Mechanism of inhibition of matrix metalloproteinase-9 induction by NO in vascular smooth muscle cells

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Gurjar, Milind V., Jason DeLeon, Ram V. Sharma, and Ramesh C. Bhalla. Mechanism of inhibition of matrix metalloproteinase-9 induction by NO in vascular smooth muscle cells. J Appl Physiol 91: 1380–1386, 2001.—Vascular smooth muscle (VSM) cell migration is a critical step in the development of a neointima after angioplasty. Matrix metalloproteinases (MMPs) degrade the basement membrane and extracellular matrix, facilitating VSM cell migration. Recently, we demonstrated that nitric oxide (NO) inhibits interleukin-1β (IL-1β)-stimulated MMP-9 induction in rat aortic VSM cells. In this study, we examined the hypothesis that NO inhibits MMP-9 induction by attenuating superoxide generation and extracellular signal-regulated kinase (ERK) activation. Stimulation of VSM cells with IL-1β significantly (P < 0.05) increased superoxide production, ERK activation, and MMP-9 induction. Pretreatment of VSM cells with the NO donor DETA NONOate significantly (P < 0.05) decreased IL-1β-stimulated superoxide generation. In addition, pretreatment of VSM cells with a specific ERK pathway inhibitor, PD-98059, or DETA NONOate inhibited IL-1β-stimulated ERK activation and MMP-9 induction. Direct exposure of VSM cells to increased superoxide levels by treatment with xanthine/xanthine oxidase increased ERK activation and MMP-9 induction, whereas pretreatment of cells with PD-98059 significantly (P < 0.05) inhibited xanthine/xanthine oxidase-stimulated ERK activation and MMP-9 induction. We conclude that NO inhibits IL-1β-stimulated MMP-9 induction by inhibiting superoxide generation and subsequent ERK activation.

reactive oxygen species; matrix metalloproteinases; extracellular signal-regulated kinase; vascular smooth muscle cells; interleukin-1β; nitric oxide

VASCULAR SMOOTH MUSCLE (VSM) cell migration plays an important role in the pathogenesis of atherosclerosis and restenosis after vascular injury (37). On injury, VSM cells migrate from the tunica media to the intima, leading to neointima formation (37). VSM cell migration requires breakdown of extracellular matrix (8, 28, 32). One possible mechanism by which VSM cells break down extracellular matrix is by secreting matrix metalloproteinases (MMPs) (8, 23, 32, 34). MMPs are increased at the site of vascular injury (2, 16), whereas naturally occurring tissue inhibitors of MMPs decrease VSM cell migration both in vitro and in vivo after vascular injury (12, 42). In this study, we investigated the signaling pathways involved in the interleukin-1β (IL-1β)-stimulated MMP-9 induction in VSM cells in vitro.

Elevated levels of cytokines and reactive oxygen species (ROS) are the characteristic features associated with atherosclerosis and restenosis after vascular injury (reviewed in Refs. 18, 21, 24). Cytokines, such as IL-1β, are released at the site of vascular injury (7, 13), stimulating superoxide generation in VSM cells (3). This increased superoxide generation plays an important role in the ongoing inflammation (21, 24, 26). Experimental models of hypercholesterolemia, hypertension, diabetes, and balloon-injured coronary arteries all show that ROS generation is enhanced in the blood vessel wall (19, 25, 30, 31). ROS generation has been further localized to the tunica media, suggesting a prominent role for VSM cells in their production (33). Directly exposing VSM cells to ROS-generating systems stimulates migration, proliferation, and growth, implicating ROS in these processes (18, 21, 24, 35). ROS activate several signaling pathways including Akt, mitogen-activated protein kinases (MAPK), nuclear factor-κB, and caspases in VSM cells (18, 21, 24, 27). Taken together, these data suggest that overproduction of ROS in vasculoproliferative diseases may promote VSM cell migration and proliferation.

We have shown that exogenous expression of endothelial nitric oxide (NO) synthase (eNOS) gene inhibits VSM cell migration and proliferation in vitro (10, 20, 38) and neointima formation in balloon-injured carotid arteries in vivo (11). eNOS gene expression and NO donors inhibited IL-1β-stimulated MMP-9 induction in VSM cells (20). Similar to our observations, other investigators recently demonstrated that NO inhibits IL-1β-stimulated MMP-9 induction in rat mesangial cells (9). How IL-1β stimulates MMP-9 induction and how NO inhibits this process in VSM cells is not fully understood. Recently, the MAPK pathway has been implicated in MMP-9 induction in breast epithelial cells (36). In addition, a recent study has shown that...
ROS stimulate MMP-9 induction in human fetal membranes (5). In this study, we addressed the role of ROS and the underlying signaling pathway(s) involved in MMP-9 induction in VSM cells.

Specifically, we investigated the role of the superoxide-ERK pathway in IL-1β-stimulated MMP-9 induction and the inhibitory effects of NO on this signaling cascade. To examine the mechanism of NO inhibition of MMP-9 induction, we treated cells with NO donor DETA NONOate, before IL-1β stimulation. MMP-9 induction was measured by gelatin zymography and RT-PCR. ERK activation was analyzed by Western blot analysis using a phosphorylated ERK (pERK)-specific antibody that binds only to activated ERK. To study the role of ERK in IL-1β-stimulated MMP-9 induction, cells were treated with the specific ERK pathway inhibitor PD-98059. Cells were treated with xanthine/xanthine oxidase to confirm the role of superoxide in ERK activation and MMP-9 induction. Our results show that IL-1β-stimulates MMP-9 induction via a superoxide-ERK-dependent pathway, and NO inhibits this pathway to inhibit MMP-9 induction in VSM cells.

MATERIALS AND METHODS

Materials. Chemicals and materials were obtained from the following sources: 10% gelatin zymography precast gels, renaturing buffer, developing buffer, and Seabluue molecular weight markers from NOVEX; human rIL-1β from R&D Systems; rabbit and mouse anti-mouse IgG-HRP conjugate and rabbit anti-ERK 1/2 antibody from Santa Cruz Biotechnology; PD-98059, mouse anti-pERK antibody, and mouse anti-phospho-p38 antibody from Cell Signaling Technology; SB-203580 from Upstate Biotechnology; DETA NONOate from Cyanam Chemicals; dihydroethidium (HE) from Molecular Probes; supersignal chemiluminescence detection kit, elution buffer from Pierce. Supercrypt one-step RT-PCR kit, cell culture media, and additives not listed were of the highest grade available from Life Technologies; chemicals not listed were of the highest grade available from Sigma Chemical.

Cell culture. Rats were maintained and used in compliance with the principles set forth in the “Guide for Care and Use of Laboratory Animals” and approved by the University of Iowa Animal Care and Use Committee. VSM cells were cultured from Wistar male rat (8–10 wk old; 200–300 g body wt) thoracic aorta as described previously (20, 38). Briefly, cultured from Wistar male rat (8–10 wk old; 200–300 g body wt) thoracic aorta as described previously (20, 38). Briefly, cells were grown in DMEM with low glucose supplemented with 10% fetal bovine serum and antibiotics (100 U/ml) for 48 h before stimulation and then divided into control and stimulated groups. Cells were treated with DETA NONOate (500 μM) or ERK pathway inhibitor PD-98059 (50 μM) or p38 MAPK pathway inhibitor SB-203580 (10 μM) dissolved in DMEM-0.1% BSA 1 h before IL-1β stimulation. After 1 h, serum-free DMEM-0.1% BSA was added to all cell culture dishes. IL-1β was added to the stimulated group at a final concentration of 5 ng/ml. Xanthine (100 μM) and xanthine oxidase (5 μU/ml) were added to dishes to generate superoxide. After 24 h, conditioned medium was collected, centrifuged to remove cell debris, and stored in aliquots at −80°C for future use.

Estimation of intracellular ROS. Intracellular ROS generation was determined by use of the redox-sensitive probe HE that is oxidized to ethidium by superoxide. The ethidium is detected as red nuclear fluorescence after binding to DNA (4). VSM cells were subcultured to 60–70% confluence on circular 25-mm glass coverslips in a six-well tissue culture dish and were serum starved in DMEM-0.1% BSA. Serum-starved cells were stimulated with IL-1β for 90 min in the presence or absence of DETA NONOate (500 μM). Cells were incubated for 30 min with 5 μM HE in DMEM-0.1% BSA and thereafter rinsed with DMEM-0.1% BSA and mounted in a temperature-controlled chamber, and fluorescence was detected by confocal laser scanning microscopy (Zeiss confocal laser scanning microscope). Excitation wavelength was 488 nm, and emission wavelength was 650 nm. HE did not produce significant autofluorescence in unstimulated cells. Images were collected and analyzed by using the Confocal Assistant Program.

Zymography. Gelatinase activity in conditioned media collected from cell cultures was measured by using zymography as previously described (20, 22). Equal amounts of conditioned media (10 μl) were subjected to electrophoresis using Novex 10% zymography gels containing 0.1% gelatin. Gels were washed with renaturing buffer (Novex) for 30 min and incubated at 37°C for 20 h in developing buffer (Novex). After 20 h, gels were stained with Coomassie blue stain. All gels were calibrated by use of Seabluue molecular weight marker (Novex). Gels were scanned and quantified by using Quantity One software (Bio-Rad).

Western blot analysis for pERK. Activation of ERK was estimated by measuring ERK phosphorylation with the use of a pERK-specific antibody that recognizes only activated ERK. Semiconfluent VSM cells were serum starved for 24 h, then treated with PD-98059 (50 μM) or DETA NONOate (500 μM) for 1 h and stimulated with xanthine/xanthine oxidase (100 μM-5 μM) or IL-1β (5 ng/ml). Cell lysates were collected after either 15 min or 4 h stimulation with IL-1β as previously described (20, 38). An equal amount of total protein (15 μg) from each sample was resolved by SDS-PAGE. The samples were then electroblotted onto polyvinylidene difluoride membranes (Immobilon-P). Membranes were then serially incubated, first with blocking buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1% (vol/vol) Tween 20, and 5% (wt/vol) nonfat milk for 30 min. The second incubation (1 h) was performed with primary antibody, mouse anti-pERK (1:1,000) diluted in blocking buffer. A final incubation (30 min) was carried out with anti-mouse IgG-HRP diluted (1:2,000) in blocking buffer. Immunoreactive bands were visualized by use of a Supersignal chemiluminescence detection kit (Pierce). To determine total ERK levels, the blots were stripped by use of elution buffer (Pierce) at room temperature for 1 h and reprobed with rabbit polyclonal ERK antibody (1:2,000). Immunoreactive bands were quantified by using Fluor-S-Max Chemidoc system and Quantity One software (Bio-Rad).

One-step RT-PCR. Serum-starved semiconfluent VSM cells were pretreated with PD-98059 or DETA NONOate for 1 h and stimulated with IL-1β (5 ng/ml). After 24 h, total RNA was isolated, quantitated, and stored at −80°C for future use. One-step RT-PCR was set up using MPP-9 primers 5′-CTT AGA TCA TTC TTC AGC CCT GGT CTT-3′ (sense) and 5′-GAT CCA CCT TCT GAG ACT TCA-3′ (antisense) (9). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers 5′-ATT TGG CCG TAT TGG CCG CCT-3′ (sense) and 5′-ACA GCC TTT GCA GCA CCA GTG G-3′ (antisense) were used as internal controls to normalize for the variations in RNA loading. The RT-PCR products (600 bp for GAPDH and 700 bp for MPP-9).
were resolved on 1.5% agarose gel, visualized by ethidium bromide staining, and quantified by using the Fluor-S-Max Chemidoc system and Quantity One software (Bio-Rad). MMP-9 expression was expressed as MMP-9/GAPDH product density.

Data analysis. Western blots, RT-PCR, and zymogram gels were scanned, and the relative intensity of bands was determined by densitometry using the Fluor-S-Max Chemidoc system and Quantity One software (Bio-Rad). Statistical analysis was carried out by Student’s t-test, and the difference was considered significant at $P < 0.05$. The results are presented as means ± SE; $n$ represents the number of separate experiments.

RESULTS

**ROS stimulates MMP-9 induction by an ERK-dependent mechanism.** ROS have been shown to stimulate MAPK activation in VSM cells (1, 21, 24). Recent studies have reported that ROS stimulate MMP-9 induction in human fetal membranes (5). To determine whether ROS also stimulate MMP-9 induction in VSM cells, we treated cells with xanthine/xanthine oxidase to increase superoxide levels. In unstimulated cells, MMP-9 was undetectable or present at very low levels (Figs. 1, 4, and 7). Treatment of cells with xanthine/xanthine oxidase resulted in a significant ($P < 0.05$) increase (50-fold) in MMP-9 induction in VSM cells (Fig. 1). Addition of xanthine or xanthine oxidase alone did not increase MMP-9 induction in VSM cells. Adenovirus-mediated overexpression of human manganese superoxide-dismutase gene in VSM cells inhibited xanthine/xanthine oxidase-stimulated MMP-9 induction, suggesting that superoxide produced by simultaneous addition of xanthine/xanthine oxidase is responsible for MMP-9 induction (M. V. Gurjar, J. DeLeon, R. V. Sharma, and R. C. Bhalla, unpublished data).

To determine whether ERK activation is involved in ROS-stimulated MMP-9 induction, cells were treated with xanthine/xanthine oxidase in the presence or absence of a specific ERK-pathway inhibitor PD-98059 (29). Zymography revealed that pretreatment of cells with PD-98059 for 1 h significantly ($P < 0.05$, $n = 4$) increased ERK activation, and PD-98059 significantly ($P < 0.05$) decreased X/XO-stimulated ERK activation.

**IL-1β stimulates MMP-9 induction by ERK-dependent mechanisms.** Recent studies have shown that IL-1β activation is involved in MMP-9 induction in VSM cells. Serum-stimulated VSM cells were either left untreated ($-$) or pretreated ($+$) with PD-98059 for 1 h. Cells were then treated without or with X/XO to generate ROS. Cell lysates were collected after 15 min, and equal amounts of total protein (15 μg) were analyzed by Western blotting. A: representative Western blot demonstrating phosphorylated (pERK) and total ERK levels. B: summary data from densitometric analysis (means ± SE; $n = 4$). X/XO treatment significantly (*a, $P < 0.05$, $n = 4$) increased ERK activation, and PD-98059 significantly (*b, $P < 0.05$) decreased X/XO-stimulated ERK activation.
Stimulation of VSM cells with IL-1β (5 ng/ml) significantly increased ERK activation as demonstrated by an increase in phospho-ERK levels (Fig. 3). In unstimulated cells, phospho-ERK levels were undetectable or present at very low levels. Pretreatment of cells with PD-98059 (50 μM) 1 h before IL-1β stimulation significantly decreased phospho-ERK levels (Fig. 3). IL-1β treatment of VSM cells markedly increased MMP-9 mRNA levels, which was significantly inhibited by PD-98059 treatment (Fig. 4A). To determine whether the absence of IL-1β-stimulated MMP-9 levels due to an increase in mRNA levels, we used RT-PCR to quantify the MMP-9 mRNA levels, which was significantly increased in IL-1β-stimulated cells and was undetectable in unstimulated cells (Fig. 4B). Inhibition of the ERK-pathway by PD-98059 inhibited the IL-1β-stimulated MMP-9 induction (Fig. 4B). These results suggest that IL-1β-stimulated MMP-9 induction requires ERK activation. Although IL-1β also produced a marked increase in p38 MAPK activation (a six- to eightfold increase in phosphorylated p38 MAPK over basal levels; data not shown), inhibition of this pathway by the specific inhibitor SB-203580 (10 μM) did not inhibit MMP-9 induction (Fig. 4A).

**NO attenuates IL-1β-stimulated superoxide levels, ERK 1/2 phosphorylation, and MMP-9 induction.** IL-1β stimulates ROS production in VSM cells (3). NO reacts with superoxide (6) and has been shown to inhibit IL-1β-stimulated MMP-9 induction (20). Therefore, we investigated the effects of NO on IL-1β-stimulated superoxide generation using HE. IL-1β stimulation resulted in the generation of superoxide, evidenced by an increase in red nuclear fluorescence due to breakdown of HE to ethidium (Fig. 5B), compared with unstimulated cells (Fig. 5A). Treatment of cells with the NO donor DETA NONOate almost completely abolished the IL-1β-stimulated increase in superoxide levels (Fig. 5D). DETA NONOate had no effect on HE fluorescence in unstimulated cells (Fig. 5C). These data indicate that NO treatment attenuates the IL-1β-stimulated increase in superoxide levels.

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**Fig. 3. Interleukin-1β (IL-1β) activates ERK in VSM cells.** Serum-starved cells were either left untreated (−) or pretreated (+) with PD-98059 for 1 h. Cells were then stimulated without or with IL-1β (5 ng/ml) for 15 min, and cell lysates were prepared for Western blotting. **A:** representative Western blot demonstrating phospho-ERK and total ERK levels. **B:** summary data from densitometric analysis (means ± SE; n = 4). IL-1β stimulation markedly increased phospho-ERK levels compared with undetectable levels in unstimulated cells. Pretreatment of VSM cells with PD-98059 significantly decreased IL-1β-stimulated phospho-ERK levels.

**Fig. 4. IL-1β stimulates MMP-9 induction through the ERK but not p38 mitogen-activated protein kinase (MAPK) pathway.** Serum-starved VSM cells were either left untreated (−) or pretreated (+) with PD-98059 (50 μM) or SB-203580 (10 μM) for 1 h. Cells were then stimulated without or with IL-1β (5 ng/ml), and conditioned medium and total RNA were collected after 24 h. **A:** summary data from densitometric analysis of MMP-9 levels estimated by zymography (means ± SE; n = 5). IL-1β treatment significantly increased MMP-9 induction compared with undetectable levels in unstimulated cells. PD-98059 significantly decreased IL-1β-stimulated MMP-9 induction compared with untreated cells, whereas SB-203580 had no significant effect. **B:** representative RT-PCR from 3 similar experiments showing MMP-9 (700 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 600 bp) PCR products. IL-1β treatment markedly increased MMP-9 message levels in all 3 experiments compared with unstimulated cells. Treatment of cells with PD-98059 markedly inhibited IL-1β-stimulated MMP-9 induction. GAPDH message levels were used as internal controls for RT-PCR and were similar in all lanes.
Next, we investigated whether NO inhibits IL-1β-stimulated MMP-9 induction by inhibiting ERK activation. Treatment of cells with the NO donor DETA NONOate significantly ($P < 0.05$) reduced the level of pERK at both early (15 min) and late (4 h) time intervals compared with cells treated with IL-1β alone (Fig. 6). Significantly, DETA NONOate treatment also inhibited IL-1β-stimulated MMP-9 induction estimated by both zymography (Fig. 7A) and RT-PCR (Fig. 7B).

**Fig. 5.** NO inhibits IL-1β-stimulated superoxide generation in VSM cells. Serum-starved VSM cells grown on 25-mm coverslips were either left untreated (−) or pretreated (+) with DETA NONOate for 1 h and then stimulated without or with IL-1β (5 ng/ml). Cells were then loaded with dihydroethidium (5 μM), and confocal images were captured 90 min after stimulation with IL-1β. Excitation wavelength was 488 nm, and emission wavelength was 650 nm. Representative images are presented from 3 similar experiments. IL-1β stimulation markedly increased superoxide production in VSM cells as evidenced by dramatic increases in red nuclear fluorescence due to breakdown of dihydroethidium to ethidium (B) compared with unstimulated cells (A). Treatment of cells with DETA NONOate almost completely abolished IL-1β-stimulated superoxide production (D). DETA NONOate had no effect on dihydroethidium fluorescence in unstimulated cells (C).

**Fig. 6.** NO inhibits IL-1β-stimulated ERK activation. Serum-starved VSM cells were either left untreated (−) or pretreated (+) with DETA NONOate for 1 h and then stimulated without or with IL-1β (5 ng/ml) for 15 min and 4 h, and cell lysates were prepared for Western blotting. Summary data from densitometric analysis of the pERK bands are presented as means ± SE ($n = 4$). IL-1β stimulation significantly increased pERK levels at both 15 min and 4 h compared with unstimulated cells. Pretreatment of cells with DETA NONOate significantly ($*P < 0.05$, $n = 4$) decreased IL-1β-stimulated pERK levels both at 15 min and at 4 h compared with IL-1β-stimulated controls.

**Fig. 7.** NO inhibits IL-1β-stimulated MMP-9 induction. Serum-starved VSM cells were either left untreated (−) or pretreated (+) with DETA NONOate for 1 h and then stimulated without or with IL-1β (5 ng/ml). Conditioned medium was collected after 24 h treatment with IL-1β and total RNA isolated. A: summary data from densitometric analysis of MMP-9 levels estimated by zymography (means ± SE; $n = 4$). IL-1β treatment dramatically increased MMP-9 levels in the conditioned media compared with undetectable levels in unstimulated cells. Pretreatment of cells with DETA NONOate resulted in a significant ($*P < 0.05$) decrease in MMP-9 levels compared with untreated cells. B: summary data from densitometric analysis of the MMP-9 RT-PCR product presented as MMP-9/GAPDH (means ± SE; $n = 4$). Pretreatment of cells with DETA NONOate significantly ($*P < 0.05$) inhibited IL-1β-stimulated increase in MMP-9 message levels.
DISCUSSION

The major findings of this study are that 1) ROS stimulates MMP-9 induction in VSM cells, 2) ERK activation is required for ROS- and IL-1β-stimulated MMP-9 induction, 3) NO inhibits IL-1β-stimulated increase in superoxide levels, and 4) NO inhibits IL-1β-stimulated ERK activation and MMP-9 induction. These findings provide a mechanistic explanation for NO-mediated inhibition of VSM cell migration and suggest the ROS-ERK signaling pathway as a possible target to inhibit MMP-9 induction and thus VSM cell migration.

Results from our laboratory have shown that NO donors or expression of eNOS gene in VSM cells inhibits cell migration in vitro and neointima formation in vivo (10, 11, 20, 38). DETA NONOate inhibits VSM cell migration and proliferation in a dose-dependent fashion, with the maximal effects at 500 μM (10). At 500 μM, DETA NONOate inhibits MMP-9 induction by 50–80% whereas eNOS gene expression inhibits MMP-9 induction by 20–30% (20). This discrepancy in the inhibition of MMP-9 induction is most likely due to the amount of NO produced by the two treatment protocols. Treatment of VSM cells with 500 μM DETA NONOate results in the release of almost 100-fold more NO (500 nmol of nitrite production per milliliter medium after 24 h) than that observed in eNOS-transfected cells stimulated with growth factors (~6 nmol of NO produced per million cells per milliliter; Ref. 20). Thus, in the current study, we used DETA NONOate to produce more robust and reproducible inhibition of MMP-9 induction.

ROS are produced by a variety of cells in the vessel wall, including endothelial and VSM cells, and have been shown to regulate VSM cell migration (6, 18, 21, 24, 39). Recent studies have demonstrated the importance of ROS as second messengers in growth factor and cytokine regulation of VSM cell functions including migration (reviewed in Refs. 18, 21, 24). NO has been shown to react with superoxide and also to modulate cellular signaling pathways (6, 40). We have recently shown that NO inhibits MMP-9 induction and VSM cell migration (20). However, the molecular mechanisms involved in NO-mediated inhibition of MMP-9 induction are not well understood. Results presented in this study suggest that inhibition of ROS levels and/or ROS signaling may be how NO inhibits MMP-9 induction and VSM cell migration. In support of this, our data demonstrate that generation of superoxide by xanthine/xanthine oxidase stimulates MMP-9 induction in VSM cells. Exogenous expression of the SOD gene in VSM cells abolished xanthine/xanthine oxidase-stimulated MMP-9 induction in VSM cells. Exogenous expression of the SOD gene in VSM cells abolished xanthine/xanthine oxidase-stimulated MMP-9 induction, suggesting that superoxide is required for MMP-9 induction (M. V. Gurjar, J. DeLeon, R. V. Sharma, and R. C. Bhalla, unpublished data). Furthermore, treatment of cells with a NO donor attenuated IL-1β-stimulated superoxide generation and MMP-9 induction in VSM cells. Overall, our results suggest that NO interferes with IL-1β-stimulated ROS signaling to inhibit MMP-9 induction. Our findings are in agreement with recent studies showing that an increase in ROS in human fetal membrane increases MMP-9 synthesis and secretion (5). ROS scavenging agents inhibit the matrix-degrading capacity of macrophage-derived foam cells, further implicating ROS in increasing MMP activity and/or levels (14).

IL-1β has been shown to stimulate superoxide production in VSM cells and is one of the most potent stimuliators of MMP-9 induction (3, 15). Earlier studies have also shown that ROS stimulate ERK activation in VSM cells (1) and that ERK activation plays a critical role in VSM cell migration (17, 29). In addition, ERK activation is involved in MMP-9 induction in other cell types (36, 41). However, our findings are the first direct evidence demonstrating the role of ROS-ERK signaling in MMP-9 induction in VSM cells. Our results show that treatment of VSM cells with NO-donor DETA NONOate inhibited IL-1β-stimulated MMP-9 induction. In support of our findings, a recent study has shown that NO inhibits angiotensin II-stimulated ERK activation (40).

In conclusion, we have demonstrated that NO inhibits IL-1β-stimulated superoxide-ERK signaling to decrease MMP-9 induction in VSM cells. Although the present study demonstrates that NO attenuates superoxide levels in VSM cells in response to IL-1β stimulation, future studies are required to investigate whether NO acts as superoxide scavenger or inhibits the superoxide production by inhibiting the ROS-generating enzymes.

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