-nitrobenzoic acid), as described in our previous studies.

**Measurement of Enzyme Activities**

The activities of glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-Red), and glutathione S-transferase (GST) were measured as described in our previous studies, with protein concentrations determined by bicinchoninic acid procedure, using bovine serum albumin as a reference.

**Western Blot Analysis**

Nuclear and cytoplasmic extracts from VSMCs were obtained using the NE-PER Nuclear and Cytoplasmatic Extraction Kit (Pierce). As described in our previous studies, samples containing equal amounts of proteins were separated on polyacrylamide gels using a mini-vertical electrophoresis system. After electrophoresis, proteins were transferred onto polyvinylidene membranes. The membranes were then incubated with anti–NF-κB p65 monoclonal antibody (Transduction Laboratories) at a dilution of 1:750, with anti–IκBα polyclonal antibody (Santa Cruz) at 1:750, with anti–IκBβ polyclonal antibody (Santa Cruz) at 1:750, with anti–ICAM-1 monoclonal antibody (PharMingen) at 1:4000, and with anti–β-actin antibody (Sigma) overnight at 4°C. The proteins were visualized using horse radish peroxidase (HRP)-conjugated secondary antibodies and the Chemiluminescent Substrate Kit (Amersham) and quantified using UN-SCAN-IT gel Automated Digitizing System (version 5.1, Silk Scientific Inc). Protein levels were normalized to the expression levels of β-actin (arbitrary units).

**Data Analysis**

Data are expressed as mean±SEM and analyzed using Student’s t test or ANOVA in conjunction with the Newman-Keuls test where applicable. Differences between groups were considered statistically significant when P<0.05.

**Results**

**Cellular MG Levels and MG-Induced AGE Formation**

The cellular level of MG was significantly higher in VSMCs from SHR than from WKY (Figure 1A). To determine whether an elevated MG level resulted in increased AGE formation, we measured AGE levels in VSMCs from SHR and WKY. Figure 1B shows a significantly higher basal level
of AGEs in VSMCs in SHR VSMCs compared with WKY (note first column in each of the WKY and SHR groups).

After exposure to MG (100 to 500 \(\mu\)mol/L) for 24 hours, marked significant increases in AGE formation were observed in VSMCs from SHR and WKY (n=5). Cellular levels of GSSG were measured after the cells were treated with or without MG or with MG and NAC for 24 hours in SHR and WKY (n=3). Values are given in GSH-equivalents. \(^{*}P<0.05\) vs cells from the same rat strain in the absence of agents; \(^{+}P<0.05\) for SHR vs WKY.

### Effects of MG on Oxidative Stress

The basal level of oxidative stress, as measured by DCFH oxidation, was significantly higher in VSMCs from SHR than from WKY (Figure 2A). A significant increase in the oxidative stress was observed after VSMCs were treated for 24 hours with MG at different concentrations (50 to 500 \(\mu\)mol/L), with SHR showing a significantly greater oxidative stress response to MG treatment compared with that of WKY (Figure 2A). At the concentration of 100 \(\mu\)mol/L, for instance, MG increased the oxidative stress level by 2-fold (from 27±2 to 56±6) in SHR and by 1.57-fold (from 19±1.7 to 30±4.8) in WKY. In both WKY and SHR, VSMC maximal oxidative stress was seen at a MG concentration of 100 \(\mu\)mol/L.

Strong oxidant scavenging results in the oxidation of GSH to GSSG; hence, another measure of oxidative stress is the GSH/GSSG ratio. Under basal culture conditions, GSSG levels were 0.58±0.05 nmol/mg protein in WKY VSMCs and 1.44±0.2 nmol/mg protein in SHR VSMCs (Figure 2B). Basal levels of GSH were significantly lower in SHR (7.5±0.8 nmol/mg protein) than in WKY (17.6±1.0 nmol/mg protein) VSMCs (Figure 3A). Thus, the GSH/GSSG ratios were 30.3 and 5.0, respectively, for WKY and SHR.

The addition of MG (300 \(\mu\)mol/L) for 24 hours resulted in a large increase in GSSG in both SHR and WKY, indicative that MG increases oxidative stress. This interpretation is reinforced by the findings that MG-enhanced GSSG formation was significantly inhibited after the cells from both strains were pretreated with N-acetyl cysteine (NAC) (600 \(\mu\)mol/L) for 30 minutes (Figure 2B). The addition of MG had no effect on the content of GSH in either the SHR or WKY VSMCs until MG levels were at 500 \(\mu\)mol/L (Figure 3A), suggesting that the ability to synthesize GSH and/or reduce GSSG was little affected until MG levels were 500 \(\mu\)mol/L.

We also examined whether MG could affect the activities of 2 critical antioxidant enzymes that are known to be susceptible to glycation inactivation, i.e., GSH-Px and GSSG-Red. GSH-Px activity is significantly lower in VSMCs from
Expression of ICAM-1 of GST in VSMCs from SHR and WKY (Figure 3D). There were no significant differences in basal or MG-induced activities of VSMCs from both SHR and WKY (Figure 3C). There were no differences in cytoplasmic levels of IkBα in the absence or the presence of MG in VSMCs from SHR and WKY (P>0.05, n=4 for each group; data not shown).

The basal expression level of ICAM-1 protein was significantly higher in VSMCs from SHR than from WKY (Figure 4C). The expression levels of ICAM-1 were increased in VSMCs from both rat strains after the treatment with MG (300 μmol/L) for 24 hours, but with a greater increase in SHR (Figure 4C). A 30-minute pretreatment with NAC (600 μmol/L) before MG addition significantly decreased the MG-induced expression of ICAM-1 in VSMCs from SHR (Figure 4C).

Oxidative stress has been linked to the pathological processes of hypertension. We have recently reported the age-related increase in vascular oxidative stress in association with the rise of blood pressure in SHR. The observations from our previous studies, and those of others, indicate that free radicals, such as superoxide anion, increase IP3 formation and decrease cellular cGMP in SHR VSMCs, thereby causing a significant vasoconstriction and increased peripheral resistance. The cellular level of oxidative stress is dependent on the balance of oxidant production and scavenging. Enhanced oxidative stress occurs because of either overproduction of strong oxidants or decreased strong oxidant-scavenging capacity, such as an impaired GSH system.

In the present study, we demonstrate for the first time that VSMCs from SHR have a significantly greater content of GST in VSMCs from SHR and WKY (Figure 3D). There were no significant differences in basal or MG-induced activities of GST in VSMCs from SHR and WKY (Figure 3D).

Discussion

Effects of MG on Nuclearily Localized NF-κB and Expression of ICAM-1

The basal level of nuclear NF-κB p65 in VSMCs from SHR was significantly higher than that in the cells from WKY (Figure 4A). After treatment with MG (300 μmol/L) for 3 hours, a large significant increase in the nuclear level of NF-κB p65 was observed in VSMCs from SHR but not from WKY. Moreover, the MG-induced increase in nuclearily localized NF-κB p65, indicating activation of the NF-κB, was partially attenuated by 30-minute pretreatment with NAC (P<0.05, 1-tailed Student’s t test), indicating that oxidative stress is likely involved in the MG-induced activation of NF-κB in VSMCs of SHR.

To further define the mechanisms of the MG-induced NF-κB activation, the cytoplasmic levels of IkBα (IkBα and IkBβ) were determined after the cells were treated with MG. In VSMCs from SHR, the basal cytoplasmic level of IkBα was significantly higher compared with that from WKY (Figure 4B). In SHR VSMCs, a significant decrease in the IkBα level was observed after the treatment of cells with MG (300 to 500 μmol/L) or H2O2 (300 μmol/L) for 3 hours (Figure 4B). In contrast, neither MG nor H2O2 had an effect on IkBα in VSMCs from WKY (Figure 4B). There were no differences in nuclear levels of IkBα in the absence or the presence of MG in VSMCs from SHR and WKY (P>0.05, n=4 for each group; data not shown).

The basal expression level of ICAM-1 protein was significantly higher in VSMCs from SHR than from WKY (Figure 4C). The expression levels of ICAM-1 were increased in VSMCs from both rat strains after the treatment with MG (300 μmol/L) for 24 hours, but with a greater increase in SHR (Figure 4C). A 30-minute pretreatment with NAC (600 μmol/L) before MG addition significantly decreased the MG-induced expression of ICAM-1 in VSMCs from SHR (Figure 4C).

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The possible mechanisms by which MG enhanced oxidative stress include (1) MG-enhanced AGE formation that in turn can activate RAGE, thereby promoting superoxide anion radical production as previously shown by others,24 and (2) the MG-induced impairment of GSH-Px and GSSG-Red in VSMCs from SHR. The evidence for the former is that MG caused an increase in GSSG content in both WKY and SHR VSMCs, and this increase in GSSG was counteracted by NAC. MG-induced impairment of GSSG-Red and GSH-Px would also result in increased oxidative stress because GSSG-Red plays an important antioxidant defense role by reducing GSSG to GSH, whereas GSH-Px scavenges peroxides, which can be converted to very reactive free radicals by using GSH as the electron donor.25 The MG-induced impairment of enzymatic activity was not seen with GST, suggesting that GSSG-Red and GSH-Px activities were inactivated by glycation, because these proteins are known to be susceptible to glycation inactivation.8,9

Addition of MG to the medium had no effect on GSH content until MG concentrations were increased to 500 μmol/L. This decrease does not seem to be caused by cell damage because GST activity was unaffected by this concentration of MG. Furthermore, visual inspection of the cultures did not suggest that there was damage. Because MG at 100 to 300 μmol/L decreased GSSG-Red activity, which correlated with the increased GSSG formation but had no effect on GSH concentrations, it suggests that the GSH synthetic enzymes are unaffected by MG at concentrations up to 300 μmol/L.

We next questioned whether an elevated MG in VSMCs might be involved in activation of NF-κB. Activation of the transcription factor NF-κB and induction of the associated genes have been suggested to be responsible for inflammatory vascular responses.26 In our studies, high basal levels of nuclearly localized NF-κB p65 with associated increased ICAM-1 expression was found in SHR VSMCs. An increased basal cytoplasmic level of 1κBα (inhibitory protein for NF-κB) was also observed, which likely represents an adaptive response to an enhanced activation of NF-κB in VSMCs from SHR because the 1κBα gene has κB elements in its promoter region.27 There were no differences in the cytoplasmic levels of 1κBβ between SHR and WKY VSMCs. Activation of NF-κB involves degradation of 1κBα, followed by translocation of the p65/p50 heterodimer to the nucleus.28 We found that within 3 hours of treatment of SHR VSMCs with 300 μmol/L MG, there was a significant increase in the nuclear level of NF-κB p65 and a decrease in cytoplasmic level of 1κBα protein (but no change in the cytoplasmic levels of 1κBβ); this is indicative of NF-κB activation. Although 300 μmol/L MG did cause a small but significant rise in cellular oxidative stress as measured by DCFH oxidation and GSSG-Red and GSH-Px inactivation in WKY VSMCs, it did not result in NF-κB activation, as no change was seen in nuclearly localized NF-κB p65 or in cytoplasmic 1κBα. The activation of NF-κB in SHR VSMCs was associated with a large increase of ICAM-1 24 hours after exposure to 100 to 300 μmol/L MG. A smaller but significant increase in ICAM-1 was also seen in WKY VSMCs 24 hours after exposure to 300 μmol/L but not to 100 μmol/L MG.

The activation of NF-κB by oxidants such as superoxide and H2O2 has been observed in human endothelial cells.29,30 In the present study, we show that hydrogen peroxide can induce degradation of 1κBα protein in SHR VSMCs but not in WKY VSMCs. Several lines of evidence indicate that MG-induced activation of NF-κB is likely mediated through production of strong oxidants. First, MG caused a significant increase in cellular oxidative stress and increased GSSG content that was counteracted by NAC. Second, MG caused a significantly elevated activation of NF-κB only in SHR VSMCs, cells with a much higher level of endogenous oxidative stress than that of WKY VSMCs. Third, NAC pretreatment decreases the extent of MG-induced activation of NF-κB in SHR VSMCs and decreased the extent of the associated induction of ICAM-1.

In summary, our studies suggest that elevated cellular levels of MG and the MG-induced multiple alterations in VSMC functions may play a role in the pathogenic process of hypertension in SHR.

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