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Effects of Oxidative Stress on the Expression of Aldose Reductase in Vascular Smooth Muscle Cells

Hyo Jung Kim, Ki Churl Chang, and Han Geuk Seo

Department of Pharmacology, Cardiovascular Research Institute, College of Medicine, Gyeongsang National University, Chinju 660-751, Korea

Oxidative stress and methylglyoxal (MG), a reactive dicarbonyl metabolites produced by enzymatic and non-enzymatic reaction of normal metabolism, induced aldose reductase (AR) expression in rat aortic smooth muscle cells (SMC). AR expression was induced in a time-dependent manner and reached at a maximum of 4.5-fold in 12 h of MG treatment. This effect of MG was completely abolished by cyclohemide and actinomycin D treatment suggesting AR was synthesized by *de novo* pathway. Pretreatment of the SMC with *N*-acetyl-L-cysteine significantly down-regulated the MG-induced AR mRNA. Furthermore, DL-Buthionine-(*S,R*)-sulfoximine, a reagent which depletes intracellular glutathione levels, increased the levels of MG-induced AR mRNA. These results indicated that MG induces AR mRNA by increasing the intracellular peroxide levels. Aminoguanidine, a scanvenger of dicarbonyl, significantly down-regulated the MG-induced AR mRNA. In addition, the inhibition of AR activities with statil, an AR inhibitor, enhanced the cytotoxic effect of MG on SMC under normal glucose, suggesting a protective role of AR against MG-induced cell damages. These results imply that the induction of AR by MG may contribute to an important cellular detoxification of reactive aldehyde compounds generated under oxidative stress in extrahepatic tissues.

Key Words: Oxidative stress, Aldose reductase, Diabetic complication, Methylglyoxal, Polyol pathway

INTRODUCTION

Aldose reductase (EC 1. 1. 1. 21; AR), a member of NADPH-dependent aldo-keto reductase family, has been studied for its implication in the development of various diabetic complication. AR catalyzes the reduction of glucose to sorbitol with NADPH as a cofactor, which is converted to fructose by sorbitol dehydrogenase with NAD^+. This alternative route of glucose metabolism is accelerated under hyperglycemia and known as so-called polyol pathway. Activation of the polyol pathway under hyperglycemic condition has been implicated to contribute to the development of diabetic complication. AR exhibits broad substrate specificity for a variety of aldehydes including the aldehyde form of glucose and catecholamine metabolites. 4-hydroxy-2,3-trans-nonenal (HNE), a reactive aldehydes produced by peroxidation of polyunsaturated fatty acids (Spycher et al, 1996), hydrogen peroxide (Spycher et al, 1997), and acrolein (Kolb et al, 1994) are also reduced by AR. Recently, we found that AR mRNA is induced in a dose- and time-dependent manner by nitric oxide in rat aortic smooth muscle cells (SMC) (Seo et al, 2000). These results suggested that AR play a role for modulation of vascular remodeling in diseases condition such as diabetes, hypertention, atherosclerosis. Other group also reported the induction of AR gene by HNE and hydrogen peroxide, suggesting a potential roles of AR for cellular antioxidant defense mechanism against oxidative stress-induced cell death (Spycher et al, 1997).

Methylglyoxal (2-oxopropanol; MG) is formed by the enzymatic and non-enzymatic reaction of normal

Corresponding to: Han Geuk Seo, Department of Pharmacology, College of Medicine, Gyeongsang National University, 92 Chilam-Dong, Chinju 660-751, Korea. (Tel) 82-55-751-8742, (Fax) 82-55-759-0609, (E-mail) hgseo@nongae.gsnu.ac.kr

metabolism (Lyles & Chalmers, 1992) and glycolysis bypass (Phillips & Thornalley, 1993). The major source of MG formation in physiological systems is the nonenzymatic fragmentation of triosephosphate (Phillips & Thornalley, 1993). The rate of MG formation in normal healthy volunteer is approximately 120 μM/day and is increased under hyperglycemic conditions and by addition of triosephosphate precursor such as glucose, fructose (Thornalley, 1988; Phillips & Thornalley, 1993). The high serum level of MG was observed with 5-6- and 2-3-fold increase in patients with insulin-dependent diabetic mellitus and non-insulin-dependent diabetic mellitus, respectively (McLellan et al, 1994). MG is catalyzed to D-lactate via the intermediate *S*-D-lactoylglutathione by the glyoxalase system using reduced glutathione as a cofactor (Thornalley et al, 1990). But, it is now receiving renewed interest as a substrate for AR, although the detoxification rate of MG by AR is 10-40-fold lesser than glyoxalase system in almost human tissues except for kidney medulla (Grimshaw, 1992; Vander Jagt et al, 1992).

Here, we report that oxidative stress and MG induce expression of AR gene, which is implicated in a defense mechanism against oxidative damages. To explore the roles of AR on oxidative stress and the cytotoxic effects of MG, SMC was treated with statil, a AR-specific inhibitor. The inhibition of AR activity enhanced the cytotoxic effect of MG on SMC, suggesting a protective role of AR against MG-induced cell damages. These results indicate that AR functions as a detoxification system that degrades toxic aldehydes generated during hyperglycemic condition or normal cellular metabolism.

METHODS

Reagents

Cycloheximide, actinomycin D, aminoguanidine, SNAP, TPA, methylglyoxal (MG), MTT, and HNE were from Sigma Chemical Co. (St. Louis, MO, USA). BSO and NAC were from Aldrich Chem. Co. (Milw, USA). Goat anti-rabbit IgG antibody was Jacson Immuno Research. Anti-rat AR antibody and Statil [3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazine-1-yl-acetic acid; ICI 128436] were a kind gift from Dr. C. Yabe-Nishimura (Kyoto Prefectural Univ. of Medicine, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from ICN Pharmaceuticals. Other reagents were of the highest grade available.

Cell culture

Aortic smooth muscle cells (SMC) from rat were isolated from free-floating explants of aorta as previously described (Seo et al, 2000). Briefly, thoracic aorta dissected from adult male Sprague-Dawley rat was cut longitudinally and the endothelial cells were removed. The isolated medial membranes were cut into small pieces and incubated for a day. After supplementation with fresh medium, the tissues were again incubated for a few days. SMC was removed by trypsinization and maintained in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% heat-inactivated fetal bovine serum at 37°C under an atmosphere of 95% air and 5% CO_2.

Northern blot analysis

Total RNA was isolated by extraction with acid guanidium thiocyanate-phenol-chloroform as described (Chomczynski & Sacchi, 1987) and quantified by measuring the absorbance at 260 nm. Five micrograms of total RNA, heat-denatured at 65°C for 15 min in gel running buffer [40 mM 3-N(morpholino) propanesulfonic acid, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0] containing 50% formamide, was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred onto a Hybond-N$^+$ nylon membrane and immobilized. After hybridization with a ^{32}P-labeled rat AR cDNA probe using QuikHyb solution at 68°C, the membrane was washed and the radioactivity on the membrane was detected by autoradiography. The blot was stripped and rehybridized with a murine GAPDH cDNA probe. The radioactivity of the signals was quantified by a Bio-Rad image analyzer and plotted as a percent control of AR to GAPDH mRNA ratios, respectively

Aldose reductase activity

Cells treated with 500 μM MG for the indicated time periods were washed with ice-cold PBS (pH 7.4) and harvested by scraping. The cell suspension was homogenated with a glass dounce homogenizer in 20 mM sodium phosphate buffer (pH 7.0) containing 2

mM dithiothreitol, 5 μM leupeptin, 2 μM pepstatin, and 20 μM phenylmethylsulfonyl fluoride. After centrifugation of the homogenate for 10 min at 2,000 $\times$ g, the supernatant fraction was supplied for the enzyme analyses. The activity of AR was determined in a reaction mixture containing 0.1 M sodium phosphate buffer (pH 6.2), 150 μM NADPH, 10 mM DL-glyceraldehyde, and the enzyme solution in a total volume of 1 ml (Nishimura et al, 1991). The activity was measured spectrophotometrically by estimating NADPH oxidation from a decrease in absorbance at 340 nm.

Western blot analysis

Thirty micrograms of protein in the supernatant fraction of the cell homogenate was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane for 1 h at 20 V. The membrane was blocked by incubation with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h at room temperature, and reacted with anti-rat AR antibody in TBS containing 1% BSA and 0.05% Tween-20 for overnight at 4°C. Then, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG antibody diluted to 1 : 3000 in TBS containing 1% BSA and 0.05% Tween-20 for 2 h at room temperature. After washing, immunoreactive bands were detected with ECL kits.

Cytotoxicity assay

Cell viability was determined according to the method previously described (Seo et al, 2000). Briefly, cells were cultured in 24-well plates for overnight and and various concentrations of MG were added to the medium of cells with or without statil. After 24 h incubation, MTT (final 0.1 mg/ml) was added to the medium of SMC and incubated for an additional 4 h. The formazan crystals formed in the living cells were solubilized in acidified isopropanol and measured spectrophotometrically at 570 with background subtraction at 650 nm.

Statistical analysis

The paired Student's *t* test was used to compare the significance of the difference between data. All data are expressed as means $\pm$ S.D.

RESULTS

Induction of AR expression by oxidants and MG

Using northern blot analysis, we examined the effects of oxidants and MG on transcriptional levels of AR in SMC. Oxidants such as 1 mM SNAP, 10 μM HNE significantly induced the AR mRNA (Fig. 1). After treatment of SMC with 500 μM MG, the levels of AR mRNA was also increased at 6 h and reached a maximum 4.5-fold induction at 12 h (Fig. 2A). The enhanced AR transcripts declined to the base line after 24 h (Fig. 2A). Whether the accumulation of AR mRNA by MG results in elevation of activity and protein levels of AR, we examined the activity and protein levels in MG-stimulated SMC. A significant enhancement of AR activity was detected after 24 h incubation with MG (Fig. 2B). Elevation of AR

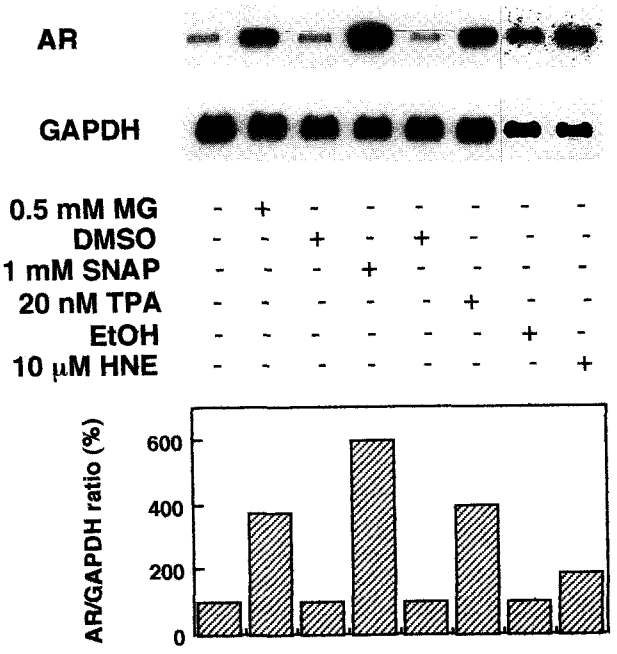

Fig. 1. Induction of aldose reductase (AR) mRNA by several oxidative agents SMC was incubated with 500 μM MG, 20 nM TPA, 1 mM SNAP, 10 mM HNE for 12 h. Total RNA was extracted and fractionated by 1% agarose gel electrophoresis. Fractionated RNA was transferred onto membrane and hybridized with AR cDNA probe. Each membrane was stripped and rehybridized for GAPDH as an internal control. The radioactivity of the signals was quantified by a Bio-Rad image analyzer and plotted as a percent control of AR to GAPDH mRNA ratios, respectively.

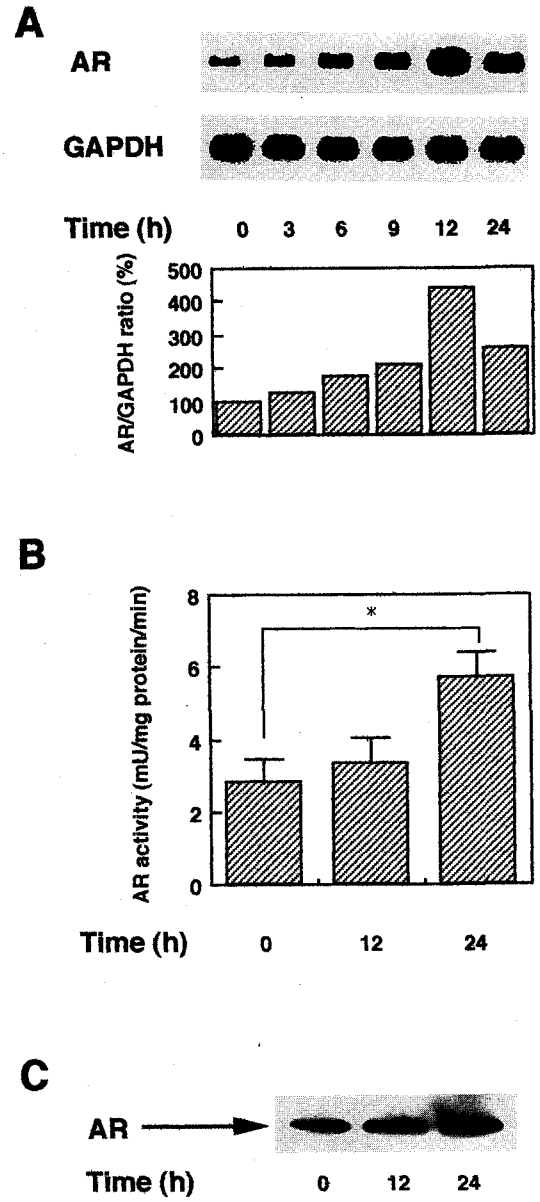

Fig. 2. Effects of methylglyoxal (MG) on the expression of aldose reductase (AR). Time-dependent induction of AR mRNA (A), AR activities (B), and AR proteins in SMC incubated with 500 μM MG for indicated time. Total RNA was extracted and subjected to northern blot analysis as described in *Methods* (A). The enzyme activity of the supernatant fraction of the homogenate was measured spectrophotometrically. Vertical columns represent the means ± S.D (n=3), $*P<0.01$ (B). The protein levels of AR in the supernatant fraction was analyzed by western blotting with anti-rat AR antibody as described in *Methods* (C).

activity was in accord with the levels of protein determined by western blot analysis (Fig. 2C). These results indicate that AR mRNA enhanced by MG

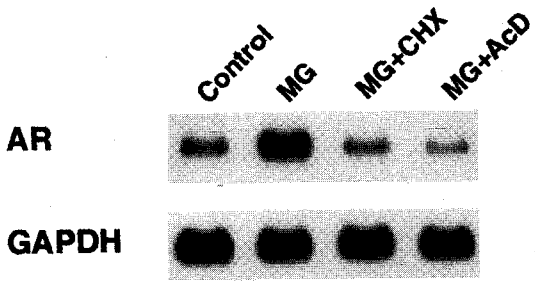

Fig. 3. Effects of actinomycin D (AcD) and cycloheximide (CHX) on the methylglyoxal-induced aldose reductase mRNA expression. SMC was incubated with 500 μM methylglyoxal for 13 h in the absence or presence of AcD (4 μM) or CHX (40 μg/ml). Total RNA was extracted and subjected to northern blot analysis as described in *Methods*.

stimulation was followed by an increase in enzyme activity and protein levels with a time lag of several hours.

Effects of cycloheximide, actinomycin D, and aminoguanidine on the enhancement of AR mRNA levels by MG

To examine whether enhancement of AR gene expression by dicarbonyl involves the synthesis of new proteins, the effects of actinomycin D (AcD) and cycloheximide (CHX) on the expression of AR mRNA were investigated. Induction of AR mRNA during treatment with the dicarbonyl was completely abolished in the presence of actinomycin D or cycloheximide (Fig. 3). These results indicate that *de novo* mRNA synthesis as well as *de novo* synthesis of proteins that act on the AR gene promotor are essential to induction of AR mRNA expression in MG-treated SMC.

We also evaluated the effects of aminoguanidine on the induction of AR mRNA by MG. As shown in Fig. 4, aminoguanidine inhibited MG-induced AR mRNA expression in SMC. This suggests that MG may directly enhance AR mRNA in SMC.

Involvement of reactive oxygen species in MG-induced AR mRNA expression

It has been reported that MG produces reactive oxygen species (ROS) (Kalapos, et al, 1993; Yim et al, 1995). To clarify the role of ROS during induction of AR mRNA by MG, the effects of NAC and

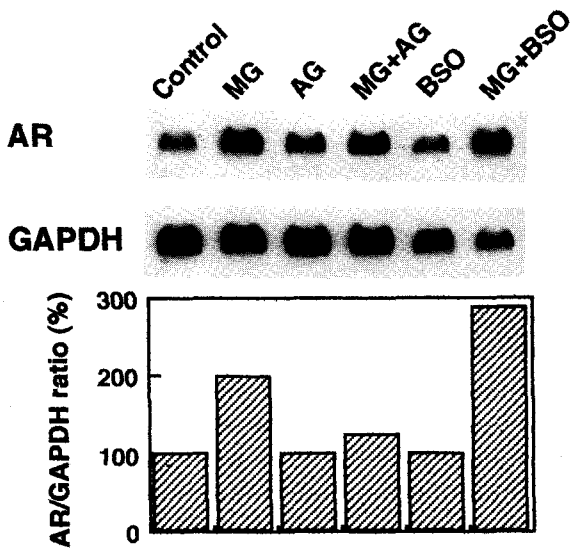

Fig. 4. Effects of aminoguanidine (AG) and BSO [DL-Buthionine-(*S,R*)-sulfoximine] on enhancement of aldose reductase mRNA level by methylglyoxal (MG). SMC was incubated with 500 mM MG for 13 h in the absence or presence of AG (5 mM) or BSO (10 μM). Total RNA was extracted and subjected to northern blot analysis as described in *Methods*.

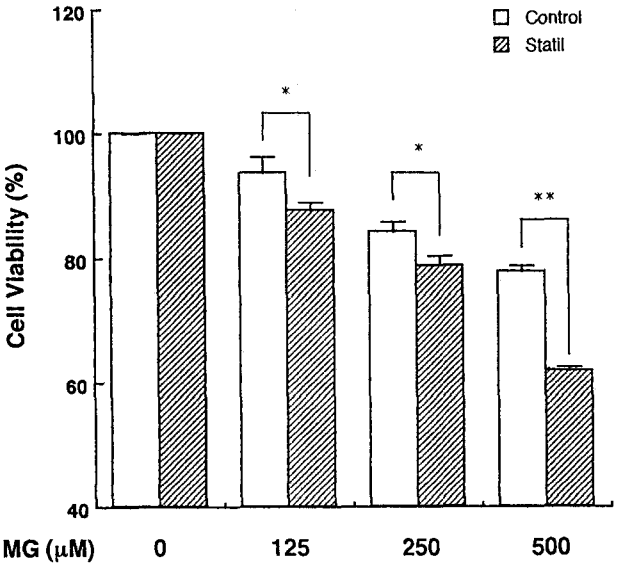

Fig. 6. Effects of methylglyoxal (MG) and aldose reductase inhibition on cell viability of SMC determined by MTT assay. Cells were incubated with various concentrations of MG in the absence or presence of 50 μM statil for 24 h. The vertical columns represent the means ± S.D. (n=4). $*P<0.05$, $**P<0.01$, compared with control.

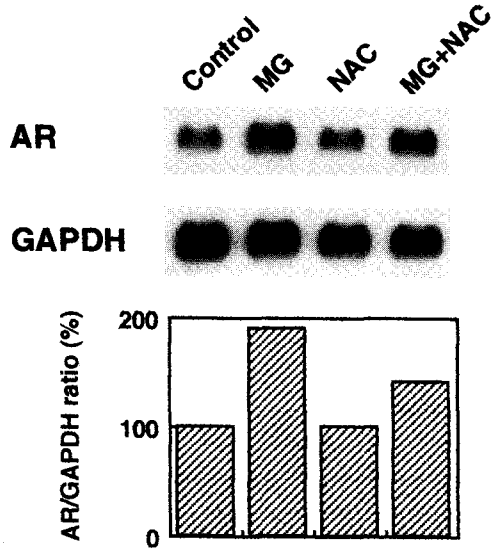

Fig. 5. Effects of N-acetyl-L-cysteine (NAC) pretreatment on the methylglyoxal-induced aldose reductase mRNA expression. SMC was preincubated in the medium containing 20 mM NAC for 24 h. SMC was rinced with PBS (−) twice and subsequently incubated in the fresh medium containing 500 μM MG for another 13 h. Total RNA was extracted and subjected to northern blot analysis as described in *Methods*.

DL-Buthionine-(*S,R*)-sulfoximine (BSO) on the induction of AR gene levels were examined. The SMC were preincubated with NAC (20 mM) or BSO (10 μM) for 24 h, and then the cells were treated with 0.5 mM MG for an additional 12 h. NAC pretreatment decreased the enhancement of MG- induced AR mRNA (Fig. 5). In contrast, pretreatment of SMC with BSO increased the levels of MG- induced AR mRNA (Fig. 4). These results, therefore, indicated that production of peroxides by MG is involved in the induction of AR in SMC.

Effect of AR inhibition on viability of SMC treated with MG

To evaluate the role of AR induction in SMC treated with MG, we performed the cell viability assay with Statil. The viability of SMC treated with various concentartion of MG was decreased in a dose-depenednt manner (Fig. 6). This MG-mediated cytotoxicity was increased in the presence of AR inhibitor. This finding suggest that the enhancement of AR expression by MG may protects the cells from the toxic effects of aldehyde compounds such as MG or MG-mediated end products.

DISCUSSION

The elevation of oxidative stress and MG generation during hyperglycemia such as diabetes mellitus is widely ap proved to be related to diabetic complications (Che et al, 1997). Recently, it has been reported that aldose reductase (AR) is induced by several oxidant such as hydrogen peroxide, HNE, and nitric oxide (Spycher et al, 1997; Seo et al, 2000). The present study was undertaken in an attempt to evaluate the effect of oxidative stress and MG on the expression levels of AR in SMC and to explore the role of AR under oxidative conditions.

We have shown that oxidative stress significantly induce AR mRNA and the elevation of AR mRNA levels by MG involved the *de novo* synthesis of new proteins that act on the AR gene promotor in SMC. Although the concentration of MG need to induce the AR was above 100 μM and appears to be slightly higher than concentrations under diabetic condition (5~10 μM). It has been published that the actual concentration of MG by judged by the ^{14}C-methylglyoxal incorporation was much lower (in the range of 2~4 μM) than added concentration (100~400 μM), indicating that MG acts at physiologically relevant concentrations (Pfeifer et al, 1996; Che et al, 1997). Lower levels of MG, as seen in patients with diabetes, would be sufficient to evoke the induction of AR mRNA in the cells.

A significant increase in intracellular reactive oxygen species (ROS) was reported in SMC (Che et al, 1997) and hepatocytes (Kalapos et al, 1993) treated with MG. It is also reported that MG induce apoptotic cell death in macrophage-derived cell lines through the production of ROS (Okado et al, 1996). Therefore, chronic exposure to high concentration of this dicarbonyl may be a causative factor in oxidative conditions such as diabetes. ROS, in fact, have been implicated in a lot of diseases including diabetic complication, aging, cancer (Halliwell et al, 1992; Baynes, 1991). The present investigation demonstrated that MG significantly increased intracellular peroxides prior to the elevation of AR mRNA levels in this study. The elevation of intracellular ROS levels by MG has several possible routes. One is that ROS are generated during the glycation reaction of amino acids by MG (Yim et al, 1995). The other one is that the elimination of ROS is not sufficient because the glutathione content of cells is depleted during MG metabolism by the glyoxalase system. Thus, MG elicits cytotoxic effects by increasing the production of intracellular peroxides during the reaction with cellular components such as amino acid or proteins.

The induction of AR expression by MG treatment was significantly suppressed when SMC was preincubated with NAC, a glutathione precursor and thiol antioxidants (Natoli et al, 1997). The effects of NAC pretreatment on the MG-induced AR expression indicated that MG may generate peroxides to exert its action on cells. In line with this result, treatment of BSO increased the levels of MG-induced AR mRNA. These results provide strong evidence that MG triggers AR mRNA induction in SMC by creating a state of oxidative stress. Our results implicated that MG elucidate physiological signalling and cytotoxic effects through producing the ROS.

MG is known to contribute to cell damages under hyperglycemic conditions such as diabetes either as a direct toxin or as a precursor for advanced glycation reaction (Beisswenger et al, 1999). To clarify the cytotoxic effects of MG in SMC, we investigated the effects of MG on viability of cultured rat SMC. As shown in Fig. 6, addition of MG in the culture medium of SMC significantly decreased cell viability on a dose-dependent manner. The cytotoxic effect of MG was increased when the activity of AR was suppressed by the inclusion of statil. This result implicated that the induction of AR activity by MG protected cells from MG-generated cell damages. It was also reported that such toxic aldehydes as HNE are good substrate for AR (Vander Jagt et al, 1995) and also induce the AR expression (Spycher et al, 1997; Rittner et al, 1999). The enhancement of AR by MG may, therefore, be a consequence of an adaptive response of SMC to detoxify cytotoxic carbonyl compound. This cytoprotective roles of AR could be attributed to the wide substrate specificity of this enzyme not only glucose but also for reactive aldehyde compounds generated in the cells subjected to oxidative stress. The underlying mechanisms on protective roles of AR were proposed that the enzyme can degrades toxic aldehydes formed during oxidative damages by exposure on external oxidants or inflammatory reactions (Vander Jagt et al, 1995; Spycher et al, 1997; Seo et al, 2000). Although the physiological roles of AR are not fully elucidated, an association with the development of diabetic complications has been suggested (Yabe-Nishimura et al, 1998). However, no therapeutic benefits from AR inhibition have

not been demonstrated in experimental therapies (Pfeifer et al, 1996). Our present studies, therefore, indicate that AR functions as a detoxification enzyme that degrades toxic aldehydes formed during oxidative stress.

Taken together, the present studies are the first evidence that MG induce AR mRNA expression and activity enhancement through ROS production by provoking oxidative stress in cultured rat vascular smooth muscle cells. The cells exposed to oxidative stress induce AR to protect the cells from the cytotoxic actions of reactive aldehyde compounds. The present findings may contribute to new physiological function of AR as a detoxifying enzyme for toxic aldehyde such as MG in modulating vascular homeostasis.

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