Nitric Oxide Inhibits Matrix Metalloproteinase-2 Expression via the Induction of Activating Transcription Factor 3 in Endothelial Cells

Hsuan-Hsu Chen and Danny Ling Wang

Graduate Institute of Life Sciences, National Defense Medical Center (H.-H.C.) and Institute of Biomedical Sciences, Academia Sinica (D.L.W.), Taipei, Taiwan

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ABSTRACT

Nitric oxide (NO) has been shown to inhibit migration of cells in which various matrix metalloproteinases (MMPs) are involved. The underlying molecular mechanisms of this inhibition remain elusive. Endothelial cells (ECs) constitutively produce MMP-2. The effect of NO on MMP-2 expression was examined. A dose-dependent inhibition of MMP-2 mRNA level was demonstrated in ECs treated with NO. ECs infected with adenovirus carrying endothelial NO synthase (Ad-eNOS) reduced MMP-2 expression. The inhibitory effect of NO on MMP-2 expression was a transcriptional event because NO reduced MMP-2 promoter activity. NO treatment of ECs consequently suppressed MMP-2 secretion revealed by zymographic assay. Functional analysis of MMP-2 promoter (1716 base pairs) indicated that the p53-binding site (−1659 to −1629) was crucial for MMP-2 promoter activity. Activating transcription factor 3 (ATF3) has been reported to act as a transcriptional repressor for p53. ECs treated with NO induced ATF3 expression. Consistently, Ad-eNOS-infected ECs showed an increase of ATF3 level. Moreover, ECs either over-expressed ATF3 or, when treated with an ATF3 activator (MG-132; carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), resulted in a repression of MMP-2 promoter activity. Because of MMP-2 suppression by NO, ECs treated with NO inhibited endothelial migration, a phenomenon similar to that of ECs treated with MMP-2 antibody or MG-132. These results indicate that NO-attenuating endothelial migration is mediated at least in part by its reduction of MMP-2 expression via the up-regulation of ATF3. This study provides a molecular basis that supports the notion that NO acts as a negative regulator in endothelial migration.

Nitric oxide (NO) produced from NO synthase is involved in various biological functions. Endothelial cells (ECs) lining the vascular wall constantly release NO, which is crucial in maintaining vascular integrity. ECs release NO mainly via the activation of endothelial NO synthase (eNOS), a constitutively expressed enzyme. The released NO has multiple functions on the vascular cells including the migration and/or proliferation of endothelial cell and smooth muscle cell (SMC), platelet aggregation, and leukocyte adhesion. However, the detailed molecular mechanism of how NO affects cellular physiology remains unclear.

Studies of NO effects on cells have yielded controversial results. NO is well known to activate intracellular guanylyl cyclase to produce cGMP, which affects cGMP-dependent protein kinase. However, the effects of NO on cells are complex, in which both the cGMP-dependent and -independent signaling pathways may be involved (Maulik et al., 1996). The consequence of NO effects on cells varies depending on the NO donor used and the duration of NO treatment (Gooch et al., 1997).

Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent enzymes that degrade various extracellular matrix proteins and are thus important during atherogenesis and/or vascular remodeling (Deryugina et al., 1998). Among those MMPs, a 72-kDa proteinase known as matrix metalloproteinase 2 (MMP-2; also called type IV collagenase or ge-

ABBREVIATIONS: NO, nitric oxide; MMP-2, matrix metalloproteinase-2; ATF3, activating transcription factor 3; EC, endothelial cell; eNOS, endothelial nitric-oxide synthase; Ad-eNOS, adenovirus carrying eNOS; NOC18, 2,2′-(hydroxynitrosohydrazino)bis-ethanamine; DEA/NO, $(C_2H_5)_2N[N(O)NO]^-Na^+$; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; KT5823, (9S,10R,12R)-2,3,9,10,11,12, hexahydro-10-methoxy-2,9-dimethyl-1-ox0−9.12-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-I][1,6]benzodiazocine-10-carboxylic acid methyl ester; BrdU, bromodeoxyuridine; SMC, smooth muscle cell; BAEC, bovine aortic endothelial cell; FBS, fetal bovine serum; AS, Angeli's salt; m.o.i., multiplicity of infection; M-199, medium-199; BSA, bovine serum albumin; bp, base pair(s); JNK, c-Jun NH_2-terminal kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole).

latinase) has been shown to degrade mainly collagen, gelatin, and elastin (Okada et al., 1990). MMP-2 is constitutively produced by ECs and SMCs (Hanemaaijer et al., 1993; Galis et al., 1994). MMP-2 has been known for its role in growth factor-induced angiogenesis (Haas and Madri, 1999), and its gene expression is modulated during vascular remodeling, injury, and inflammation (Owens et al., 1997). A recent study showed that coronary vessel increased MMP-2 activities if NO synthase was inhibited (Matsunaga et al., 2002). Interestingly, SMCs infected with adenovirus carrying endothelial NO synthase (Ad-eNOS) also inhibited SMC migration via the inhibition of MMP-2 activities (Gurjar et al., 1999). However, there are controversial reports on the effects of NO on endothelial migration. ECs treated with NO or infected with Ad-eNOS were shown to increase endothelial migration (Kawasaki et al., 2003). In contrast, other studies demonstrated that NO donor or increased NO production inhibited endothelial migration (Lau and Ma, 1996; Kook et al., 2003). Similarly, an induction of eNOS expression in ECs inhibited endothelial migration (Tan et al., 1999). Thus, the detailed molecular mechanisms of how NO affects endothelial migration remain elusive.

Activating transcription factor 3 (ATF3), a member of the ATF/cAMP responsive element binding protein family of transcription factors, is a stress-inducible transcriptional repressor (Hashimoto et al., 2002). ATF3 is rapidly induced in response to stress in ECs, SMCs, and macrophages (Hai et al., 1999). In tumor necrosis factor α-induced apoptosis of ECs, ATF3 functions as a cell survival factor by down-regulating the transcription of p53 gene (Kawauchi et al., 2002). MMP-2 promoter contains a conserved consensus p53-binding site previously shown to up-regulate MMP-2 expression (Bian and Sun, 1997). Recent studies have shown that MMP-2 gene expression is down-regulated by ATF3 via the suppression of p53 *trans*-activation on the MMP-2 promoter region (Yan et al., 2002). This transcriptional repression is achieved by ATF3 interfering with p53-dependent *trans*-activation via a mechanism in which p53 transcriptional activity, but not DNA binding, is attenuated.

In this study, the role of ATF3 in the inhibitory effect of NO on MMP-2 expression was examined. Our results indicate that the inhibition of endothelial MMP-2 expression by NO is mediated at least in part via the induction of ATF3. Our study strongly indicates that NO exerts its role as a negative regulator during endothelial migration by inhibiting MMP-2 expression.

Materials and Methods

Reagents. A full-length human ATF3 cDNA subcloned into the pCGN vector, a cytomegalovirus promoter-driven expression vector, was a kind gift from Dr. Tsonwin Hai, Columbus, OH (Chen et al., 1994). DEA/NO [$(C_2H_5)_2N[N(O)NO]^-Na^+$, ($t_{1/2}$ ~17 min) and Angeli's salt (AS) ($O[N(O)NO]_2^-Na_2$) were kindly provided by Dr. David Wink, National Cancer Institute, Bethesda, MD (Wink et al., 1998). In contrast to nitric oxide (NO·) generated by NO donor, AS produced nitroxyl anion (NO$^-$), which is one electron reduction product of NO·. AS in saline was allowed to decompose for 72 h at room temperature before administration and was used as a control. In other experiments, another long-half-life NO donor (NOC18; $t_{1/2}$ ~57 h) was used. NOC18, MG-132, and a cGMP-dependent protein kinase inhibitor (KT5823) were obtained from Calbiochem (San Diego, CA). Bromodeoxyuridine (BrdU) was purchased from Roche (Mannheim, Ger-

many). The anti-p53, anti-eNOS, and anti-BrdU were obtained from BD Biosciences PharMingen (San Diego, CA). The recombinant MMP-2 was purchased from Oncogene Science (Cambridge, MA). Rabbit anti-ATF3 antibody (C-19) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to MMP-2 and tubulin were purchased from Lab Vision (Fremont, CA).

Endothelial and Saos-2 Cell Culture. Human umbilical cord vein ECs were isolated as described previously(Chiu et al., 1999). ECs were cultured in medium 199 (M-199) (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT). Second passage of confluent ECs was used for all experiments. Confluent ECs were treated with an ATF3 activator (MG-132) and a NO donor (DEA/NO or NOC18). Ninety percent of confluent ECs were used for infection with adenovirus carrying eNOS. For promoter assay, bovine aortic endothelial cells (BAECs) with no more than 17 passages and Saos-2 cells were used. BAECs were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human osteosarcoma p53-deficient Saos-2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 15% FBS.

Preparation of Adenovirus Carrying eNOS. The eNOS expression plasmid was kindly provided by Dr. P. F. Chen (University of Texas, Houston, TX). Replication-defective adenoviruses were produced as described elsewhere (Wung et al., 2001). Adenovirus shuttle plasmid vector pAd-CMV was kindly provided by Dr. S. H. Chen of Mount Sinai School of Medicine (New York, NY). The recombinant adenovirus carrying eNOS was prepared as described previously (Wung et al., 2001).

MMP-2 Promoter Construction and Deletion. Two oligonucleotide primers, 5'-cacacccaccagacaagcct-3' and 5'-aagccccagatgcgcagcct-3', were synthesized by the ABI 3948 DNA Primer (Bian and Sun, 1997). The MMP-2 promoter region (from −1659 to +57 bp) was generated by polymerase chain reaction. The 1716-bp fragment of 5'-flanking region was inserted into the luciferase reporter gene vector, PGL3-basic (Promega, Madison, WI). The 5'-deletion constructs of reporter genes were generated by restriction enzyme digesting and subcloning of DNA fragments from −1629, −1462, −1230, −908, and −240 to +57 into the SmaI and BglII sites of the pGL3-basic vector.

Gene Transfer. For adenovirus gene transfer study, ECs (1.0 × 10^7 cells) were infected with 100 plaque-forming units of virus per cell [multiplicity of infection (m.o.i.) 100] in serum-free M-199 for 2 h, followed by 48-h incubation with M-199 containing 20% FBS.

Measurement of Nitric Oxide Released from NO donor. Nitric oxide release was measured with the ISO-NOP, a Clark-type electrode (World Precision Instruments, Inc., Sarasota, FL). This electrode, converting a NO concentration (nanomolar) to an electric current (picoamperes), was connected to an amplifier (ISO-NO Mark II) and a data acquisition system (Duo-18), which were purchased from World Precision Instruments, Inc., for on-line recording and data processing. A chemically generated NO was used as a standard for the calibration of NO concentration in medium. Briefly, the standard curve of NO concentration was obtained based on the following reaction: $2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + 2H_2O + 2K_2SO_4$, where a series of KNO_2 concentrations was used and all chemicals were from Sigma-Aldrich (St. Louis, MO).

RNA Isolation and Northern Blot Analysis. Confluent ECs (10^6 cells/cm^2) were cultured in M-199 containing 0.1% BSA, as a control, and 0.1% BSA with NOC18 for 24 h. Total RNA from ECs was isolated using RNAzol B (Tel-Test Inc., Friendswood, TX). Each RNA sample (15 μg) was separated by electrophoresis and transferred onto a nylon membrane, Nytran (Schleicher & Schuell, Keene, NH), by a vacuum blotting system, VacuGene XL (Amersham Biosciences, Piscataway, NJ). Two oligonucleotide primers, 5'-tgggagcatggcgatggata-3' and 5'-acagtggacatggcggtctca-3', were synthesized (Nie et al., 1999) and a 493-bp fragment of MMP-2 cDNA was generated by polymerase chain reaction. After hybridization with an

[α-^{32}P]dCTP-labeled MMP-2 cDNA probe, the nylon membrane was washed with 1× standard saline citrate containing 1% SDS and then exposed to X-ray film (Kodak X-Omat-AR; Eastman Kodak, Rochester, NY). Autoradiographic results were scanned and analyzed using the UMAX 1220S scanner with a photo-mask (UMAX Corp., Hsin-Chu, Taiwan) and the AlphaEase 3.3 Image System (Alpha Innotech, San Leandro, CA), respectively.

DNA Fragmentation Assay. Confluent ECs (10^6 cells/cm^2) were cultured in M-199 containing only 0.1% BSA, 0.1% BSA with NOC18 (500 μM), and 2% FBS for 24 h. For DNA fragmentation assay, ECs were pelleted and resuspened in lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100). After incubation at 56°C with RNase A (100 μg/ml) and then with proteinase K (200 μg/ml) (Roche), the cell lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v/v). The DNA was precipitated with 100% ethanol and subsequently washed with 70% ethanol. The DNA was pelleted by centrifugation and was dissolved in 1× Tris/EDTA buffer. DNA was separated by horizontal electrophoresis on 1.5% agarose gel and then stained with ethidium bromide.

Zymographic Analysis of MMP-2. Confluent ECs (10^6 cells/cm^2) were cultured in serum-free M-199 containing only 0.1% BSA with or without NOC18 for 24 h. An equal volume (20 μl) of this conditional medium from ECs was applied to gel. The activities of MMP-2 were analyzed by using gelatin zymography. In brief, the conditional medium was applied to 10% SDS-polyacrylamide gel containing 0.1% gelatin. Commercially available MMP-2 was used as a reference. After electrophoresis, gels were incubated in 2.5% Triton X-100 for 1 h and then incubated in enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM CaCl$_2$, and 0.02% Brij-35) for 24 h. The gels were stained with 0.2% Coomassie Brilliant blue and destained with a solution containing 30% methanol and 10% acetic acid. The gels were scanned using the UMAX 1220S scanner with a photo-mask (UMAX Corp.).

Luciferase Assay. The constructs of pSV-β-galactosidase plasmid, pCGN-ATF3, PGL3-MMP-2, pCDNA3-wtp53, pCDNA3-mtp53 (R248W) (Di Como et al., 1999), and 5'-deletion DNA plasmids were purified by an EndoFree Plasmid Maxi Kit of the DNA Purification System (QIAGEN, Valencia, CA). Constructs were transfected into BAECs and Saos-2 cells using the LipofectAMINE method (Invitrogen). The transfected cells were seeded on 12-well culture plates (BAECs) and 60-mm Petri dishes (Saos-2) for 24 h. The cell extract was assayed for luciferase activity using the Luciferase Assay System (Promega). The pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency.

In Vitro Assay for Endothelial Migration. ECs in monolayer were disrupted using an ~0.5-mm cell scraper. ECs then were treated with either a NO donor (NOC18, 500 μM) or the monoclonal antibody to tubulin, as a control, MMP-2 (5 μg/ml), and MG-132 (5 μM) in M-199 containing only 0.1% BSA for 24 h. Endothelial migration was observed and photographed under a microscope. The results were photographed at 24 h after the injury using a Phase Contrast Video-microscope System (Nikon DIAPHOT, Hamamatsu C2400, and Sony Color Video Printer). To study the endothelial proliferation, BrdU (100 μM) was preincubated with ECs, followed by fixation with 50% methanol and 50% acetone. Incorporated BrdU was detected with mouse anti-BrdU antibody followed by goat anti-mouse fluorescein isothiocyanate-conjugated antibody. The BrdU staining in ECs was photographed under a microscope (BX60; Olympus, Tokyo, Japan) using a digital camera (Kodak DCS420).

Immunoblot Analysis. ECs were lysed with buffer containing SDS and β-mercaptoethanol and subjected to 10% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane (Schleicher & Schuell). The membrane was probed using antibody to ATF3, eNOS, MMP-2, p53, and tubulin and then analyzed using horseradish peroxidase-labeled rabbit anti-mouse IgG. Results were detected using an ECL Detection System (Amersham Biosciences). Autoradiographic results were scanned using the UMAX 1220S scanner with a photo-mask.

Statistical Analysis. Statistical analyses were performed using the paired Student's t test for experiments consisting of two groups or by analysis of variance for experiments consisting of more than two groups. Data are presented as mean ± S.E.M. Statistical significance was defined as probability values (P) < 0.05.

Results

NO Donor Releases NO and Is Noncytotoxic to ECs. ECs constantly release a basal level of NO, which is important for maintaining vascular integrity. The molecular mechanism of NO effects on vascular cells remains unclear. To study the direct effects of NO on ECs, NO donor was used in this study. To assess the NO concentration in the medium to which ECs were actually exposed, NO released from a NO donor (DEA/NO or NOC18) into cultured medium was analyzed. Using KNO$_2$-generated NO as a standard, the standard curve of NO concentrations was obtained (Fig. 1A). NO released into medium was thus determined to be NO donor concentration-dependent (Fig. 1B). Although each NO donor was added initially with the same concentration, the actual NO released into cultured medium was distinctly different by nearly 10-fold between DEA/NO ($t_{1/2}$ ~17 min) and NOC18 ($t_{1/2}$ ~57 h). As expected, NOC18 released NO with a slower but sustained rate. To avoid the potentially oxidative damage of ECs exposed to a high concentration of NO, NOC18 was thus used for the remaining experiments. ECs treated with NOC18 (up to 500 μM) for 24 h did not show any DNA fragmentation (Fig. 1C). These ECs remained viable after trypan blue staining (data not shown). Although NO has been shown to increase migration of ECs (Kawasaki et al., 2003), NO inhibition of endothelial migration has also been reported (Lau and Ma, 1996; Kook et al., 2003). Thus, the detailed mechanisms of NO effects on endothelial migration remain to be defined.

Endothelial NO Suppresses MMP-2 Activity via the Inhibition of MMP-2 Gene Expression. To elucidate whether NO affects MMP-2 gene expression, ECs were treated with NOC18, and MMP-2 mRNA levels were subsequently analyzed. As shown in Fig. 2A, NOC18 treatment of ECs for 24 h inhibited the MMP-2 mRNA expression in a dose-dependent manner. Similarly, ECs treated with DEA/NO for 3 h inhibited MMP-2 mRNA expression (data not shown). NO is known to increase intracellular cGMP (Kelm and Schrader, 1990). However, ECs pretreated with a cGMP-dependent protein kinase inhibitor (KT5823, 1 μM) for 30 min followed by NO treatment did not alter the inhibitory effect of NO on MMP-2 mRNA expression (Fig. 2B), indicating that cGMP-dependent protein kinase was not involved. In addition, the suppression of MMP-2 promoter activity was shown in ECs treated with NOC18 or DEA/NO. In contrast, ECs treated with Angeli's salt, which contains the same functional group as DEA/NO but does not yield NO (Wink et al., 1998), exhibited no inhibitory effect on MMP-2 promoter activity (Fig. 2C). These results strongly indicate that the NO suppression of MMP-2 gene expression is a transcriptional event. Consequently, ECs treated with NOC18 for 24 h showed a decrease of MMP-2 activity, assayed by zymography, in cultured medium compared with those ECs from controls (Fig. 2D). Furthermore, NO had no direct effect on MMP-2 enzyme activity because MMP-2 activity was not altered when collected cultured medium was

directly incubated with a NO donor (data not shown). NO donor applied to cultured medium has the drawback of a relatively short half-life. To overcome this problem and also to confirm that the NO effects are physiologically relevant, ECs were infected with replication-free Ad-eNOS that has been successfully used for the study of the biology of endothelial cells (Zanetti et al., 2000; Wung et al., 2001; Katusic et al., 2003). ECs infected with Ad-eNOS significantly increased their eNOS protein levels (Fig. 3A). Accordingly, these Ad-eNOS-infected ECs were shown to have reduced MMP-2 mRNA levels (Fig. 3B) and decreased MMP-2 protein levels (Fig. 3C). Thus, NO released from ECs reduced the MMP-2 activity via the inhibition of MMP-2 gene expression.

NO Induces ATF3 Expression and Results in the Suppression of MMP-2. To elucidate the inhibitory mechanism of NO on MMP-2, various lengths of MMP-2 promoter constructs containing luciferase were analyzed for their promoter activities by transient transfection of these constructs into bovine aortic ECs. As shown in Fig. 4A, the promoter activity was greatly reduced when the p53-binding region

$(-1629$ to $-1659)$ was deleted. These results are consistent with an earlier report which demonstrated that p53 plays an essential role in regulating basal expression of MMP-2 (Bian and Sun, 1997). ATF3 was reported to repress MMP-2 expression via the interference with the p53-dependent *trans*-activation of MMP-2 gene (Yan et al., 2002). Thus, the suppression of MMP-2 expression by ATF3 is p53-dependent. To confirm that our MMP-2 promoter construct was sensitive to ATF3 in the presence of p53 expression, human osteosarcoma p53-deficient Saos-2 cells were used to test the inhibitory effects of ATF3 on p53-dependent *trans*-activation of our MMP-2 promoter construct. As shown in Fig. 4, B and C, expression of a wild-type p53 in Saos-2 cells increased MMP-2 promoter activity. In contrast, expression of a mutant p53 did not affect the basal promoter activity of MMP-2. However, cells with ATF3 induction after treatment with an ATF3 activator (MG-132) attenuated the p53-dependent promoter activity of MMP-2. Our results confirm that our MMP-2 promoter construct is sensitive to the ATF3 expression in the presence of p53. To further assess whether ATF3
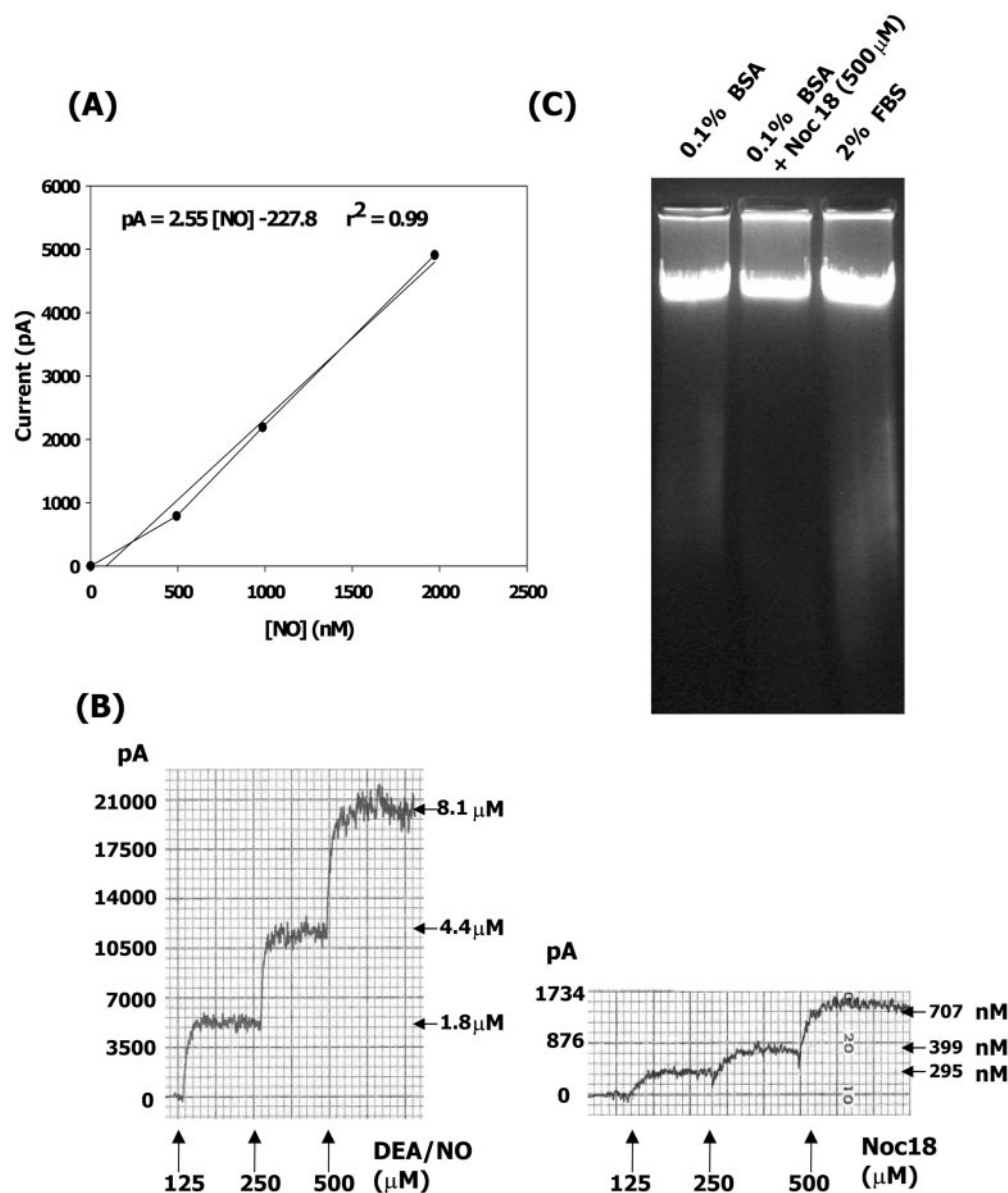
Fig. 1. NO donor releases NO in the cultured medium and its effects on cultured ECs. NO concentration was determined with a nitric oxide sensor as described under *Materials and Methods*. A, NO concentration (nanomolar) versus current (pico-amperes) standard curve was obtained with the NO generated from a series of KNO_2 concentrations. B, NO released from NO donor (DEA/NO or NOC18) at different concentrations was measured immediately after its addition into the culture medium at 37°C. C, ECs in M-199 containing 0.1% BSA (2% FBS as a reference control) were incubated with or without NOC18 (500 μM) for 24 h, and DNA was collected and separated on agarose gel and then stained with ethidium bromide.
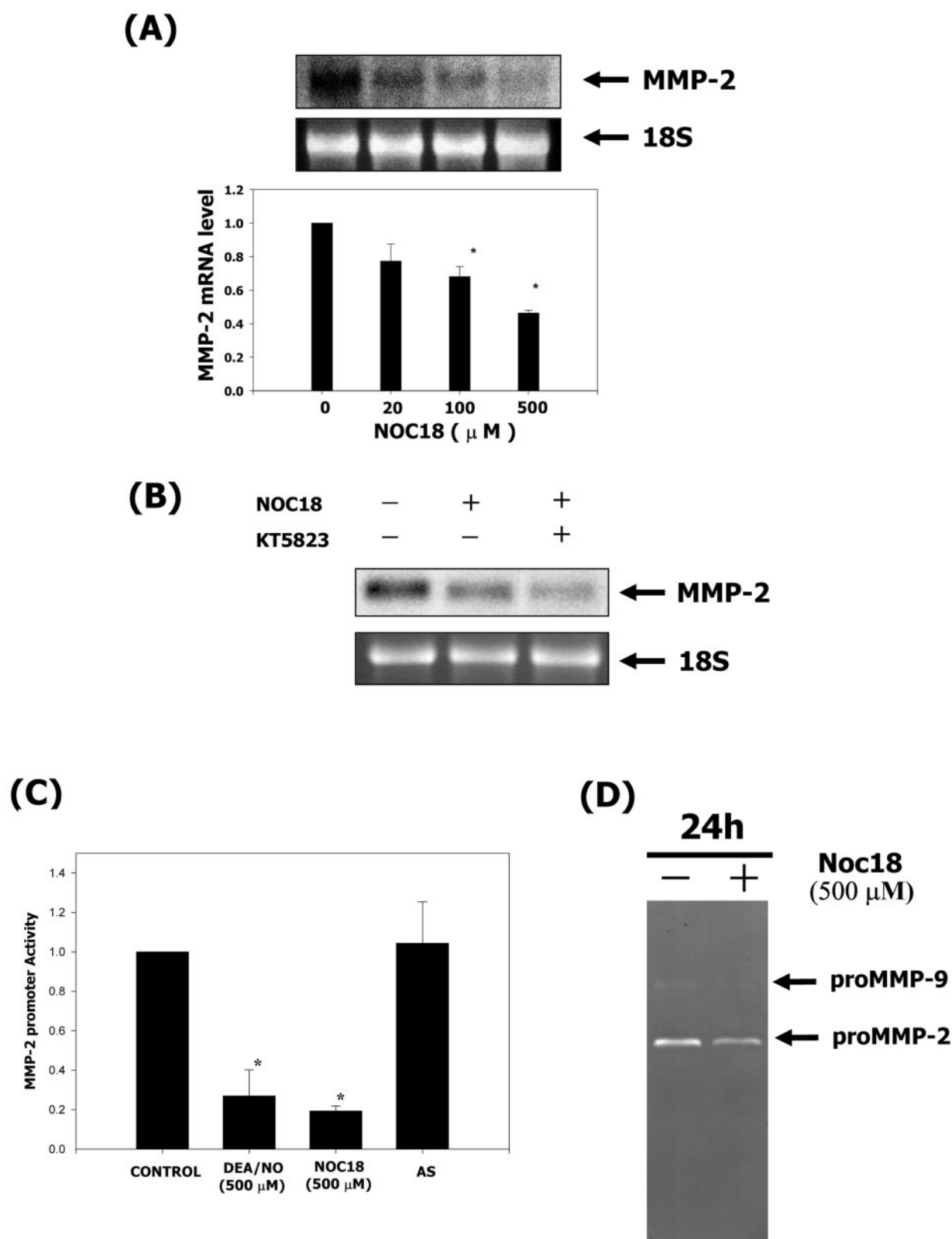
Fig. 2. NO inhibition of MMP-2 gene expression is a transcriptional event and this inhibitory effect is cGMP-independent. Total RNA (15 μg/lane) separated on a 1% formaldehyde-agarose gel was transferred to a nylon membrane and probed with the [α-^{32}P]MMP-2 cDNA, with 18S used as an internal control. A, MMP-2 mRNA levels from ECs treated with NOC18 at various concentrations for 24 h. B, ECs were treated with NOC18 (500 μM) in the presence of KT5823 (1 μM), a cGMP-dependent protein kinase inhibitor, followed by Northern blot analysis. C, a chimera construct containing MMP-2 promoter region (1716 bp) and reporter gene luciferase were transfected into BAECs and then exposed to DEA/NO (500 μM), NOC18 (500 μM), or AS (500 μM). AS in saline was allowed to decompose for 72 h and then was applied onto cultured ECs as a control. ECs were cotransfected with pSV-β-galactosidase to normalize the transfection efficiency. Data are shown as fold induction relative to those ECs without NO treatment. D, pro-MMP-2 and pro-MMP-9 collected from cultured medium from ECs incubated with NOC18 (500 μM) for 24 h were analyzed for their pro-MMP-2 and pro-MMP-9 activity by gelatin zymography. Results are mean $\pm$ S.E.M. from at least three independent experiments. $\star$, $P < 0.05$ versus controls.

is induced in ECs after NO treatment, ECs after NO treatment were checked for ATF3 contents. NOC18 treatment of ECs induced ATF3 expression in a dose- and time-dependent manner (Fig. 5, A and B). Consistently, ECs infected with Ad-eNOS significantly increased ATF3 protein levels (Fig.

5C). To assure the inhibitory effect of ATF3 on MMP-2 promoter activity, ECs were cotransfected with MMP-2 promoter construct with pCGN vector, as a control, or ATF3 over-expression plasmid (pCGN-ATF3). ECs over-expressed with ATF3 increased ATF3 protein levels in a dose-depen-

(A)

(B)
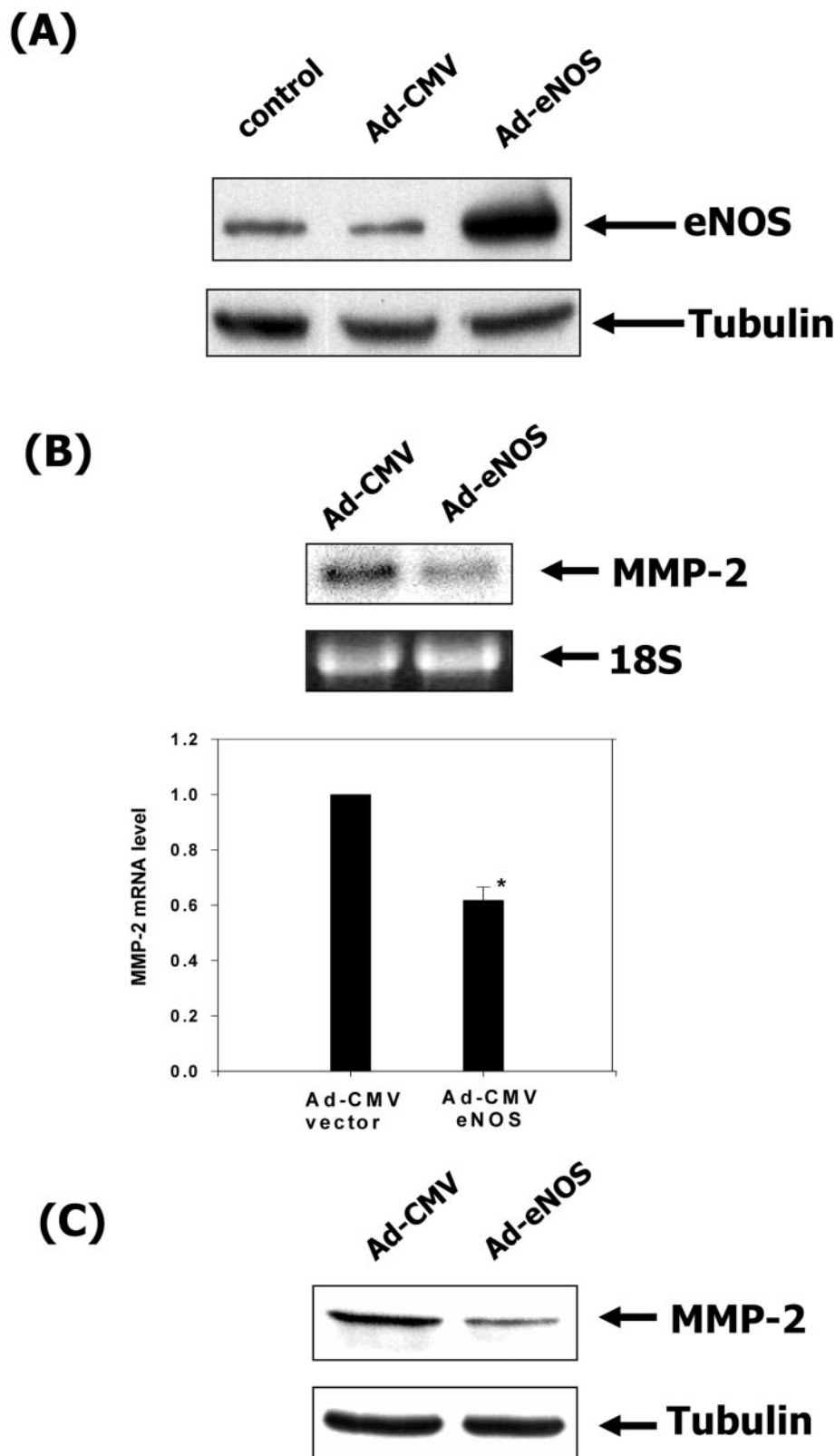
Fig. 3. ECs with eNOS over-expression inhibits MMP-2 expression. A, ECs infected with Ad-eNOS (100 m.o.i.) increased their eNOS protein level. ECs infected with adenovirus carrying empty vector (Ad-CMV) were used as controls. B, ECs infected with Ad-eNOS or Ad-CMV were incubated for 48 h. Total RNA samples were collected and analyzed for MMP-2 mRNA levels by Northern blot. Results are presented as ratio of controls with band density normalized to 18S RNA. C, ECs infected with Ad-eNOS or Ad-CMV were incubated for 48 h. Total protein samples were collected and analyzed for MMP-2 protein levels by Western blot. Data are shown as mean ± S.E.M. from at least three independent experiments. ⋆, $P < 0.05$ versus control ECs.

(C)

dent manner (Fig. 6A). The promoter activity of MMP-2 was suppressed by the over-expression of ATF3 in ECs (Fig. 6B). ECs treated with an ATF3 activator (MG-132) were also shown to increase ATF3 expression in a dose-dependent manner (Fig. 6C). Consistently, this ATF3 induction in ECs dose dependently inhibited the MMP-2 promoter activities (Fig. 6D). Our results clearly show that ECs exposed to NO reduce their MMP-2 expression via the induction of ATF3.

Endothelial Migration Is Inhibited by NO. A migration assay of ECs was performed by scrapping ECs at the middle of the Petri dish, leaving this area devoid of cells (Fig. 7A). This assay revealed that endothelial migration was attenuated after treating ECs with NOC18 for 24 h. This phenomenon was similar to those of ECs treated with either a monoclonal antibody to MMP-2 or the ATF3 activator (MG-132) to induce ATF3 expression. In contrast, ECs treated with a monoclonal antibody to tubulin as a negative control did not affect migration. Using BrdU to identify the newly synthesized nuclear DNA, no apparent difference was found between the control and NO-treated ECs (Fig. 7B) in the
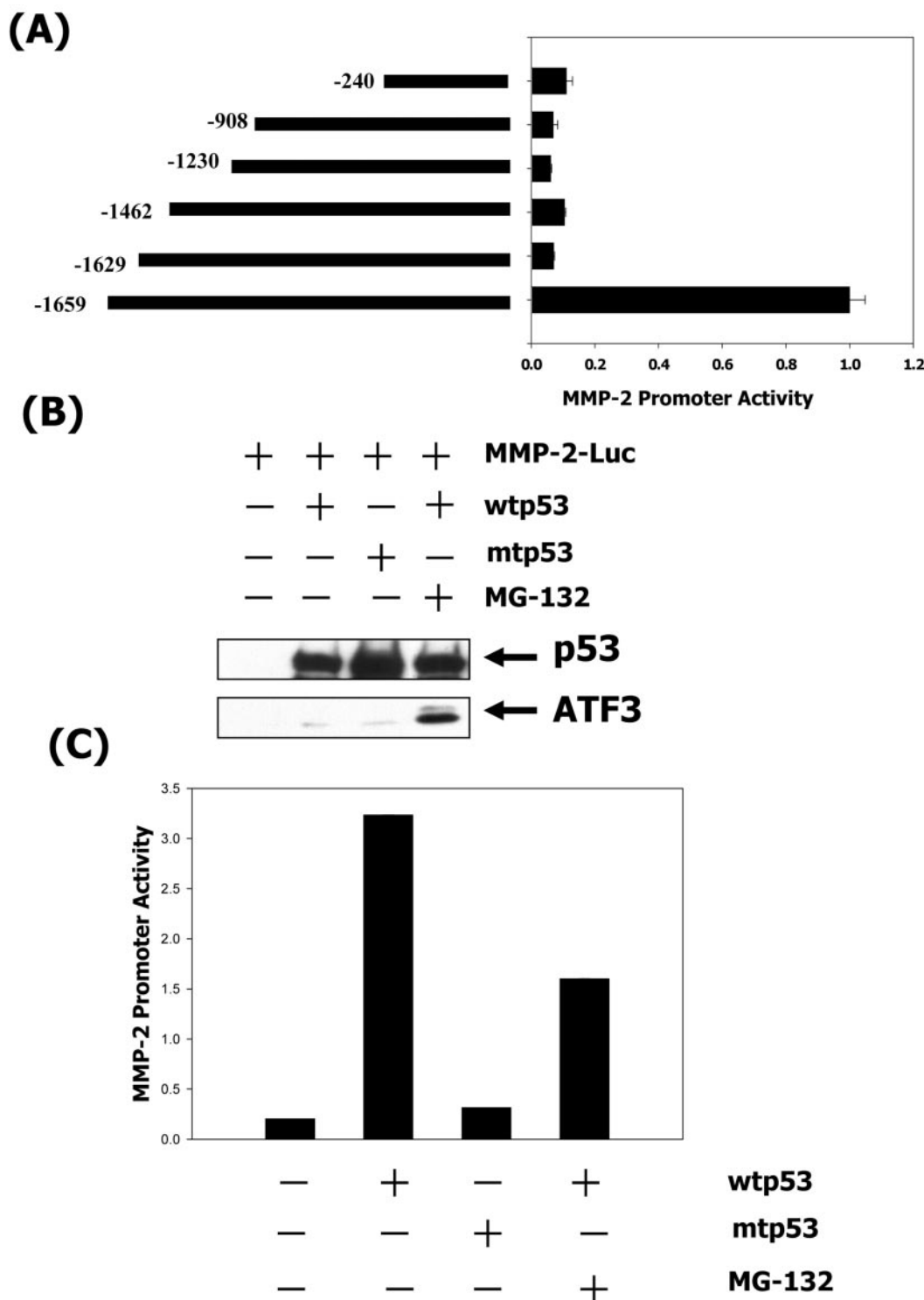

Fig. 4. The p53-binding region is essential for MMP-2 promoter activity. A, the MMP-2 promoter activities from a series of deletion constructs are shown and were transiently transfected into BAECs. The p53-binding region is located between −1659 and −1629. B, osteosarcoma p53-deficient Saos-2 cells transfected with pSV-β-galactosidase (0.6 μg) and PGL3-MMP-2 (1.2 μg) were transiently cotransfected with either an empty vector (pCDNA3-CMV, 35 ng), or a wild-type (pCDNA3-wtp53, 35 ng) or mutated (R248W) p53 (pCDNA3-mtp53, 35 ng). MG-132 (5 μM) was immediately introduced to cotransfected Saos-2 cells. An equal amount of total cell lysate was resolved by electrophoresis and subjected to Western blotting using either an anti-p53 or anti-ATF3 monoclonal antibody. C, the MMP-2 promoter activity was measured 24 h after transfection. Data are represented as mean ± S.E.M. from at least three experiments.

DNA synthesis near the wounded region. This suggests that NO preferentially inhibits mainly endothelial migration rather than proliferation.

In summary, this study demonstrates clearly that NO treatment of ECs inhibits basal MMP-2 expression. NO-induced ATF3 expression contributes at least in part to this MMP-2 inhibition. NO seems to exert its role as a negative regulator in regulating endothelial migration and, thus, plays an important role during vascular remodeling after vessel injuries.

Discussion

MMP-2 plays an important role during vascular remodeling, injury, and inflammation (Owens et al., 1997). The regulation of gene expression and activity of various MMPs are complex and NO seems to play a role. NO was shown to attenuate the MMP-9 activity by reducing MMP-9 expression (Eberhardt et al., 2000). MMP-2 activation is suppressed by a NO donor in human breast cancer cells (Zhang et al., 2002). However, NO has been shown to inhibit the expression of MMP-9, but not MMP-2, in rat smooth muscle cells (Upchurch et al., 2001). Earlier study demonstrated that NO stimulated the activity of a 72-kDa MMP in mesangial cells (Trachtman et al., 1996). Ad-eNOS gene transfer to increase endogenous NO inhibits SMC migration via the inhibition of
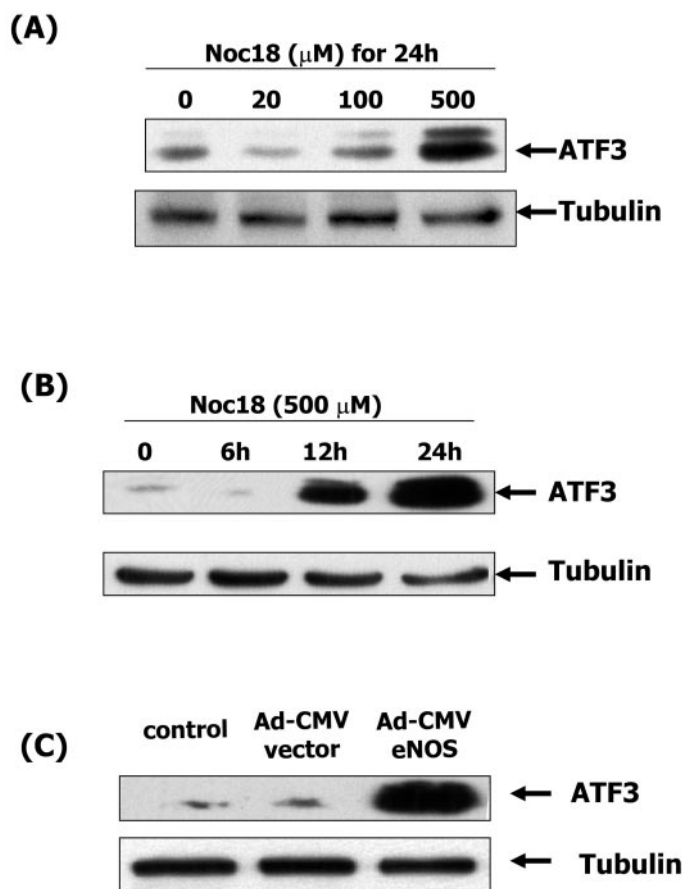
(A)

(B)

(C)

Fig. 5. ECs treated with NO induce ATF3 expression. A and B, NOC18 treatment of ECs induced ATF3 protein expression in a dose- and time-dependent manner. C, ECs infected with Ad-eNOS (100 m.o.i.) increased ATF3 protein expression. ECs infected with an adenovirus carrying empty vector (Ad-CMV) were used as controls.

MMP-2 and MMP-9 activities (Gurjar et al., 1999). Furthermore, mice with genetic deletions of either MMP-2 or MMP-9 resulted in significant impairment of SMC migration (Johnson and Galis, 2004). In the present study, NO did attenuate basal secretion of pro-MMP-2 in ECs. The discrepancy regarding the effects of NO on MMPs is not clear; it might be attributable to the different cell types or might reflect the local NO concentration to which cells are exposed after NO influx. Various NO donors with distinct NO production rate may also trigger different cellular responses (Gooch et al., 1997; Thomas et al., 2002). In this study, NO was studied for its effect on basal MMP-2 expression in ECs and was shown to inhibit MMP-2 expression via the induction of ATF3. This inhibitory effect of NO on MMP-2 expression was shown in ECs either treated with a NO donor or infected with adenovirus carrying eNOS. The NO donor (NOC18) used here has a long half-life. NO released from NOC18 into culture medium was measured and was shown to be released at a stable rate with a comparable concentration within a normal physiological range. This NO donor applied to ECs neither caused DNA fragmentation nor induced apparent morphological changes. The effects of NO on cellular physiology are complex and can be mediated via cGMP-dependent and/or -independent pathways. However, the inhibitory effect of NO on endothelial migration in this study seemed to be mediated predominantly via a cGMP-independent pathway. This is in agreement with our previous report showing that NO attenuating the shear stress-induced Egr-1 expression is not mediated via cGMP (Chiu et al., 1999). A recent study showed that activation of MMP-9, but not MMP-2, via a direct S-nitrosylation by NO led to neuronal cell death (Gu et al., 2002). In the present study, NO donor incubated with collected culture medium did not change the MMP-2 activity. The present study shows that NO inhibition of endothelial migration is mediated at least in part via the suppression of basal MMP-2 expression. Several lines of evidence support this notion. First, NO donor dose dependently inhibited the basal MMP-2 expression. Second, NO reduced the MMP-2 promoter activity. Third, NO treatment resulted in a decrease of basal MMP-2 secretion into the culture medium. Consistently, ECs infected with adenovirus carrying eNOS showed an attenuation of MMP-2 expression. Finally, NO inhibited endothelial migration, a phenomenon similar to those of ECs treated with antibody to MMP-2 or with an ATF3 activator (MG-132). It was also noted, based on the in vitro migration assay, that the NO donor-treated group showed no significant difference in DNA synthesis near the wounded region compared with the control group, indicating that NO has minor effects on endothelial proliferation. Our results thus strongly support that the inhibitory effect of NO on endothelial migration is acting on basal expression of MMP-2.

We further demonstrated that NO induced a dose- and time-dependent induction of ATF3 expression and that this ATF3 induction resulted in the inhibition of MMP-2 promoter activity. ATF3 is an immediate early gene that can repress genes by binding to the cis-element of target genes (Hai et al., 1999). Kawauchi et al. (2002) clearly demonstrated that ATF3 acts as a transcriptional repressor for p53 and ATF3 protects ECs from tumor necrosis factor-induced apoptosis. A recent study shows that ATF3 suppresses $trans$-activation of a p53-binding region on MMP-2 promoter (Yan
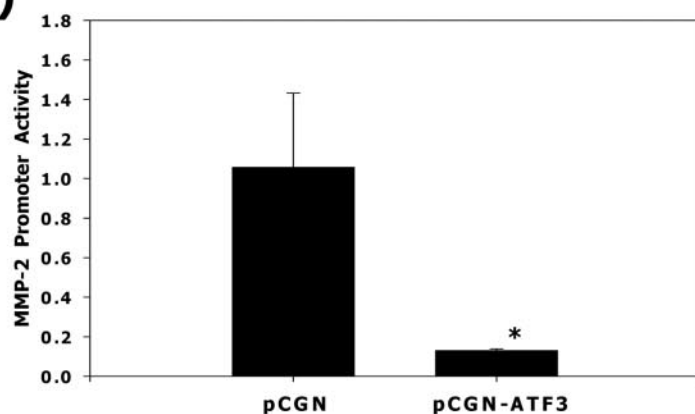
et al., 2002). ATF3 thus antagonizes p53-dependent MMP-2 promoter activity. In this study, the promoter activity of MMP-2 was greatly reduced when the p53-binding region (-1629 to -1659) was deleted, illustrating the importance of p53 in the regulation of MMP-2 expression on ECs. However, there was no significant difference regarding the p53 binding
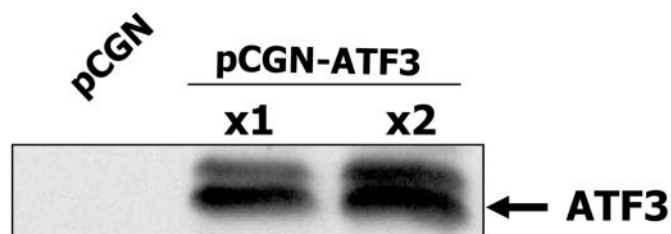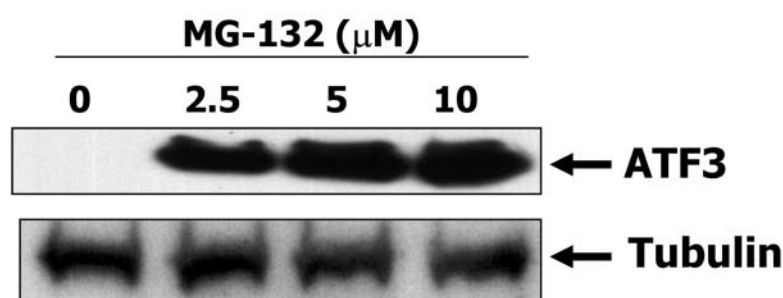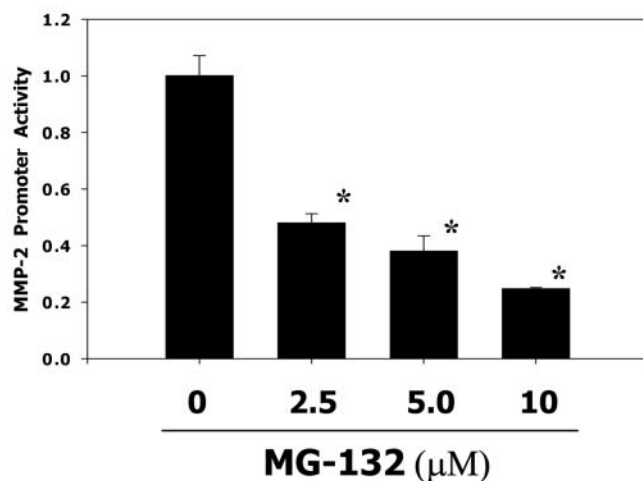

Fig. 6. ECs with ATF3 over-expression inhibit MMP-2 expression. ECs were cotransfected with MMP-2 promoter construct with pCGN vector, as a control, or ATF3 over-expression plasmid (pCGN-ATF3). A, ECs over-expressing ATF3 increased ATF3 protein levels. B, the promoter activity of MMP-2 was suppressed by the over-expression of ATF3 in ECs. C, ECs transfected with pGL3-MMP-2 and then treated with different doses of ATF3 activator (MG-132) up-regulated ATF3 protein levels. D, MMP-2 promoter activity is shown in a dose-dependent inhibition by MG-132. Data are represented as mean $\pm$ S.E.M. from at least three experiments. ⋆, $P < 0.05$ versus control ECs.

activity to the corresponding oligonucleotides in the MMP-2 promoter with the nuclear extracts from either control or NO-treated ECs (H.-H. Chen and D. L. Wang, unpublished observations). In this study, ECs treated with NO increased ATF3, a phenomenon similar to that of ECs treated with an ATF3 activator (MG-132). Both treatments induced ATF3 expression and resulted in a suppression of MMP-2 promoter activity. The ATF3 induction by NO and the subsequent MMP-2 suppression are in agreement with that earlier report showing that ATF3 represses p53 transactivation (Yan et al., 2002).

The detailed mechanisms of ATF3 induction by NO are not clear. The c-Jun NH$_2$-terminal kinase (JNK) signaling pathway has been shown to participate in the induction of ATF3 (Cai et al., 2000). Stress signals via p38 kinase also induce ATF3 (Ichijo, 1999). ECs transiently transfected with mitogen-activated protein kinase kinase kinase (MEKK-1), upstream of the JNK pathway, induced ATF3 expression (H.-H. Chen and D. L. Wang, unpublished observations). H$_2$O$_2$-induced ATF3 is mediated via the JNK pathway (Shtil et al., 1999). However, ECs pretreated with p38 inhibitor (SB203580) attenuate the H$_2$O$_2$-mediated ATF3 induction in ECs (H.-H. Chen and D. L. Wang, unpublished observations). Thus, cells subjected to redox changes or under oxidative stress trigger the induction of ATF3. The ATF3 induction

varies among tissues and is shown to be stress kinase-dependent (Yin et al., 1997). Our results indicate that NO-induced ATF3 expression may be mediated via stress kinase activation. Furthermore, we have shown that the attenuation of redox-sensitive monocyte chemotactic protein-1 expression by NO is mediated by the suppression of reactive oxygen species produced by Rac-dependent NADPH oxidase (Wung et al., 2001). Rac, a member of the Rho family of proteins, has been suggested to modulate MMP-2 activity (Zhuge and Xu, 2001). Alternatively, NO may induce ATF3 expression via a Rac-dependent mechanism. The local NO concentration and the redox status as a result of the interplay between reactive oxygen species and reactive nitrogen species may determine the consequence of cellular responses including MMP-2 expression.

Our results suggest that arterial wall under oxidative stress or after injuries that consequently release insufficient NO may accelerate endothelial migration. In contrast, an increased NO production via the activation of eNOS by shear flow decreases endothelial migration. Our results demonstrate that NO reduces MMP-2 production by inhibiting MMP-2 gene expression via the induction of ATF3. The detailed signaling mechanisms of NO effects on ECs and how the ATF3 expression is regulated in ECs remain important questions and warrant further study.

Fig. 7. NO inhibits endothelial migration. A, endothelial migration was carried out by scraping ECs from the middle of a Petri dish, leaving an area with 500 μm (width) devoid of cells. ECs were washed and cultured in M-199 with 0.1% BSA either alone (Control) or with NO donor (NOC18). Some ECs were treated with antibody to MMP-2 (5 μg/ml) and MG-132 (5 μM). The results were photographed at 24 h after the injury using a Phase Contrast Video-microscope System. B, ECs without or with NOC18 treatment after 24 h of injury were fixed and analyzed for endothelial proliferation with BrdU incorporation. BrdU (100 μM) was preincubated with ECs followed by fixation. Incorporated BrdU was detected with mouse anti-BrdU antibody followed by goat anti-mouse fluorescein isothiocyanate-conjugated antibody. Results were photographed under a microscope using a digital camera.

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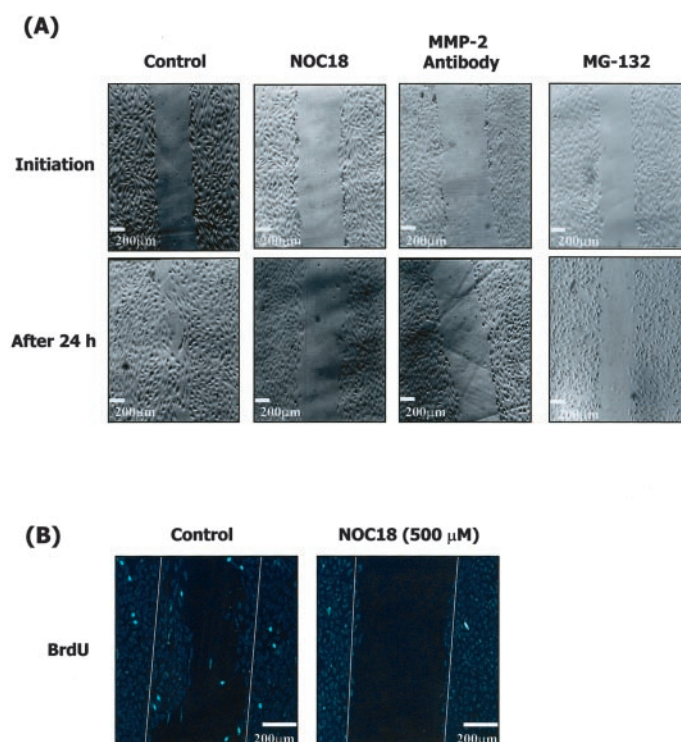
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Address correspondence to: Dr. Danny Ling Wang, Cardiovascular Division, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan 11529. E-mail: lingwang@ibms.sinica.edu.tw