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Substrate-Induced Up-Regulation of Aldose Reductase by Methylglyoxal, a Reactive Oxoaldehyde Elevated in Diabetes

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ABSTRACT

Methylglyoxal (MG), a reactive dicarbonyl produced during glucose metabolism, induced a dose- and time-dependent increase in aldose reductase (AR) mRNA level in rat aortic smooth muscle cells (SMCs). AR has been implicated in the pathogenesis of diabetic complications, whereas the clinical efficacy of AR inhibitors has not been unequivocally proven. The enzyme catalyzes the reduction of glucose in the polyol pathway, as well as that of MG, which is known to be a preferred substrate of AR. A maximum of 4.5-fold induction of AR mRNA by MG was accompanied by elevated enzyme activity and protein levels and was completely abolished in the presence of cycloheximide or actinomycin D. Pretreatment of SMCs with *N*-acetyl-L-cysteine significantly suppressed the MG-induced AR expression, whereas DL-buthionine-(*S,R*)-sulfoximine further augmented the MG-induced increase in AR mRNA level. Intracellular levels of reactive oxygen species determined using 2′,7′-dichlorofluorescein diacetate were significantly elevated in SMCs treated with MG, suggesting the involvement of oxidative stress in this process. However, inconsistent with our previous findings on oxidative stress-induced up-regulation of AR, the inhibition of extracellular signal-regulated kinase by 2′-amino-3′-methoxyflavone (PD98059) did not affect MG-induced AR expression, whereas blockade of the p38 mitogen-activated protein kinase pathway by 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazol (SB203580) significantly suppressed the induction. The cytotoxic effect of MG on SMCs was significantly enhanced in the presence of the AR inhibitor ponalrestat, indicating a protective role of AR against MG-induced cell damage. Taken together, these observations indicated that substrate-induced induction of AR by MG during hyperglycemic conditions may hinder vascular remodeling and accelerate the development of vascular lesions in diabetes.

Methylglyoxal (2-oxopropanol; MG) is a highly reactive dicarbonyl that reacts on and modifies cellular proteins to form cross-links of amino groups, generating so-called advanced glycation end products. This modification has been implicated in the development of diabetic complications (Odani et al., 1999). MG is known to have multiple metabolic origins. It is formed by the enzymatic and nonenzymatic elimination of phosphate from triose phosphate (Pompliano et al., 1990) and by oxidation of aminoacetone formed in the catabolism of threonine (Reichard et al., 1986; Lyles and Chalmers, 1992). Under physiological conditions, the major source of MG is the nonenzymatic fragmentation of triose phosphate (Phillips and Thornalley, 1993), indicating that increased levels of precursors of triose phosphate, such as glucose or fructose, would augment MG formation. In fact, the rate of MG formation during hyperglycemic conditions was reported to increase (Phillips and Thornalley, 1993), and high serum levels of MG were observed in patients with either type 1 or type 2 diabetes (McLellan et al., 1994).

Two enzyme pathways are known to participate in the catabolism of MG. The glyoxalase pathway converts MG to D-lactate via the intermediate (*S*)-D-lactoylglutathione using reduced glutathione as a cofactor (Thornalley, 1990). The other pathway, currently attracting renewed interest, is mediated by aldose reductase (EC 1.1.1.21; AR), which converts MG to acetol using NADPH as a cofactor (Vander Jagt et al., 1992). In the liver, in which high levels of glutathione and low levels of AR are present, the glyoxalase system is the major catabolic pathway of MG. On the other hand, the AR

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ABBREVIATIONS: AR, aldose reductase; MG, methylglyoxal; SMCs, aortic smooth muscle cells; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SSC, standard saline citrate; BSA, bovine serum albumen; TBS, Tris-buffered saline; BSO, DL-buthionine-(*S,R*)-sulfoximine; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazol; PD98059, 2′-amino-3′-methoxyflavone; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NAC, *N*-acetyl-L-cysteine; AGE, advanced glycation end products; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; GSH, glutathione.

pathway becomes more significant in other tissues, including the cardiovascular-renal system, in which AR is abundant (Gui et al., 1995; Nishimura et al., 1988; Nishimura et al., 1993; Vander Jagt et al., 2001). Also, a recent report demonstrated that over-expression of the gene encoding AR increases the tolerance against MG in *Saccharomyces cerevisiae* (Aguilera and Prieto, 2001).

AR, designated AKR1B1 as a member of NADPH-dependent aldo-keto reductase family (Jez et al., 1997), has been studied for its involvement in the pathogenesis of various diabetic complications (Kinoshita and Nishimura, 1988; Yabe-Nishimura, 1998). AR catalyzes the reduction of the aldehyde form of glucose to sorbitol, which is subsequently converted to fructose by NAD^+-dependent sorbitol dehydrogenase. This alternative route of glucose metabolism, known as the polyol pathway, is accelerated during hyperglycemia to elicit impaired ion transport (Greene, 1988), altered redox status (Tilton et al., 1992), deranged osmotic balance (Kinoshita and Nishimura, 1988), and enhanced oxidative stress (Gonzalez et al., 1986). Although numerous AR inhibitors have been developed as new therapeutic agents for diabetic complications, their clinical efficacy has not been proven unequivocally. On the other hand, AR exhibits broad substrate specificity for a variety of aldehydes, including catecholamine and steroid metabolites such as 3,4-dihydroxyphenylglycoaldehyde, isocorticosteroids, isocaproaldehyde, progesterone, and 17α-hydroxyprogesterone (Yabe-Nishimura, 1998). Along with MG, acrolein and 4-hydroxy-2,3-*trans*-nonenal, reactive aldehydes of exogenous origin or produced during lipid peroxidation, are also good substrates for AR. Under physiological conditions, AR may therefore serve as an extrahepatic detoxification enzyme against endogenous and xenobiotic aldehydes.

In our previous publication, we reported that nitric oxide up-regulates AR expression in aortic smooth muscle cells and in a macrophage cell line (Seo et al., 2000). Because suppression of AR activity augmented the cytotoxic effect of nitric oxide, the increased expression of AR seemed to serve as a self-defense mechanism against the nitric oxide–induced cell death. These findings therefore suggested a potential role of AR as a detoxification enzyme in modulating the survival of cells exposed to various noxious stimuli. To elucidate the cytoprotective role of AR against reactive aldehydes elevated in the presence of high ambient glucose concentrations, we investigated the effects of MG on the expression of AR and cell viability. Here, we report that MG up-regulated AR gene expression in vascular smooth muscle cells, and the cytotoxic effect of MG was augmented when the enzyme activity was suppressed by an AR inhibitor. Vascular remodeling, propagated not only by cellular proliferation and migration but also by apoptosis, is impaired when cell death is hindered. These results therefore suggest that AR activity may be up-regulated in the vascular tissue of diabetic patients, and this may act against vascular remodeling to accelerate the development of vascular lesions in diabetes.

Materials and Methods

Reagents. Methylglyoxal, aminoguanidine, actinomycin D, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and cycloheximide were obtained from Sigma-Aldrich (St. Louis, MO). DL-Buthionine-(*S,R*)-sulfoximine (BSO) and *N*-acetyl-L-cysteine (NAC) were from Aldrich Chemical Company (Milwaukee, WI). 4-(4-Fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole (SB203580), 2′-amino-3′-methoxyflavone (PD98059), and 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) were from Calbiochem (San Diego, CA). Goat anti-rabbit IgG antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Ponalrestat [3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazine-1-yl-acetic acid; ICI 128 436] was a kind gift from Dr. T. G. Flynn (Queen's University, Kingston, Canada). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Other reagents were of the highest grade available.

Cell Culture. Rat aortic smooth muscle cells (SMCs) were isolated from free-floating explants of aortae as described previously (Seo et al., 2000). Briefly, thoracic aortae dissected from adult male Sprague-Dawley rats were cut longitudinally, and the endothelial cells were removed. The isolated medial membrane was cut into small pieces and incubated for 1 day. After supplementation with fresh medium, the tissue was again incubated for 2 to 3 days. SMCs were 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 in an atmosphere of 95% air and 5% CO_2.

Northern Blot Analysis. Total RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform as described previously (Chomczynski and Sacchi, 1987) and quantified by measuring the absorbance at 260 nm. Aliquots of 5 μg of total RNA, heat-denatured at 65°C for 15 min in gel-running buffer [40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0] containing 50% formamide, were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. The size-fractionated RNA was transferred onto Hybond-N nylon membranes (Amersham, Uppsala, Sweden) overnight by capillary action and was immobilized using a UV Stratalinker (Stratagene, La Jolla, CA). After hybridization with a ^{32}P-labeled rat AR cDNA probe at 68°C in QuikHyb solution (Stratagene), the membranes were washed once with 2× sodium chloride-sodium citrate buffer (SSC; 1 × SSC, 15 mM sodium citrate, and 150 mM NaCl, pH 7.5) containing 0.1% SDS for 30 min at room temperature and then twice with 2× SSC containing 0.1% SDS for 30 min at 55°C. The membrane was exposed for 1 to 3 days with intensifying screens at −70°C and the radioactivity was detected by autoradiography. The blots were stripped by boiling in 0.1× SSC containing 0.1% SDS and were rehybridized with a murine GAPDH cDNA probe.

Aldose Reductase Activity. Cells treated with 500 μM MG for the indicated times were washed with ice-cold PBS, pH 7.4, and harvested by scraping. The cell suspensions were homogenized 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 2000*g*, the supernatant fraction was used for enzyme analysis. The activity of AR was determined in reaction mixtures 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 reaction was started by the addition of enzyme, and activity was measured spectrophotometrically by estimating NADPH oxidation from the decrease in absorbance at 340 nm. Assays were carried out at room temperature with an appropriate blank subtracted from each reaction to correct for nonspecific oxidation of NADPH during the measurement. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol/min NADPH under the present assay conditions.

Western Blot Analysis. Aliquots of 30 μg of protein from the supernatant fraction of the cell homogenate were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Membranes blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 2 h at room temperature were reacted with anti-rat AR

antibody in TBS containing 1% BSA and 0.05% Tween 20 overnight at 4°C, and then incubated with peroxidase-conjugated goat anti-rabbit IgG antibody diluted to 1:3000 for 2 h at room temperature. After washing in TBS containing 0.1% BSA and 0.1% Tween 20, immunoreactive bands were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Cytotoxicity Assay. Cell viability was determined according to the method described previously (Seo et al., 2000). Briefly, cells were seeded in 24-well plates and treated with various concentrations of MG with or without AR inhibitor for 24 h. MTT (final concentration, 0.1 mg/ml) was added to the culture medium, and cells were incubated for an additional 4 h. After removal of the medium, the formazan crystals formed after the reduction of MTT by mitochondrial dehydrogenases in the living cells were solubilized in acidified isopropanol and measured spectrophotometrically at 570 nm with background subtraction at 650 nm.

Laser Scanning Cytometry. To assess the levels of intracellular peroxides, laser scanning cytometry was carried out using the hydrogen peroxide–sensitive fluorophore H_2DCF-DA. SMCs incubated with 5 μM H_2DCF-DA for 30 min were harvested and washed twice with ice-cold phosphate-buffered saline. The cells were immediately observed using a laser scanning cytometer and analyzed for fluorescence intensity.

Statistical Analysis. Student's t test was used for comparison of means. All data are expressed as means ± S.D. or S.E.

Results

Induction of AR mRNA in MG-Treated SMCs. When SMCs were incubated in medium containing 500 μM MG, the level of AR mRNA increased significantly, reaching a maximum of 4.5-fold after 12 h (Fig. 1A). When these cells were exposed to various concentrations of MG for 12 h, a dose-dependent increase in AR mRNA was observed (Fig. 1B).

Enzyme Activity and Protein Level of AR in MG-Treated SMCs. To verify whether increased AR mRNA results in the elevation of enzyme activity and protein, we determined the activity and the level of AR protein in MG-treated SMCs. A significant increase in AR activity was detected after 24 h of incubation with MG and continued for up to 48 h (Fig. 2A). These findings were in accordance with the protein levels determined by Western blot analysis (Fig. 2B). Quantitation of the protein bands indicated that the levels of AR protein increased 1.6- and 1.45-fold over controls after 24 and 48 h, respectively. The MG-induced increase in AR mRNA was thus followed by increases in enzyme activity and protein level in the cells with a time lag.

Effects of Cycloheximide and Actinomycin D on MG-Induced Increase in AR mRNA. To examine the mechanisms involved in the MG-induced increase in AR mRNA level, the effects of actinomycin D and cycloheximide were investigated. Induction of AR mRNA by MG was completely abolished in the presence of actinomycin D or cycloheximide (Fig. 3). These results indicated that de novo synthesis of mRNA as well as de novo synthesis of protein(s) that act on the AR gene promoter are essential to the induction of AR mRNA observed in MG-treated SMCs.

Involvement of Reactive Oxygen Species in MG-Induced Increase in AR mRNA. As shown in Fig. 4, aminoguanidine, a scavenger of dicarbonyl compounds, completely abolished MG-induced AR mRNA expression. This finding suggested that MG may directly initiate the up-regulation of AR mRNA as a reactive dicarbonyl. On the other hand, pretreatment of SMCs with BSO, a reagent that depletes intracellular glutathione (GSH), further augmented the MG-induced increase in AR mRNA. In contrast, NAC pretreatment dose-dependently suppressed MG-induced expression of AR mRNA (Fig. 5). NAC is known to increase the cellular pool of GSH, a free-radical scavenger (Aruoma et al., 1989), as well as acting as a cofactor of the glyoxalase system. Thus, one possible interpretation of these data is that increased oxidative stress may take part in the up-regulation of AR in MG-treated cells.

Consistent with this assumption, MG elicited a significant increase in intracellular reactive oxygen species (ROS) as

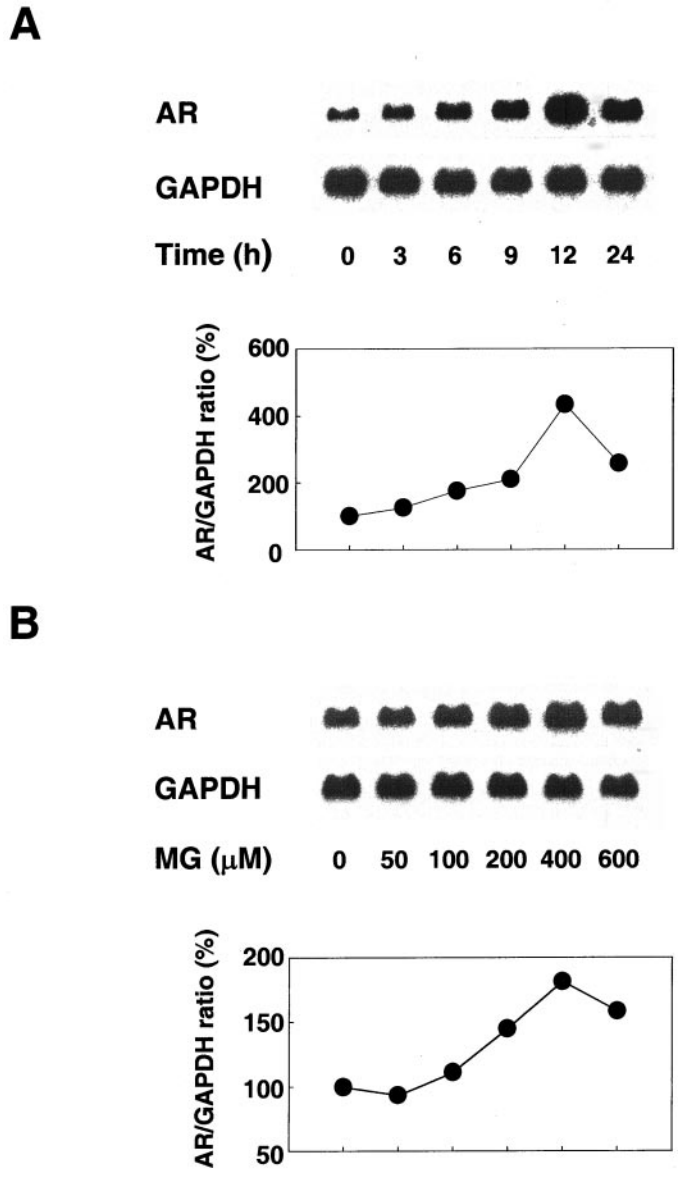

Fig. 1. Induction of AR mRNA in SMCs by MG. A, cells were incubated with 500 μM MG for the indicated times. B, cells were incubated with various concentrations of MG for 13 h. Total RNA was extracted and fractionated by 1% agarose gel electrophoresis. Fractionated RNA was transferred onto Hybond-N nylon membranes and hybridized with an AR cDNA probe. Each membrane was stripped and rehybridized for GAPDH as an internal control. The radioactivity of the signals was quantified by an image analyzer and plotted as a percentage of AR-to-GAPDH mRNA ratio relative to the control. Data are representative results from three or four experiments.

early as 1 h before the induction of AR mRNA (Fig. 6A). This increase in intracellular peroxides was abolished when SMCs were preincubated with 20 mM NAC (Fig. 6B). Accordingly, a rapid increase in intracellular ROS triggered by MG may mediate the up-regulation of AR mRNA in SMCs.

Involvement of p38 MAP Kinase Pathway in MG-Induced Increase in AR mRNA. To clarify the signal transduction pathway(s) involved in the induction of AR mRNA by MG, the effects of specific inhibitors of MAP kinase cascades were investigated. As shown in Fig. 7A, the MG-induced increase in AR mRNA level was significantly suppressed by SB203580, a specific inhibitor of p38 MAP kinase, which alone did not affect the basal level of AR mRNA. This effect of SB203580 was dose-dependent over the range of 10 to 50 μM. Similar results were obtained with SB202190, another p38 MAP kinase inhibitor (data not shown). On the other hand, PD98059, an inhibitor of ERK kinase (MEK1), did not affect the induction of AR mRNA by MG (Fig. 7B). The viability of the cells treated with SB203580 or PD98059 was >90%, as assessed by MTT assay (data not shown).

Effects of AR Inhibition on Viability of SMCs Treated with MG. To evaluate the role of increased AR activity in MG-treated SMCs, cell viability assay was performed in the presence or absence of ponalrestat, a specific inhibitor of AR. The viability of SMCs treated with various concentrations of MG declined in a dose-dependent manner (Fig. 8). This MG-mediated cytotoxicity was significantly enhanced in the presence of ponalrestat. The augmented expression of AR elicited by its substrate MG may therefore

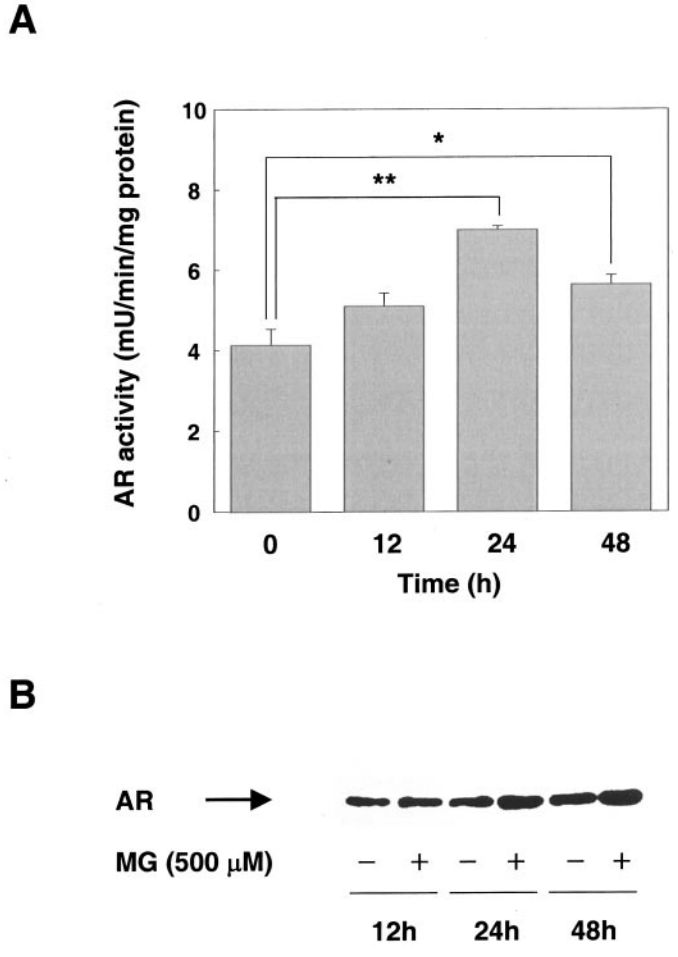

Fig. 2. Effects of MG on AR activity and protein expression in SMCs. A, activity of AR in SMCs treated with 500 μM MG. The enzyme activity in the supernatant fraction of the cell homogenate was measured spectrophotometrically. Vertical columns represent the means ± S.D. (n = 3); *$P < 0.05$, **, $P < 0.01$. B, protein levels of AR in SMCs treated with 500 μM MG. AR protein in the supernatant fraction was analyzed by the use of Western blot with anti-rat AR antibody as described under *Materials and Methods* .

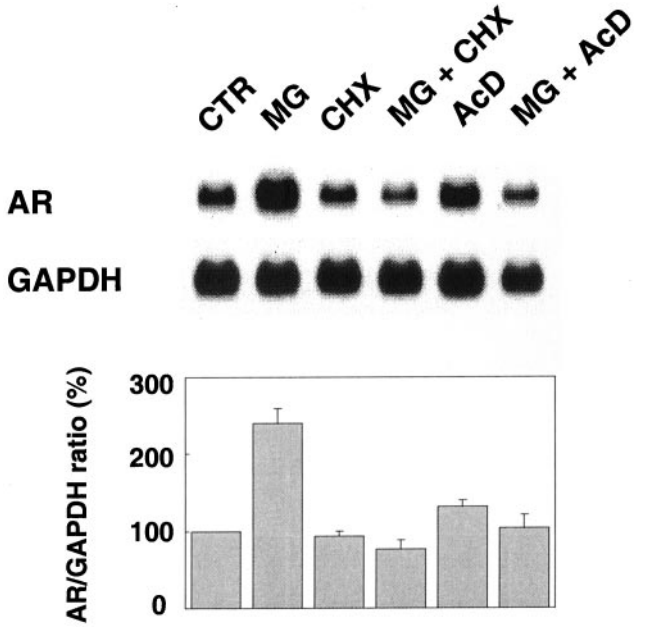

Fig. 3. Effects of actinomycin D and cycloheximide on MG-induced AR mRNA expression. SMCs were incubated with 500 μM MG for 13 h in the absence or presence of cycloheximide (CHX; 40 μg/ml) or actinomycin D (AcD; 4 μM). Total RNA was extracted and subjected to Northern blot analysis. Vertical columns represent the means ± S.E. (n = 3). CTR, control.

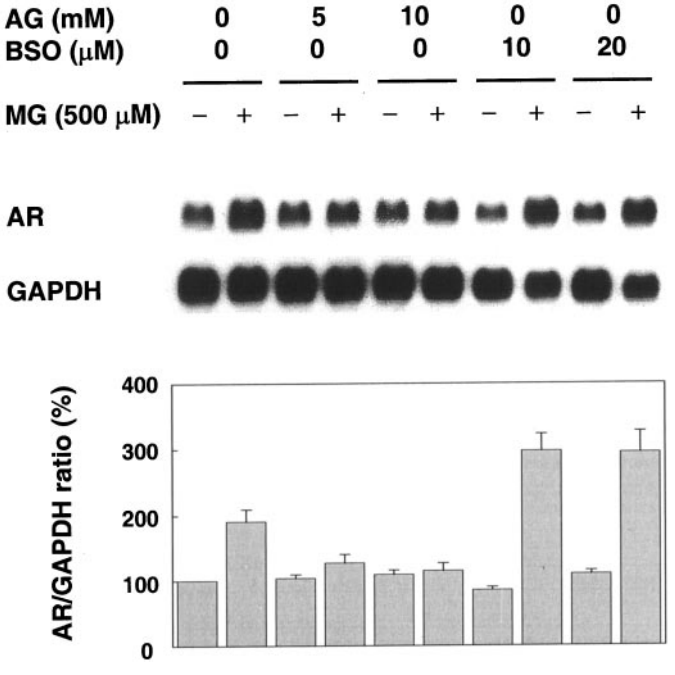

Fig. 4. Effects of aminoguanidine and DL-buthionine-*(S,R)*-sulfoximine on MG-induced AR mRNA expression. SMCs were incubated with 500 μM MG for 13 h in the absence or presence of aminoguanidine (AG) or DL-buthionine-*(S,R)*-sulfoximine (BSO). Vertical columns represent the means ± S.E. (n = 3).

protect the cells against the toxic effects of aldehydes such as MG or MG-related products.

Discussion

This study demonstrated that MG induced the expression of AR, an enzyme involved in metabolic conversion of MG and glucose. Elevated levels of cytotoxic aldehyde MG have been reported during hyperglycemic conditions and have been implicated in the vascular complications of diabetes (Vander Jagt et al., 1992; Yamada et al., 1994; Okado et al., 1996; Che et al., 1997). In the tissues of healthy subjects, the estimated rate of formation of MG was approximately 125 μM/day, whereas the concentration of serum MG in patients with diabetes was reported to be elevated up to severalfold (McLellan et al., 1994). Because MG is membrane-permeable, the level of free MG in the extracellular space can be expected to reflect the level in the cell. The concentration of MG (100–600 μM) that induced AR expression in this study was within the range of MG (310 μM) recently identified in Chinese hamster ovary cells (Chaplen et al., 1998). Accordingly, the concentration of extracellular MG that elicits AR induction demonstrated in this study seems to be physiologically relevant. Increased levels of MG may thus affect the vascular cells directly as well as indirectly via the induction of AR, and these mechanisms coupled with the increased flux through the polyol pathway may participate in the development of diabetic vascular complications. This setting is in agreement with our previous observation, in that advanced glycation end products (AGE) induced the augmented expression of AR, leading to the notion of a reciprocal relationship between augmented activity of AR and AGE to promote the development of vascular lesions in diabetes (Nakamura et al., 2000).

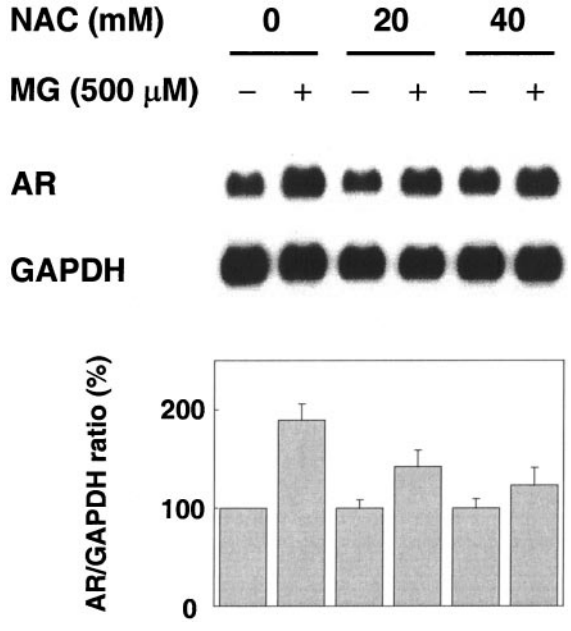

Fig. 5. Effects of *N*-acetyl-L-cysteine pretreatment on MG-induced AR mRNA expression. After preincubation with 20 or 40 mM *N*-acetyl-L-cysteine (NAC) for 24 h, SMCs were rinsed twice and subsequently incubated in fresh medium containing 500 μM MG for another 13 h. Vertical columns represent the means ± S.E. (n = 3).

Long-term exposure to high concentrations of MG may be among the causative factors that accelerate cellular oxidative stress under such pathological conditions as diabetes (Baynes, 1991). Induction of AR expression by MG was significantly suppressed when SMCs were preincubated with NAC, a thiol antioxidant as well as a precursor of GSH (Aruoma et al., 1989). On the other hand, pretreatment of SMCs with BSO, a reagent that depletes intracellular GSH, further augmented the MG-induced increase in AR mRNA level. Because GSH also serves as a cofactor of the glyoxalase system that catalyzes the metabolic disposal of MG, interpreting these observations is a complex procedure. However, the determination of intracellular levels of ROS using a peroxide-sensitive fluorophore demonstrated clearly that MG induced a significant increase in intracellular ROS level before the elevation of AR mRNA. This finding is in line with

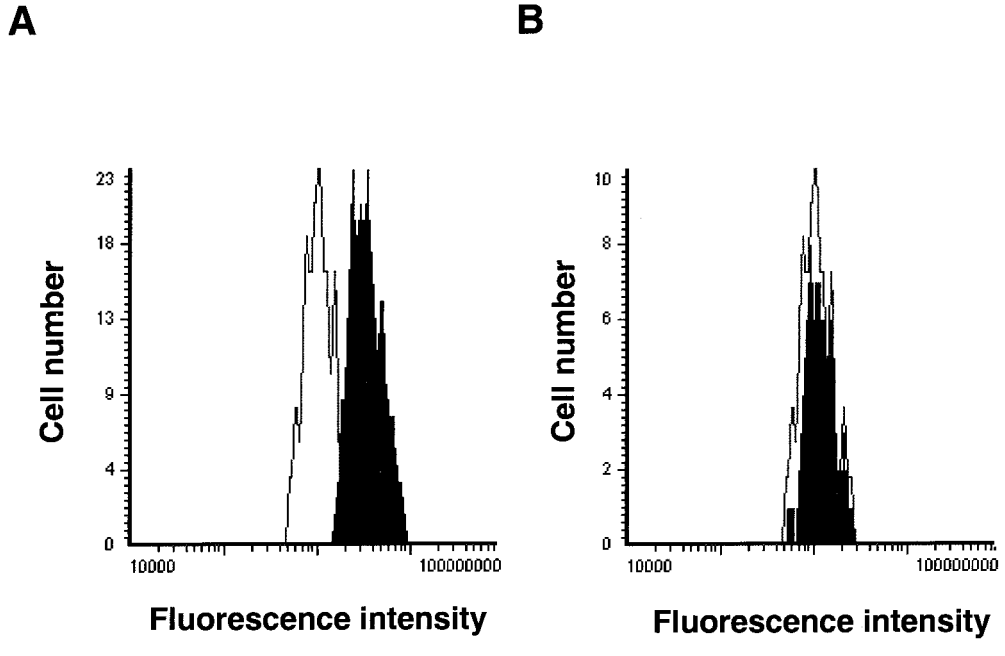

Fig. 6. Intracellular peroxide production by MG and inhibition by *N*-acetyl-L-cysteine in SMCs. A, cells incubated with (filled area) or without (open area) 500 μM MG for 1 h, were treated with a peroxide-sensitive dye, H_2DCF-DA (5 μM), during the final 30 min of incubation. Relative levels of ROS were quantified by laser scanning cytometer. B, cells pretreated with 20 mM NAC for 24 h were incubated with (filled area) or without (open area) 500 μM MG for 1 h. Data are representative results from three experiments.

previous studies in which a significant increase in intracellular ROS was demonstrated in hepatocytes and SMCs treated with MG (Che et al., 1997). In macrophage-derived cell lines, MG induced apoptotic cell death through the production of ROS (Okado et al., 1996). However, the molecular mechanism(s) underlying the MG-induced augmentation of intracellular oxidative stress remains unclear. A partial explanation for this phenomenon is that ROS are generated during a glycation reaction between MG and amino acid residues in proteins to form fluorescent products leading to cross-linking and denaturation of proteins. On the other hand, the depletion of GSH caused by increased metabolic load of MG through the glyoxalase system may decelerate the elimination of ROS in the cell. Our observation that intracellular ROS were elevated before the change in AR mRNA levels suggested that the effect of MG on AR expression may be attributable to increased oxidative stress caused by MG treatment. In fact, we and others recently demonstrated that the expression of AR was up-regulated by such oxidants as hydrogen peroxide, superoxide anion, and nitric oxide (Spycher et al., 1997; Seo et al., 2000). MG may therefore exert its action on the AR gene through ROS, which are physiological signaling molecules that activate redox-sensitive kinases and modulate gene expression.

The elevated expression of AR induced by MG was significantly suppressed by SB203580, an inhibitor of p38 MAP kinase, whereas PD098059, an inhibitor of MEK1, did not affect the MG-induced increase in AR mRNA. Accordingly, the p38 kinase pathway seems to play an important role in mediating the MG-induced AR gene expression. This is in contrast to our recent findings in hydrogen peroxide–induced up-regulation of AR in the vascular smooth muscle cell line A7r5 (Nishinaka and Yabe-Nishimura, 2001). Although the inhibition of p38 kinase by SB203580 partially suppressed the induction, the epidermal growth factor receptor–ERK pathway was the major signaling pathway involved in the

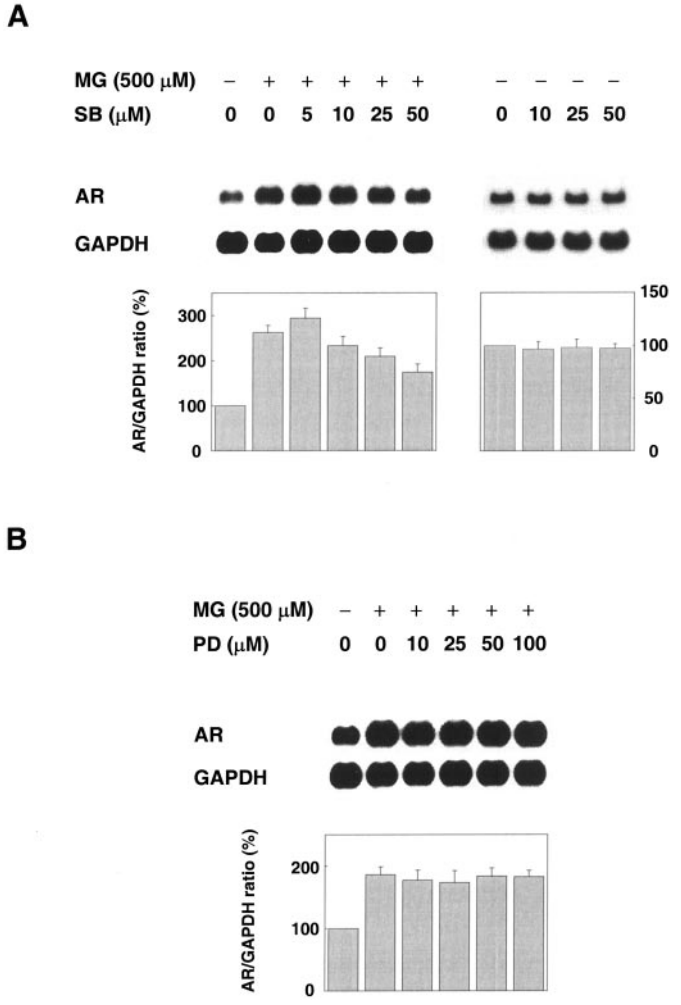

Fig. 7. Effects of inhibitors of MAP kinase cascades on MG-induced AR mRNA expression. Cells pretreated with various concentrations of the p38 inhibitor SB203580 (A) or MEK1 inhibitor PD98059 (B) for 30 min were incubated with 500 μM MG for 13 h. Vertical columns represent the means ± S.E. (n = 3).

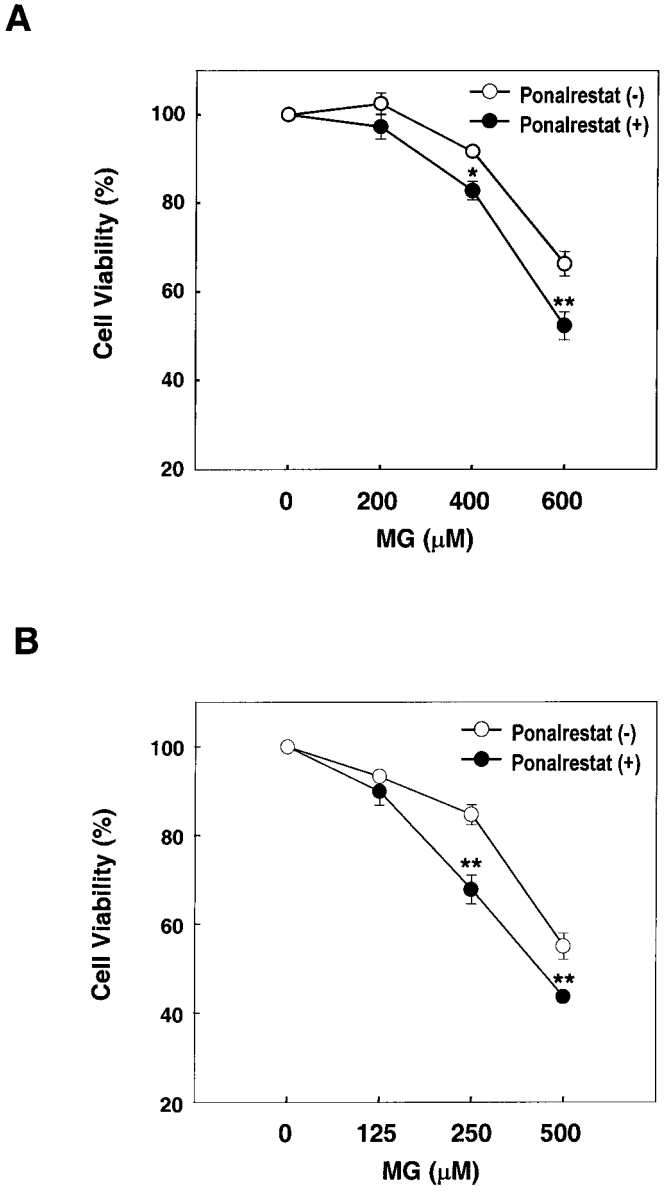

Fig. 8. Effects of MG and AR inhibition on cell viability of SMCs. Cells were incubated with various concentrations of MG in the absence or presence of 50 μM ponalrestat for 24 h, in high serum (A, 10%)- or low serum (B, 0.5%)-containing medium. Cell viability was determined by MTT assay as described under *Materials and Methods*. The vertical columns represent the means ± S.D. (n = 4). *, $P < 0.05$, **, $P < 0.01$ compared with control.

induction AR gene expression in A7r5 under oxidative stress. It should be noted that ROS have been implicated in transducing the up-regulation of AR expression regardless of whether cells were treated with MG or hydrogen peroxide. However, the major signaling cascade that mediates the induction of the AR gene seemed to differ. ROS transduce their signals via MAP kinases in many cellular processes including proliferation, differentiation, and apoptosis (Davis, 1993; Lopez-Ilasaca, 1998). MG has been reported to induce phosphorylation of not only p38 kinase but also JNK and ERKs in human umbilical vein endothelial cells (Akhand et al., 2001). In Jurkat leukemia T cells, MG also activated JNK to induce apoptosis (Du et al., 2000). Thus, the possibility remains that the JNK pathway may also participate in MG-induced AR expression in SMCs. No conclusive data are available at present, although the induction of AR by MG was not affected in the presence of curcumin even at concentrations as high as 50 μM, at which concentration it has been reported to interfere with the JNK signaling pathway (Chen and Tan, 1998; H. G. Seo, unpublished observations). Further studies are necessary to clarify the alternative signal transduction pathway(s) involved in the up-regulation of AR by MG.

A dose-dependent decrease in the viability of SMCs exposed to MG was demonstrated by MTT assay. The cytotoxic effect of MG was augmented when the activity of AR was suppressed by the inclusion of ponalrestat, an inhibitor of AR. MG contributes to a variety of detrimental processes under such pathological conditions as diabetes, either by reacting directly with proteins and DNA or by initiating AGE formation (Beisswenger et al., 1999). The present findings indicate that the induction of AR by MG is a consequence of an adaptive response of SMCs to detoxify cytotoxic carbonyl compounds and to protect SMCs from MG-induced cell death. The cytoprotective role of AR could be attributable to the broad substrate specificity of this enzyme not only for glucose, but also for reactive aldehydes generated in the cells. In fact, the functional significance of AR against cytotoxic aldehydes has been suggested in hepatoma cell lines (Takahashi et al., 1995), in vascular smooth muscle cells (Spycher et al., 1997; Seo et al., 2000; Nishinaka and Yabe-Nishimura, 2001), and in the lesions of vasculitis (Rittner et al., 1999). These findings in conjunction with the present findings provide insight into the primary role of AR as a detoxification enzyme that degrades toxic aldehydes for cell survival. In this context, it is of particular interest that apoptosis of vascular smooth muscle cells was alleviated within the vessels of diabetic animals compared with nondiabetic control animals (Hall et al., 2000). Apoptosis is one of the mechanisms that maintain the balance between cell proliferation and cell death, and perturbation of this balance is a critical element in the formation of macrovascular lesions often associated with diabetes. When seen in this light, inhibition of AR activity in the vascular cells, possibly augmented during hyperglycemic conditions, may accelerate the cell death and facilitate vascular remodeling. Consequently, inhibitors of AR hold promise for therapeutic intervention in diabetic macroangiopathy.

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