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Nitric Oxide Up-Regulates Aldose Reductase Expression in Rat Vascular Smooth Muscle Cells: A Potential Role for Aldose Reductase in Vascular Remodeling

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ABSTRACT

Acceleration of the polyol pathway under hyperglycemia is among the mechanisms implicated in the pathogenesis of diabetic complications. Although aldose reductase (AR), the rate-limiting enzyme in this pathway, is a target for pharmacological intervention of diabetic complications, the clinical efficacy of AR inhibitors has not been consistently proved. Because nitric oxide (NO) plays important roles in vascular hemodynamics and inflammatory responses that are affected under diabetic conditions, the interaction of NO with AR was investigated with rat aortic smooth muscle cells. Spontaneous NO donors, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine, elicited a dose-dependent increase in AR mRNA to a maximum of 7-fold in 12 h. The activity of AR was elevated after 10 h of SNAP treatment. These effects of NO donors were suppressed by the addition of 2-(trimethylammoniophenyl)-4,4,5,5-tetramethylimidazoline-1-oxy 3-oxide, a scavenger of NO. Induction of AR mRNA by SNAP was completely abolished by actinomycin D or cycloheximide, but unaffected by guanylate cyclase inhibitors or genistein, a tyrosine kinase inhibitor. Pretreatment of the cells with *N*-acetyl-L-cysteine significantly suppressed the SNAP-induced up-regulation of AR mRNA. Under normal glucose conditions, inclusion of the AR inhibitor ponalrestat augmented the cytotoxic effect of SNAP on the cells. The level of AR mRNA also was elevated in a murine macrophage cell line RAW 264.7 stimulated with lipopolysaccharide and interferon-γ. Inhibition of NO synthesis completely abolished the increase in AR mRNA in the stimulated cells. The up-regulation of AR by NO in the vascular lesions may modulate NO-induced cell death and the ensuing vascular remodeling during inflammatory responses.

Aldose reductase (AR; EC 1.1.1.21) has been implicated in the pathogenesis of various diabetic complications (Yabe-Nishimura, 1998). The enzyme catalyzes the reduction of various aldehydes, including the aldehyde form of glucose with NADPH as a cofactor. AR converts glucose to sorbitol, which is converted to fructose by sorbitol dehydrogenase, with a cofactor NAD^+. This is the so-called polyol pathway, the alternate route of glucose metabolism. Under hyperglycemia, the acceleration of the polyol pathway leads to an overflow of the products and the depletion of NADPH and NAD^+. Such metabolic perturbation is postulated to provoke the early tissue damage in the ocular lens, retina, peripheral nerve, and renal glomerulus where insulin-independent uptake of glucose takes place. Numerous aldose reductase inhibitors of diverse chemical structures have been developed as possible therapeutic agents for diabetic complications. Although these inhibitors were effective in diabetic experimental animal models, their clinical efficacy in diabetic patients has not been proved (Pfeifer et al., 1996).

AR exhibits a broad substrate specificity for a variety of aldehydes. Among these substrates are biogenic aldehydes derived from the catabolism of catecholamines by monoamine oxidase (Tabakoff et al., 1973), isocorticosteroids, intermediates in the catabolism of the corticosteroid hormones (Wermuth and Monder, 1983), and isocaproaldehyde, a product of side chain cleavage of cholesterol (Matsuura et al., 1996). Progesterone as well as 17α-hydroxyprogesterone, a major precursor of the androgens, estrogens, and glucocorti-

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ABBREVIATIONS: AR, aldose reductase; NO, nitric oxide; iNOS, inducible form of NO synthase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; NOC 7, 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine; SIN-1, *N*-morpholino sydnonimine; TMA-PTIO, 2-(trimethylammoniophenyl)-4,4,5,5-tetramethylimidazoline-1-oxy 3-oxide; L-NMA, N^G-monomethyl-L-arginine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NAC, *N*-acetyl-L-cysteine; IFN-γ, interferon-γ; LPS, lipopolysaccharide; SMC, aortic smooth muscle cell; EGF, epidermal growth factor.

coids was reported to be an endogenous substrate for bovine AR (Warren et al., 1993). The enzyme also catalyzes the reduction of reactive aldehydes produced by oxidative damage to unsaturated fatty acids, 4-hydroxynonenal (Vander Jagt et al., 1995) and acrolein (Kolb et al., 1994). AR may thus detoxify endogenous as well as xenobiotic aldehydes in various tissues.

Nitric oxide (NO), a nitrogen-based radical, is known to mediate macrophage cytotoxicity, regulate blood pressure, and participate in neurotransmission (Moncada et al., 1991). In the vasculature, the constitutive NO synthase is mainly present in the endothelial cells, whereas expression of inducible form of NOS (iNOS) is demonstrated in macrophages, neutrophils, and smooth muscle cells. Vascular smooth muscle cells not only produce NO via the iNOS pathway in response to various cytokines but also respond to exogenously generated NO that diffuses to the cells. In addition to its vasodilatory role, NO, either generated by NO donors or synthesized by iNOS, inhibits proliferation and induces apoptosis of vascular smooth muscle cells (Zhao et al., 1997; Iwashima et al., 1998). Of particular interest is the fact that cytokine-stimulated iNOS expression was enhanced by high glucose with increased nitrite production in macrophages and mesangial cells (Sharma et al., 1995). In rats with streptozotocin-induced diabetes, the generation of NO by granulocytes was augmented (Kedziora-Kornatowska et al., 1998). Although NO production via the constitutive isoform of NOS in the endothelial cells is attenuated under high glucose conditions (Pieper, 1998), greater amounts of NO metabolites were detected in the urine of diabetic rats (Tolins et al., 1993). In addition, aminoguanidine, an inhibitor of iNOS, was demonstrated to prevent early vascular dysfunction in diabetic rats (Tilton et al., 1993). These findings suggest that the augmented release of NO derived from iNOS is involved in the development of diabetic vascular complications.

In diabetes, the generation of oxygen free radicals is enhanced by various mechanisms. Autoxidation of glucose, accelerated nonenzymatic glycation, and attenuated availability of glutathione under hyperglycemia were among these mechanisms (Yabe-Nishimura, 1998). Recently, hydrogen peroxide was reported to induce mRNA of rat AR, AKR1B4 (Jez et al., 1996), in A7r5 cell line derived from rat vascular smooth muscle (Spycher et al., 1997). NO is a reactive free radical whose generation may be enhanced in the vascular tissue under hyperglycemic conditions. This led us to investigate whether NO affects the expression of AR in aortic smooth muscle cells. The present study illustrated that NO up-regulated AR expression to mitigate the cytotoxic action of NO. The findings provide insight into the novel role for AR in modulating the NO-induced cell death and the ensuing vascular remodeling.

Materials and Methods

Reagents. *S*-nitroso-*N*-acetylpenicillamine (SNAP), 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC 7), *N*-morpholino sydnonimine (SIN-1), 2-(trimethylammoniophenyl)-4,4,5,5-tetramethylimidazoline-1-oxy 3-oxide (TMA-PTIO), N^G-monomethyl-L-arginine (L-NMA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Dojindo Laboratories (Kumamoto, Japan). Xanthine, xanthine oxidase, methylene blue, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), *N*-acetyl-L-cysteine (NAC), actinomycin D, and cycloheximide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Interferon-γ (IFN-γ) was purchased from Genzyme (Cambridge, MA), whereas goat anti-rabbit IgG antibody and lipopolysaccharide (LPS) were from Sigma Chemical Co. (St. Louis, MO). Anti-rat AR antibody was a gift from Dr. N. Iwata (RIKEN, Wako, Japan) and ponalrestat {3-[(4-bromo-2-fluorophenyl)methyl]-3,4-dihydro-4-oxo-1-phthalazineacetic acid; I.C.I.} was from Dr. T.G. Flynn (Queen's University, Kingston, Canada). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from ICN Pharmaceuticals (Tokyo, Japan). Other reagents were of the highest grade available.

Cell Culture. Aortic smooth muscle cells (SMCs) from rat were isolated from free-floating explants of aorta as previously described (Seo et al., 1997). 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 a day. After supplementation with fresh medium, the tissue was again incubated for a few 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 20% heat-inactivated fetal bovine serum at 37°C under an atmosphere of 95% air and 5% CO_2. RAW 264.7, a mouse macrophage-like cell line transformed with the Abelson leukemia virus, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% heat-inactivated calf donor 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 and 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 RNAs were transferred onto a Hybond-N nylon membrane (Amersham, Uppsala, Sweden) overnight by capillary action, and immobilized by UV Stratalinker (Stratagene, La Jolla, CA). After hybridization with a ^{32}P-labeled probe at 42°C in the presence of 50% formamide, the membrane was washed twice 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 at 55°C for 60 min. The final wash at high stringency was in 0.2 × SSC containing 0.1% SDS for 30 min at 60°C. The radioactivity on the membrane was detected by a Fuji BAS 2000 Bioimaging Analyzer (Tokyo, Japan). The cDNA probes used in the hybridization was 10Q, originated from rat lens AR (AKR1B4) for SMCs (Nishimura et al., 1988), and KE2, originated from mouse kidney AR (AKR1B3) for RAW 264.7 (Gui et al., 1995). The blots were stripped by boiling in 0.1 × SSC containing 0.1% SDS, and rehybridized with a human GAPDH cDNA probe.

Nitrite Assay. NO formation was measured spectrophotometrically as its stable oxidative metabolite, nitrite, formed during incubation with the Griess reagents (Green et al., 1982). Briefly, 100-μl aliquots were removed from the culture medium and incubated with 100 μl of the Griess reagent [1 part 1% sulfanilamide in 0.1 M HCl and 1 part 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride] at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Bio-Rad, Richmond, CA). The nitrite concentration was determined with a curve calibrated on sodium nitrite standards. The background NO_2^- levels in the culture media were subtracted from each sample.

Aldose Reductase Activity. Cells treated with 1 mM SNAP 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 2000*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 reaction was started by the addition of enzyme and activity was measured spectrophotometrically by estimating NADPH oxidation from a 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 is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH/min under the present assay conditions. The protein concentration was determined by the method of Bradford (Bradford, 1976).

Western Blot Analysis. Fifty micrograms of protein in the supernatant fraction of the cell homogenate was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated with anti-rat AR antibody and then with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. Immunoreactive bands were detected with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chloride developing reagent (Life Technologies, Gaithersburg, MD).

Cytotoxicity Assay. The cell viability was determined by two methods. First, methylene blue content retained in the surviving cells fixed with glutaraldehyde was determined according to the method previously described (Yamazaki et al., 1986). Briefly, cells were seeded in 12-well plates and treated with various concentrations of SNAP with or without an inhibitor of AR for 24 h. The surviving cells in each well were fixed with glutaraldehyde solution (final 4.2%) added to the medium. After 15 min, the dead cells were removed by washing with PBS (pH 7.4), and the remaining adherent cells were stained with 1 ml of 0.05% methylene blue for 15 min. The excess dye was rinsed away and the plates were dried. Following the extraction of methylene blue with 2 ml of 0.33 N HCl, the optical density of the extract was read at 665 nm. Second, MTT assay was performed as described previously (Mosmann, 1983). After incubation with SNAP with or without 50 μM ponalrestat for 24 h, MTT (final 0.45 mg/ml) was added to the culture medium of SMCs seeded in 96-well plates. Cells were incubated for an additional 4 h, and the medium was removed. The formazan crystals formed after the reduction of MTT by mitochondrial dehydrogenases in the living cells were solubilized in dimethyl sulfoxide and examined spectrophotometrically at 550 nm with background subtraction at 655 nm.

Results

Induction of AR Gene Expression in SMCs by NO Donor SNAP. The expression of AR transcript, ~1.4 kb, was detected in SMCs cultured in control medium containing 5.6 mM glucose. When these cells were exposed to various concentrations of SNAP for 8 h, a concentration-dependent increase in AR mRNA was observed (Fig. 1A). In SMCs exposed to 1 mM SNAP, a concentration that elicited the maximal induction of AR mRNA, an increase in expression was apparent at as early as 3 h. The levels of AR transcript (~7-fold) peaked at 12 h after the addition of SNAP (Fig. 1B).

Effects of Various NO Donors and NO Scavenger on AR Gene Expression. To verify whether NO derived from SNAP caused the augmented expression of AR mRNA, SMCs were exposed to various NO donors and the effects of TMA-PTIO, a NO scavenger, were examined. As shown in Fig. 2A, TMA-PTIO suppressed the SNAP-induced increase in AR mRNA. A different type of NO donor, NOC 7, that releases two equivalents of NO at a time, elicited a large increase in AR transcript in SMCs. This effect of NOC 7, more pronounced than that of SNAP, was effectively suppressed by TMA-PTIO. Increased AR gene expression was similarly demonstrated in the cells treated with 1 mM SIN-1. However, TMA-PTIO did not affect the SIN-1-induced increase in AR mRNA. SIN-1 is known to decompose and yield NO and superoxide anion radicals that form peroxynitrite. Because TMA-PTIO failed to suppress the effect of SIN-1, peroxynitrite, or hydroxyl radicals derived from peroxynitrite, may not primarily contribute to the augmented expression of the AR gene. Yet the possibility remained that superoxide anions similarly induce AR gene expression in SMCs.

To examine this possibility, SMCs were incubated in a medium containing xanthine and xanthine oxidase, which generate superoxide anion radicals. As shown in Fig. 2B, approximately a 2.2-fold induction of AR mRNA was demon-

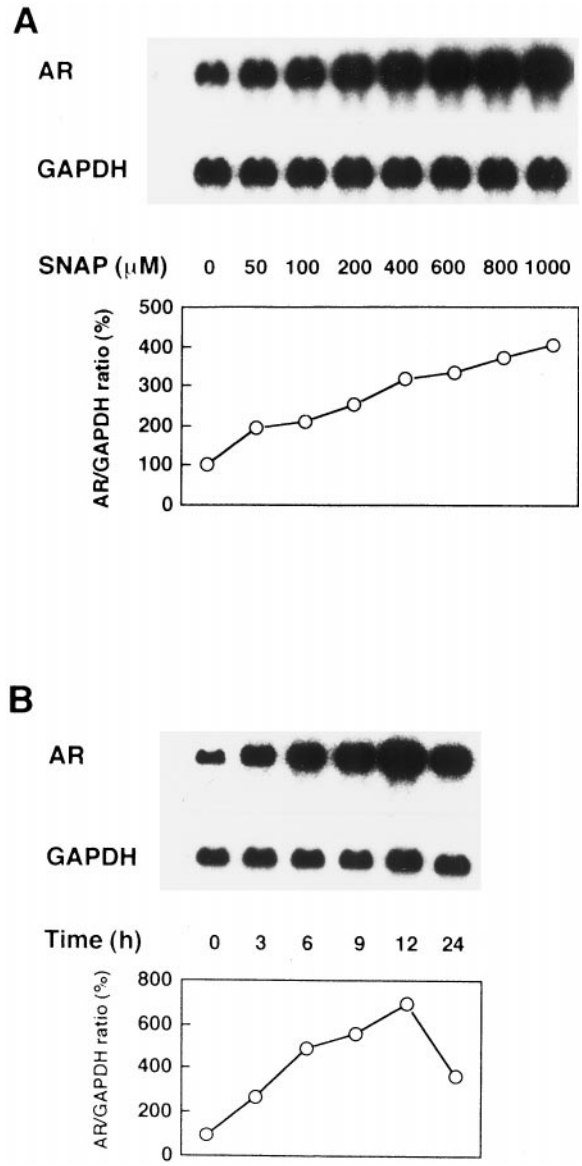

Fig. 1. Induction of AR mRNA in SMCs by the NO donor SNAP. A, cells were incubated in the absence or presence of various concentrations of SNAP for 8 h. B, cells were incubated with 1 mM SNAP for the indicated time periods. Total RNA was extracted and analyzed by Northern blotting with AR cDNA probe. Each membrane was stripped and rehybridized for GAPDH as an internal control. The radioactivity of the signals was quantified by an imaging analyzer and plotted as the percentage of control of AR to GAPDH mRNA ratios, respectively.

strated after an 8-h induction in the medium with 100 μg/ml xanthine and 58 milliunits/ml xanthine oxidase. Thus, induction of AR mRNA by superoxide anion as well as by NO or NO-derived chemical species generated from NO donors was indicated.

Mechanisms Involved in NO-Induced AR Gene Expression. To clarify whether the increase in AR mRNA elicited by NO donors was mediated by the activation of guanylate cyclase, the effects of guanylate cyclase inhibitors were investigated. The SNAP-induced AR expression was not altered by addition of 10 μM methylene blue (data not shown), nor by another selective guanylate cyclase inhibitor, ODQ (Fig. 3A). Induction of AR-like protein by growth factors that act through membrane receptor-associated tyrosine kinase activity was previously described. Fibroblast growth factors and epidermal growth factor (EGF) were reported to induce AR mRNA and protein in rat astrocytes (Laeng et al., 1995; Jacquin-Becker and Labourdette, 1997). We also observed a dose- and time-dependent increase in AR mRNA in SMCs treated with EGF (unpublished data). To examine whether NO donors increased AR gene expression by modulating the intracellular signaling pathway including tyrosine kinase, the effect of genistein, an inhibitor of tyrosine kinase, was investigated. In the presence of 50 μM genistein, however, the level of AR transcript in SNAP-treated SMCs was not affected. As demonstrated in Fig. 3B, in contrast, pretreatment of SMCs with up to 40 mM NAC, which increases the cellular pool of free-radical scavenger glutathione, significantly suppressed the level of AR mRNA in SNAP-treated

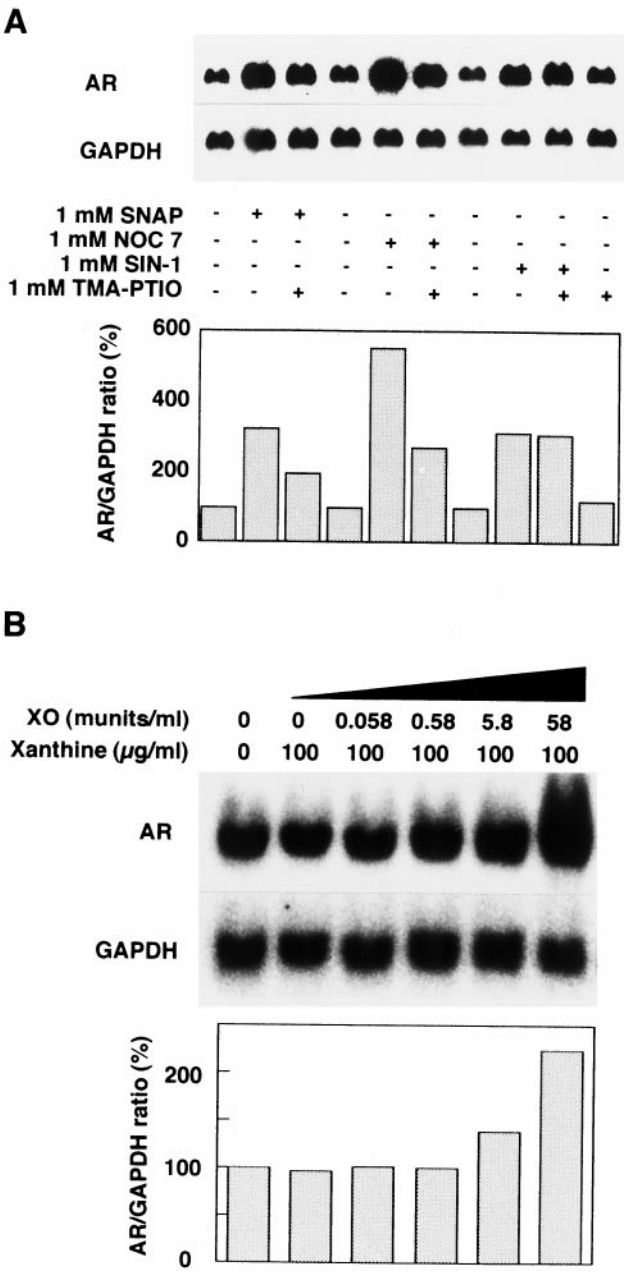

Fig. 2. A, effects of various NO donors and a NO scavenger on AR mRNA in SMCs. Top, cells were exposed to different NO donors with or without the NO scavenger TMA-PTIO for 8 h. Each membrane probed for AR was stripped and rehybridized for GAPDH. Bottom, radioactivity of the signals was quantified and plotted as the percentage of control of AR to GAPDH mRNA ratios. Results are from a single experiment and are representative of two or three experiments. B, induction of AR mRNA in SMCs by O_2^- generated by the xanthine-xanthine oxidase system. Cells were incubated in 100 μg/ml xanthine and the indicated amount of xanthine oxidase for 8 h. XO, xanthine oxidase.

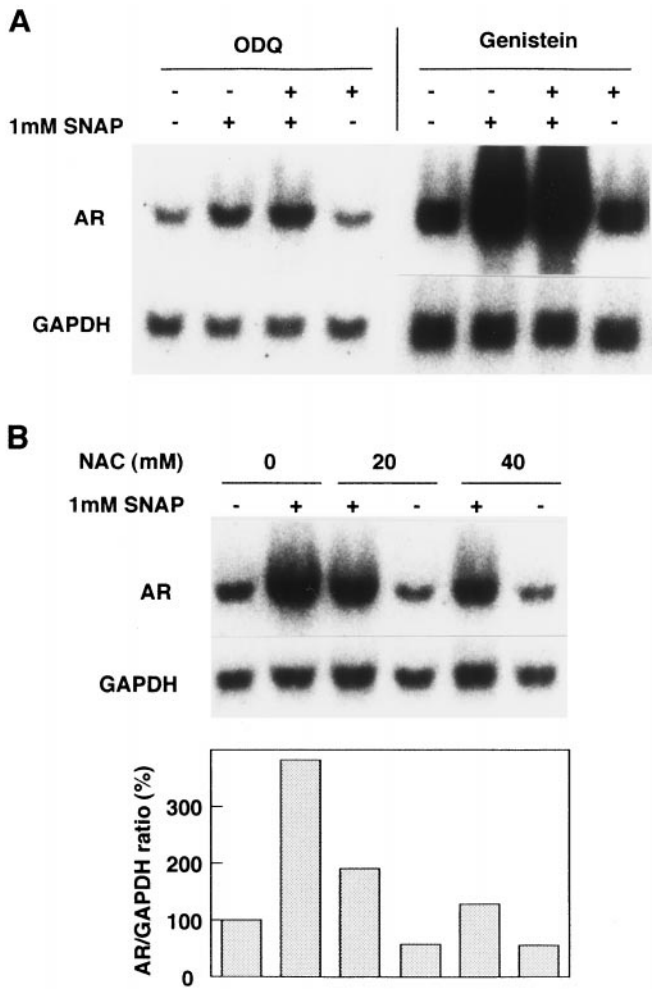

Fig. 3. A, effects of ODQ and genistein on SNAP-induced AR mRNA expression. SMCs were incubated with 1 mM SNAP for 8 h in the absence or presence of the indicated concentrations of ODQ or genistein. B, effects of NAC pretreatment on SNAP-induced AR mRNA expression. Cells were preincubated in medium containing 20 or 40 mM NAC for 24 h. Cells were rinsed and subsequently incubated in fresh medium containing 1 mM SNAP for another 8 h. Total RNA was extracted and subjected to Northern blot analysis. The results are representative of two experiments.

cells. The finding indicated that the intracellular antioxidant was effective in blocking the induction of AR mRNA by exogenously generated NO.

The SNAP-induced AR mRNA expression was completely abolished in the presence of actinomycin D or cycloheximide (Fig. 4). This indicates that de novo mRNA synthesis as well as de novo synthesis of protein(s) that may act on the AR gene promoter are involved in the increased expression of AR mRNA in SNAP-treated SMCs.

Enzyme Activity and Protein Level of AR in SNAP-Treated SMCs. To examine whether the induction of AR mRNA was accompanied by an increase in enzyme activity and protein, the activity and the level of AR in SNAP-treated SMCs was measured. As illustrated in Fig. 5A, a significant elevation in AR activity was first detected after 10 h of exposure to 1 mM SNAP. The enzyme activity was further elevated after 24 h, and these findings were in accord with the level of protein determined by Western blot analysis (Fig. 5B). The SNAP-induced increase in AR mRNA was thus followed by an increase in enzyme activity and protein with a time lag of several hours.

Effects of Endogenously Generated NO on AR Gene Expression in RAW 264.7 Cells. To further clarify whether endogenously generated NO elicits the increase in AR mRNA, we used a murine macrophage cell line RAW 264.7 that highly up-regulates the expression of iNOS on stimulation with LPS plus IFN-γ. Consistent with the enhanced production of nitrite (Fig. 6C), an endproduct of NO, a marked increase in AR transcript was demonstrated in RAW cells treated with LPS plus IFN-γ (Fig. 6, A and B). Inclusion of L-NMA, an inhibitor of NOS, suppressed the production of nitrite and completely blocked the increase in AR mRNA in the cells treated with the cytokines. These results indicated that NO generated via the endogenous iNOS pathway also induced the expression of AR.

Effects of AR Inhibition on Viability of SMCs Treated with SNAP. A dose-dependent decline in cell viability was demonstrated in SMCs treated with various concentrations of SNAP for 24 h (Fig. 7, A and B). This SNAP-mediated cytotoxicity was significantly enhanced in the presence of AR inhibitors with different chemical structures, ponalrestat and a derivative of spirohydantoin. The representative effects of ponalrestat, one of the specific inhibitors of AR (Ward et al., 1990), on the viability of SNAP-treated SMCs are illustrated in Fig. 7. The cytotoxic effect of SNAP was significantly accelerated under high glucose conditions when a higher rate of cell proliferation was denoted (Fig. 7C). In contrast with the data for the 5.6 mM glucose medium, the viability of SNAP-treated SMCs was unaltered by ponalrestat when it was included in the medium containing 30 mM glucose. These findings indicate that the up-regulation of AR by NO may protect the cells from the toxic effects of NO or NO-related chemical species, solely at normal ambient glucose concentration.

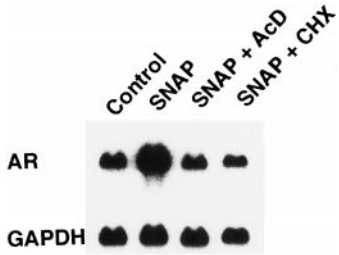

Fig. 4. Effects of actinomycin D and cycloheximide on SNAP-induced AR mRNA expression. SMCs were incubated with 1 mM SNAP for 8 h in the absence or presence of actinomycin D (AcD; 4 μM) or cycloheximide (CHX; 40 μg/ml). Total RNA was extracted and subjected to Northern blot analysis.

Discussion

The present investigation is the first to provide evidence that AR gene expression is induced by NO or NO-derived chemical species in the vascular cells and a macrophage cell line. The major findings demonstrated in this study are that: 1) NO derived from NO donors as well as from endogenous iNOS up-regulated the expression of AR; 2) this effect of NO involved de novo mRNA synthesis, whereas neither guanylate cyclase nor tyrosine kinase activity mediated the signaling pathway; and 3) inhibition of AR activity under normal glucose conditions exacerbated the cytotoxic effect of NO.

The molecular species that triggered the expression of AR mRNA appeared to be NO or NO-derived chemical species, not peroxynitrite. The findings that NOC 7, which releases two molecules of NO at a time, elicited a larger increase in AR transcript than SNAP, and that effects of these NO donors were consistently suppressed by a NO scavenger, TMA-PTIO, suggest that NO itself acts to augment the expression of AR. In the cells treated with SIN-1, the increase in AR mRNA was comparable to that in the SNAP-treated cells. SIN-1 yields the reaction product peroxinitrite by stoichio-

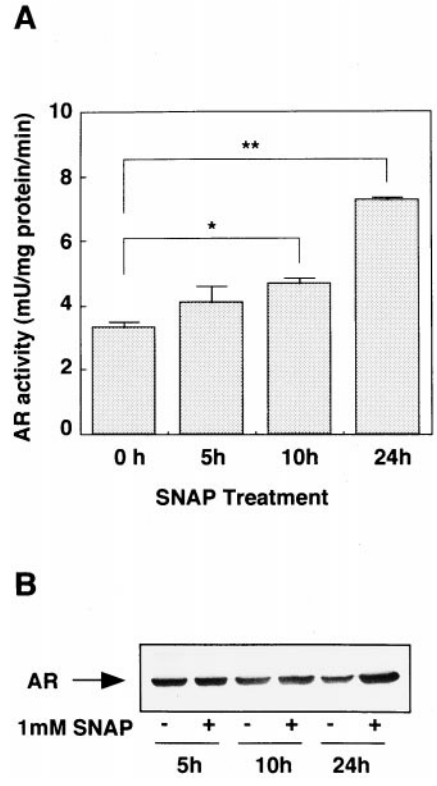

Fig. 5. A, activity of AR in SMCs treated with the NO donor SNAP. The enzyme activity in the supernatant fraction of the cell homogenate was measured spectrophotometrically. Vertical columns represent the means ± S.E. ($n = 3$). *$P < .05$, **$P < .01$. B, AR protein in SMCs treated with the NO donor SNAP. AR protein in the supernatant fraction was analyzed by Western blotting with anti-rat AR antibody as described in *Materials and Methods*.

metric generation of superoxide anion and NO. If peroxinitrite directly provokes the induction of AR, the effect of SIN-1 should have been more pronounced than that of SNAP or NOC 7. Accordingly, peroxinitrite or peroxinitrite-derived reactive species such as hydroxy radicals appeared not to be involved in the induction of AR mRNA. The failure of TMA-PTIO to suppress the effect of SIN-1 also support this assumption because this compound quenches NO, which reacts with superoxide anion. The increased AR gene expression demonstrated in SIN-1-treated cells may be partly attributed to superoxide anion radicals generated from SIN-1. In the present study, we observed augmented expression of AR mRNA in SMCs treated with xanthine and xanthine oxidase. The finding implies that superoxide anion itself also may induce AR expression in SMCs.

The up-regulation of AR by SNAP was significantly suppressed when SMCs were preloaded with NAC. NAC is a thiol antioxidant and glutathione precursor that blocks the effects of free radicals (Natoli et al., 1997). The effect of NAC pretreatment on the SNAP-induced AR expression indicated that NO, generated in the extracellular space, may permeate cell membranes to exert its action as a free radical inside the cell. In line with this assumption, intracellular NO generated in response to cytokine stimulation augmented AR expression in RAW 264.7 cells. The magnitude of AR induction in these cells was less than that in SNAP-treated SMCs, although the increase in AR mRNA was completely blocked by L-NMA, an inhibitor of NOS. NO release from NO donors may be rapid and affects all cells simultaneously, whereas NO production in response to cytokines requires a delay for transcription and protein synthesis of iNOS. This may account for the difference in the time lag and extent of AR mRNA induction between SMCs and RAW 264.7. Because superoxide anion is produced along with NO in the stimulated macrophage, this oxygen radical also may take part in the augmented expression of AR demonstrated in RAW 264.7 cells. However, the fact that the NOS inhibitor L-NMA entirely abolished the increase in AR mRNA in the stimulated cells invalidates this possibility. Collectively, our results indicate that intracellular NO or NO-derived chemical species modulate the expression of the AR gene. The lack of effect of guanylate cyclase inhibitors on the NO-induced increase in AR mRNA implies that NO up-regulates AR expression via a cGMP-independent pathway.

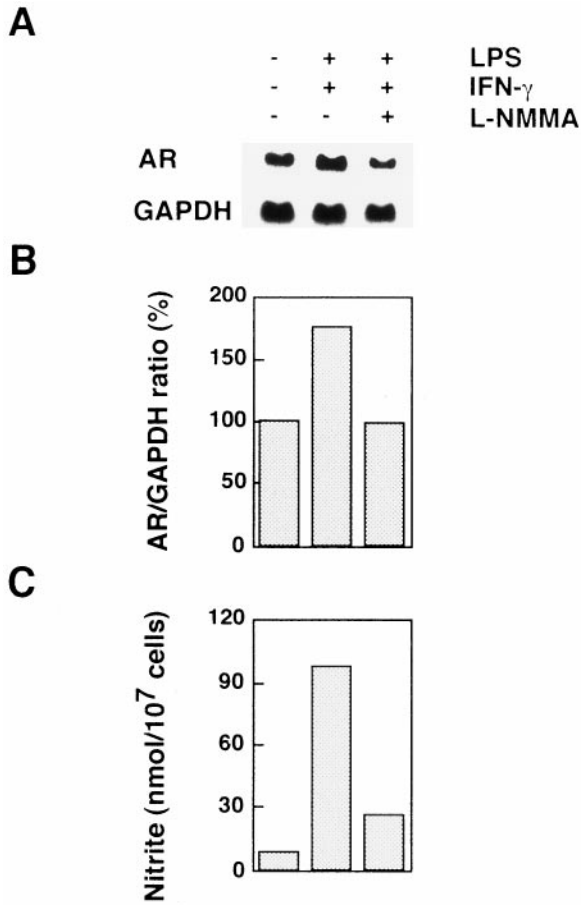

Fig. 6. Effects of endogenously generated NO on AR mRNA expression in RAW 264.7 cells. A, RAW 264.7 cells were incubated with LPS (100 μg/ml) and IFN-γ (100 U/ml), or with 3 mM L-NMA for 48 h. Total RNA was extracted and subjected to Northern blot analysis. B, radioactivity of the signals was quantified and plotted as the percentage of control of AR to GAPDH mRNA ratios. C, levels of nitrite in the culture medium harvested after the 48-h incubation. The results are representative of two experiments.

Along with the intracellular effect, NO, applied exogenously, may independently elicit the AR gene expression by acting on the cell membrane. Fibroblast growth factors, either acidic or basic, and EGF were reported to induce AR mRNA and protein in rat astrocytes (Laeng et al., 1995; Jacquin-Becker and Labourdette, 1997). We also observed an increase in AR mRNA in SMCs treated with EGF (in preparation). These findings suggest the involvement of the membrane receptor-linked tyrosine kinase signaling pathway in the induction of AR by growth factors. To clarify whether a similar transduction pathway mediates the induction of AR by NO, the effect of an inhibitor of tyrosine kinase was examined. The NO-induced augmented expression of AR was not affected in the presence of genistein, which inhibits the autophosphorylation of EGF receptor kinase. Accordingly, the NO-induced AR gene expression may be mediated by a pathway distinct from the tyrosine kinase signaling pathway. Because NO is reported to activate membrane associated ion channels and G proteins (Lander et al., 1993; Bolotina et al., 1994), the possibility that the signaling cascade is initiated at either of these sites remains.

The NO-releasing compound SNAP caused a significant concentration-dependent decline in the viability of SMCs. Under high glucose conditions with accelerated proliferation of the cells, the cytotoxic effect of SNAP was further augmented. NO produced during inflammatory reactions is known to act as a signaling molecule as well as a toxic effector. These effects of NO are highly tissue specific. In eosinophils (Beauvais et al., 1995) and hepatocytes (Kim et al., 1997), NO inhibits the programmed cell death induced by cytokines and promotes cell survival. In contrast, cytotoxic actions of NO directed against invading microorganisms can simultaneously affect susceptible host cells. In RAW 264.7 macrophages (Messmer et al., 1995), cardiac myocytes (Ing et al., 1999), and vascular smooth muscle cells (Zhao et al., 1997; Iwashima et al., 1998), NO is known as a potent inducer of apoptosis. The present study demonstrated that the cytotoxic effect of SNAP was enhanced when the activity of AR was suppressed by the addition of AR inhibitors. This finding indicated that increased AR activity protected SMCs from NO-induced cell damage. The up-regulation of AR by

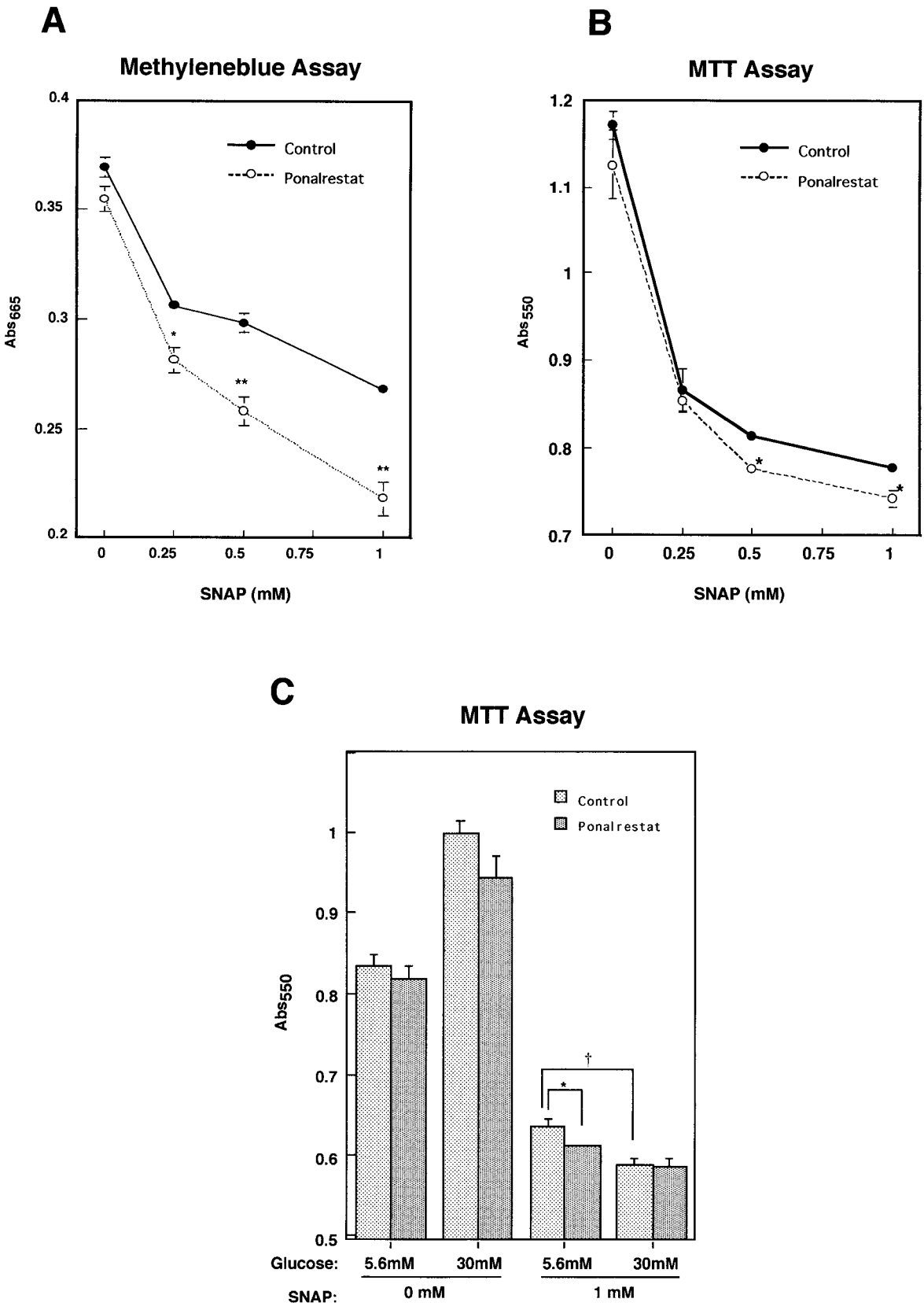

Fig. 7. Effects of SNAP and AR inhibition on cell viability of SMCs determined by the methylene blue assay (A) and MTT assay (B and C). Cells were incubated with various concentrations of SNAP in the absence (●) or presence (○) of 50 μM ponalrestat for 24 h. A, vertical columns represent the means ± S.E. (n = 3). *P < .05, **P < .01, compared with control. B, vertical columns represent the means ± S.E. (n = 5). *P < .05, compared with control. C, effect of high glucose on the cell viability of SMCs treated with SNAP. Cells were incubated with SNAP in the absence or presence of 50 μM ponalrestat for 24 h either in the low glucose- (5.6 mM) or high glucose (30 mM)-containing medium. Vertical columns represent the means ± S.E. (n = 5). *P < .05, $^{\dagger}P$ < .05.

NO may therefore be of physiological significance as a self-defense mechanism against the cytotoxic action of NO. This effect of AR can be attributed to the wide substrate specificity of the enzyme not only for glucose but also for reactive aldehydes produced in the cell. Because such toxic aldehydes as 4-hydroxynonenal, generated by oxidative damage to unsaturated fatty acids, are good substrates for AR (Vander Jagt et al., 1995), augmented expression of AR may be adapted to detoxify reactive aldehydes formed in the NO-exposed cells. Under high glucose conditions when the cytotoxic action of SNAP was augmented, however, inhibition of AR activity had no effect on the survival of SMCs. The reason for such a discrepancy in the effects of the AR inhibitor at different glucose concentrations is unclear. With increased intracellular glucose, the flux through the polyol pathway may be accelerated. If augmented flux through this pathway enhances the cell susceptibility to NO, inhibition of AR activity that blocks the pathway may set off the exacerbating effect of the AR inhibitor on cell damage elicited by NO.

A significant decrease in intracellular glutathione was reported in SMCs treated with NO donors, and restoration of the level of reduced glutathione by addition of glutathione monoethylester prevented the NO-induced programmed cell death (Zhao et al., 1997). We also observed enhanced cytotoxicity of SNAP in the SMCs pretreated with buthionine sulfoximine, which attenuates the cellular glutathione level (data not shown). In contrast, pretreatment of SMCs with a glutathione precursor NAC blunted NO-induced up-regulation of AR. These results suggest that endogenous levels of glutathione primarily affect cellular susceptibilities to NO, and increase in AR activity may be the mechanism that protects cells against NO-induced cell death. When cells are exposed to NO in high glucose-containing medium, up-regulation of AR by NO may further accelerate the flux of glucose through the polyol pathway. This may aggravate the depletion of NADPH, the cofactor for AR, to affect the activity of glutathione reductase, which maintains the level of reduced glutathione in the cell. Accordingly, the enhanced cytotoxic action of SNAP under high glucose conditions shown in this study may be attributed to the decreased levels of intracellular glutathione.

A role for NO as a proapoptotic factor for SMCs in the process of vascular remodeling has been suggested (Pollman et al., 1996; Zhao et al., 1997; Iwashima et al., 1998). Vascular remodeling is propagated not only by cellular proliferation and migration but also by apoptosis. NO-induced apoptosis may take part in arterial remodeling after vascular injury or in other pathological conditions in which cell proliferation is altered. Hypertension, atherosclerotic lesions, and diabetes are among the diseases associated with an impairment of vascular remodeling. In view of the present findings, augmented expression of AR may repress the NO-induced cell death and possibly hinder vascular remodeling. Although an association between the level of AR expression and the development of diabetic complications has been suggested (Nishimura et al., 1994; Yagihashi et al., 1996), the up-regulation of AR in vascular tissues under pathological conditions has not been taken into account until recently. The present findings may cast light on a potential role for AR in modulating vascular remodeling, and may lead to a better understanding of the clinical efficacy of AR inhibitors in diabetic patients.

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