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Involvement of integrins, MAPK, and NF-κB in regulation of the shear stress-induced MMP-9 expression in endothelial cells

Hui-Wen Sun [a,1], Chang-Jiang Li [a,1], Huai-Qing Chen [b], Hui-Li Lin [a], Hui-Xia Lv [a], Yun Zhang [a], Mei Zhang [a,*]

[a] *The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Public Health, Shandong University Qilu Hospital, Jinan, Shandong 250012, PR China*

[b] *Institute of Biomedical Engineering, West China Center of Medical Sciences, Sichuan University, Chengdu, Sichuan 610041, PR China*

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Abstract

Variations in the matrix metalloproteinase (MMP)-9 gene are related to the presence and severity of atherosclerosis. The aim of this study was to determine the signaling pathways of MMP-9 in endothelial cells subjected to low fluid shear stress. We found that low fluid shear stress significantly increased MMP-9 expression, IκBα degradation, NF-κB DNA-binding activity and phosphorylation of MAPK in cultured human umbilical vein endothelial cells (HUVECs). Inhibition of NF-κB resulted in remarkable downregulation of stress-induced MMP-9 expression. Pretreatment of HUVECs with inhibitors of p38 mitogen-activating protein kinase (MAPK) and extracellular signal-regulated kinase1/2 (ERK1/2) also led to significant suppression of stress-induced MMP-9 expression and NF-κB DNA-binding activity. Similarly, addition of integrins inhibitor to HUVECs suppressed the stress-induced MMP-9 expression, IκBα degradation, NF-κB DNA-binding activity and the phosphorylation of p38 MAPK, ERK1/2. Our findings demonstrated that the shear stress-induced MMP-9 expression involved integrins-p38 MAPK or ERK1/2-NF-κB signaling pathways.

Keywords: Matrix metalloproteinase-9; Integrins; Mitogen-activating protein kinases; NF-κB; Endothelial cells; Shear stress

Endothelial cells lining the inner vessel wall are in direct contact with flowing blood, which generates hemodynamic shear stress. Accumulating evidence indicates that mechanical forces play a key role in the pathogenesis of atherosclerosis, as atherosclerotic lesions develop mainly in the areas where blood vessels experience disturbed flow [1–5]. Endothelial cells could sense shear stress by an unidentified mechanoreceptor(s) followed by production of autocrine and paracrine factors which induce endothelium dysfunction and atherosclerosis [6–8].

MMP-9 is one kind of gelatinases that can degrade the intimal extracellular matrix, which results in leukocyte infiltration into the vessel wall and smooth muscle cell migration into the developing neointima [9,10]. These two processes are crucial in atheroma development. Genetic studies show that variations in the MMP-9 gene are related to the presence and severity of atherosclerosis [11–13].

Some studies have demonstrated that endothelial MMP-9 expression is flow-sensitive and is up-regulated by hemodynamic changes. There is considerable evidence for activation of MMP-9 by oscillatory flow in endothelial cells [14] or in arterial segments [15]. Transmural pressure activates MMP-9 in porcine carotid arteries [16]. High flow and shear stress can mediate EC and SMC to express MMP-9 [17]. Expression of proatherogenic inflammatory mediators and matrix metalloproteinase activity was high in the lowered shear stress regions [18]. However, it is still unclear about the signaling pathways of mechanical stress induced-MMP-9 expression in vascular endothelial cells.

* Corresponding author. Fax: +86 531 8616 9356.
E-mail address: daixh@vip.sina.com (M. Zhang).
[1] These authors contributed equally to this work.

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In the present study, we investigated the expression of MMP-9 in cultured HUVECs which were stimulated by low fluid shear stress. We demonstrated that low fluid shear stress induced the expression of MMP-9 mRNA and protein in endothelial cells via a mechanism that involved integrins-ERK1/2 or p38 MAPK-NF-κB signaling pathways.

Materials and methods

Materials. Monoclonal antibodies against phosphor-JNK1/2 were purchased from Upstate (Lake Placid, NY). Antibodies against phosphor-ERK1/2, phosphor-p38, β-actin and IκBα were from Santa Cruze Biotechnology (Santa Cruz, CA). Monoclonal anti-human TIMP-1 antibody was from R&D Systems (Mineapolis, MN). SB203580, SP600125 and PD98059 were from Biosource (Camarillo, CA). SN50 was from Alexis Biochemicals (Switzerland) and GRGDNP was from Bio-Mol International, L.P. (Plymouth, Meeting, PA). Recombinant human VEGF was from CHEMICON (Temecula, CA).

Cell culture. HUVECs were freshly isolated from human umbilical cord vein with 0.1% Collagenase II (Sigma, St. Louis, MO, USA), and grown in T25 flasks in M199 medium supplemented with 20% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 20 ng/ml VEGF. Confluent primary cultures were harvested using 0.25% trypsin solution (Sigma, St. Louis, MO, USA), seeded onto slides pre-coated with 1% gelatin (Sigma). After 5–8 h, complete medium was added in culture plates. The cells reached confluence in 3–4 days. HUVECs were grown in a humidified incubator in an atmosphere of 5% CO_2, 95% air.

Cell pretreatment. HUVECs, growing on slide gelatin-bottomed culture plates to 90% confluence, were 1% serum-starved for 12 h. Then GRGDNP, SP600125, PD98059, SB203580, or SN50 was added, respectively and incubated for 2 h before application of stress.

Taqman real-time quantitative RT-PCR analyses. Total RNA from HUVECs was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. The reverse transcription was performed at 42 °C for 1 h using the MLV Kit (Promega). Real-time PCR was performed on a Light Cycler (Roche Applied Science, USA). Three technical replicates were run for each gene in each sample. The primers used for MMP-9 (GenBank No. NM004994) amplification were 5′-cctggagacctgagaaccaatc-3′ (upper strand) and 5′-gatttcgactctccacgcatc-3′ (lower strand). The probe was 5′-taccgctatggttacactcgggtggc-3′. The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank M33197) were 5′-ggaaggactcatgaccacagt-3′ (upper strand) and 5′-gccatcacgccacagtttc-3′ (lower strand). The probe was 5′-tgccatcactgccacccagaagac-3′. Amplification was performed with 50 cycles and annealing 62 °C 5 s, extension at 72 °C for 10 s. The data was analyzed with Light Cycler software 4.0 (Roche Applied Science, USA). MMP-9 mRNA expression was normalized to the expressed housekeeping gene GAPDH.

SDS–PAGE zymography. The conditioned media from stressed or static HUVECs culture was concentrated 30-fold using bag filter. Protein was separated by SDS–PAGE under the non-reducing condition on 8% polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, the gel was washed at room temperature for 1 h in wash buffer (50 mM Tris–Cl, pH 7.4 and 2.5% Triton X-100) which were then incubated overnight at room temperature in 50 mM Tris–Cl, pH 7.4, 75 mM NaCl and 2.5 mM $CaCl_2$. The gels were stained with Coomassie brilliant blue R-250 and destained in 45% methanol and 10% acetic. After gel staining, MMP-9 was identified based on gelatin lysis at molecular masses 92 kDa for MMP-9. Gelatinolytic bands were quantified using Multi-Analyst densitometry software.

Western blot analysis. Protein was boiled for 5 min. Equal amounts of protein were separated with a 14% SDS–PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Following blocking with 5% non-fat milk, the blots were washed with PBS containing 0.1% Tween 20 and incubated with an appropriate primary antibody at 4 °C overnight. The blots were probed with antibodies against β-actin (rabbit, 1:1000 dilution) IκBα (mouse, 1:100 dilution), phosphor-p38 MAPK (mouse, 1:100 dilution), phosphor-ERK1/2 (mouse, 1:300 dilution), phospho JNK1/2 (rabbit, 1:100 dilution). After overnight incubation, the blots were washed with TBST and incubated with secondary antibody conjugated to HRP (Santa Cruz, 1:2000 dilution), and then washed again. The blots were then visualized with enhanced chemiluminescence (ECL).

Detection of NF-κB p65 activity. The DNA-binding activity of NF-κB p65 was detected with TransAM™ NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacture's instructions. Positive controls, blanks and samples in duplicate were tested to measure the activity of NF-κB p65. Nuclear extracts containing 20 μg of protein were incubated with binding buffer and complete lysis buffer for 1 h at 100 rpm on a rocking platform at room temperature. Then wells were washed three times and incubated with primary (1:1000 dilution for NF-κB p65) and secondary antibody for 1 h, respectively. The developing solution was added to the wells to incubate for 10 min and stop solution was added when blue color of the samples turned dark blue. NF-κB p65 was evaluated with absorbance (A) value by spectrophotometer within 5 min at 450 nm with a reference wavelength of 655 nm.

Statistical analysis. For each condition, data from at least three independent experiments were quantified and analyzed by one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. Data were presented as means ± SEM.

Results

Low fluid shear stress induces MMP-9 expression in HUVECs

To explore the effect of mechanical stress on the expression of MMP-9, HUVECs were exposed to 4 dyn/cm^2 shear stress at various time points. The level of MMP-9 mRNA maintained unchanged following 1 h of exposure. When HUVECs were subjected to shear stress for 3, 6, and 8 h, there was a significant time-dependent increase in the MMP-9 mRNA level (Fig. 1A). The medium of stressed-HUVECs was condensed 30-fold using bag filters. The MMP-9 protein activity was measured by SDS–PAGE zymography. The level of active MMP-9 protein had no obvious change after 3 h exposure to 4 dyn/cm^2 shear stress. After 6 h exposure, the level was 3.3-fold higher than that in static control ($p < 0.01$), but lower than that after 8 h (Fig. 1B). MMP-9 activity is known to be restricted by the presence of TIMP-1, a specific inhibitor of MMP-9. TIMP-1 protein levels were therefore measured by Western blot. The increased MMP-9 levels were not accompanied by a corresponding increase in TIMP-1 protein levels (Fig. 1C).

NF-κB Regulates stress-induced MMP-9 expression in HUVECs

NF-κB is an important regulator of MMP-9 induction [19,20]. To further prove NF-κB involved in stress-induced MMP-9, HUVECs were exposed to 4 dyn/cm^2 shear stress at various time points, and then total protein extracts were analyzed by Western blot. The levels of IκBα were significantly reduced after 15 and 30 min exposure to 4 dyn/cm^2 shear stress (Fig. 2A). To define the role of NF-κB in the induction of MMP-9 in stressed-HUVECs, transcription

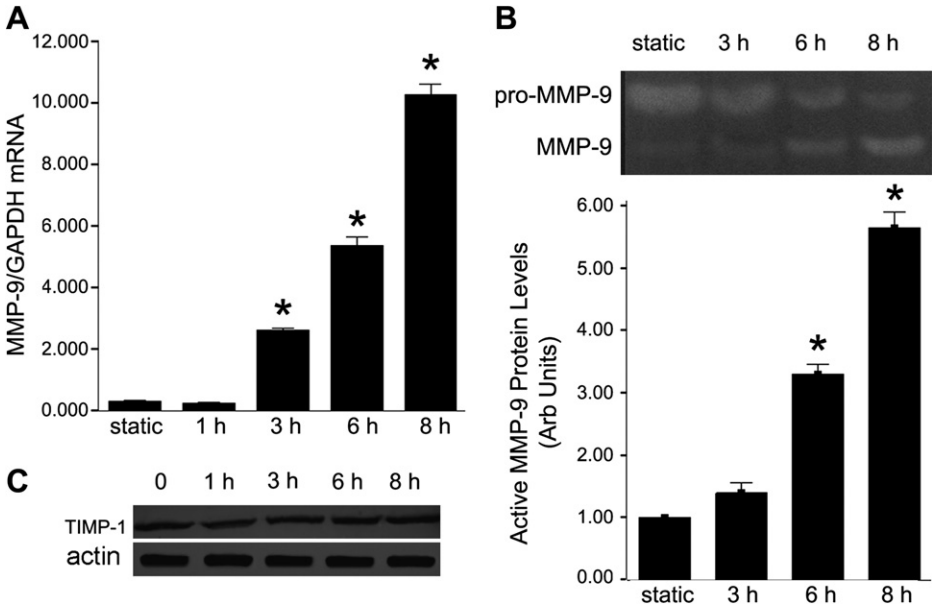

Fig. 1. Stress induces MMP-9 expression. (A) Expression of MMP-9 mRNA was measured by Taqman real-time quantitative RT-PCR. *Significant difference from static control, $p < 0.01$. (B) HUVECs were exposed to 4 dyn/cm^2 shear stress at various time points. The activity of MMP-9 protein in the cell culture medium was measured by SDS–PAGE gelatin zymography. Relative ratio in static control was expressed as 1 arbitrary unit. *Significant difference from static control, $p < 0.01$. (C) Levels of TIMP-1 protein were measured by Western blotting. Results were normalized to β-actin.

factor assay was performed. NF-κB DNA-binding activity was stronger after 1 h exposure to 4 dyn/cm^2 shear stress than that in static control ($p < 0.01$) (Fig. 2B). HUVECs were pretreated for 2 h with 18 μM SN50, a cell-permeant peptide that interrupted translocation of NF-κB, and then subjected to 4 dyn/cm^2 shear stress for 1 h. The pretreatment with SN50 efficiently inhibited NF-κB DNA-binding activity ($p < 0.05$) (Fig. 2C). In the presence of the inhibitor, both MMP-9 mRNA expression ($p < 0.01$) (Fig. 2D) and protein activity in the supernatant ($p < 0.01$) (Fig. 2E) were significantly attenuated. These results indicated that NF-κB played a crucial role in the expression of this cytokine in stressed-HUVECs.

ERK1/2 or p38 MAPK leads to stress-induced MMP-9 expression in a NF-κB-dependent manner in HUVECs

Given that low fluid shear stress induced MMP-9 expression in a NF-κB-dependent manner, we hypothesized that MAPK might be involved in stress-induced NF-κB activation and lead to MMP-9 expression. HUVECs were exposed to 4 dyn/cm^2 stress at various time points. A rapid activation of p38 MAPK, ERK1/2, and JNK1/2, as determined by phosphorylation levels, occurred after low fluid shear stress exposure in HUVECs. Phosphorylations of ERK1/2 and p38 MAPK peaked at 5 min, as did Phosphorylation of JNK1/2 at 15 min (Fig. 3A). Thereafter HUVECs were pretreated for 2 h with 20 μM PD98059, a specific ERK1/2 inhibitor, 5 μM SB203580, a specific p38 MAPK inhibitor and 18 μM SP600125, a specific JNK1/2 inhibitor, respectively. Then HUVECs were exposed to 4 dyn/cm^2 shear stress. Stress-induced ERK1/2, p38 MAPK and JNK1/2 phosphorylations were abolished by PD98059, SB203580 and SP600125 respectively (Fig. 3B). MMP-9 mRNA expression and protein activity were evidently inhibited by PD98059 and SB203580, not by SP600125 (Fig. 3C–F). However, neither SB203580 nor PD98059 was able to abrogate MMP-9 induction completely.

In order to determine the relationship between MAPK and NF-κB when MMP-9 was induced by low fluid shear stress, HUVECs were incubated with PD98059 and SB203580 for 2 h respectively before being stressed. The IκBα levels increased significantly after 15 min exposure to 4 dyn/cm^2 shear stress (Fig. 3B) and the NF-κB DNA-binding activity was depressed obviously after 1 h (Fig. 3G).

Integrins mediate stress-induced MMP-9 expression via MAPK -NF-κB signaling pathways in HUVECs

To clarify whether integrins were required for stress-induced MMP-9 expression, HUVECs were preincubated for 2 h with 50 μM GRGDNP, a synthetic peptide that could competitively inhibit integrins binding to extracellular matrix proteins containing RGD peptide, and then given 4 dyn/cm^2 shear stress for 6 h. The shear stress-induced increases in MMP-9 mRNA ($p < 0.01$) (Fig. 4A) and protein activity ($p < 0.01$) (Fig. 4B) were both significantly inhibited.

To determinate whether integrins are involved in MAPK-NF-κB signaling pathway, HUVECs were pretreated for 2 h with 50 μM GRGDNP before being projected to shear stress for 5 or 15 min. Total protein was extracted and measured by western blot. Shear stress-induced ERK1/2 and p38 MAPK phosphorylations were

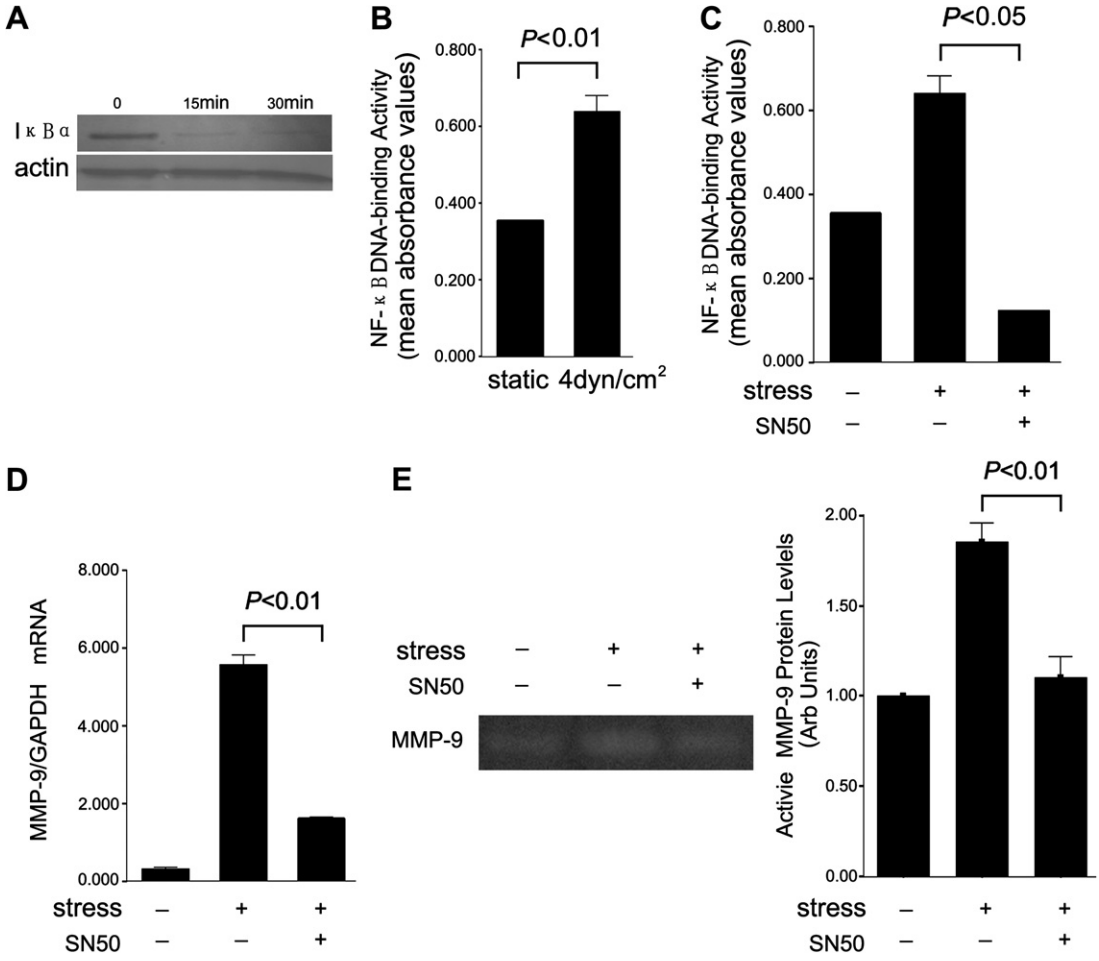

Fig. 2. NF-κB regulates stress-induced MMP-9 expression in HUVECs. (A) HUVECs were exposed to 4 dyn/cm^2 shear stress for 15 and 30 min and Western blot analysis for IκBα was performed. Results were normalized to β-actin. (B) Activity of NF-κB DNA-binding at static and 1 h after 4 dyn/cm^2 shear stress exposure. (C) Effect of 1 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 18 μM SN50 on the NF-κB DNA-binding activity in HUVECs. (D) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 18 μM SN50 on MMP-9 mRNA in HUVECs. (E) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 18 μM SN50 on MMP-9 protein activity in HUVECs.

inhibited after 5 min exposure. JNK phosphorylation was inhibited and the degradation of IκBα was reduced after 15 min (Fig. 4C).

To examine NF-κB DNA-binding activity, HUVECs were pretreated for 2 h with 50 μM GRGDNP before being exposed to 4 dyn/cm^2 shear stress for 1 h. Nuclear protein was extracted from the collected HUVECs and transcription factor assay was performed. As shown in Fig. 4D, NF-κB DNA-binding activity was depressed significantly ($p < 0.01$).

Discussion

In this study, we demonstrated that HUVECs could secrete MMP-9 protein into culture medium in response to low fluid shear stress and the elevated levels correlated with the increased MMP-9 mRNA levels. Integrins, ERK1/2 or p38 MAPK and NF-κB were involved in this signal transduction pathway.

To identify signaling pathways included in this induction, we studied the activation of NF-κB, a known transcriptional factor in response to other stimuli. NF-κB is present in the cytosol in an inactive form bound to its inhibitor IκBα. p50/p65 heterodimers are predominant in many cell types. Phosphorylation and subsequent degradation of IκBα result in the release of NF-κB dimmers which subsequently translocate to the nucleus. Then the appropriate target genes are activated.

In our experiment, we found rapid degradation of IκBα and enhancement of NF-κB DNA-binding activity under low fluid shear stress. Further evidence supporting the involvement of NF-κB activation was that SN50, a pharmacological inhibitor, could inhibit stress-induced MMP-9 expression. However, MMP-9 secretion was not completely inhibited. This suggested that MMP-9 production was regulated mainly by NF-κB, also partly by other transcriptional factors. These results clearly demonstrated the contribution of NF-κB to stress-induced MMP-9 expression.

MAPK pathways have been shown to be important mediators of signal transduction, which are involved in multiple intracellular signaling cascades and activated by

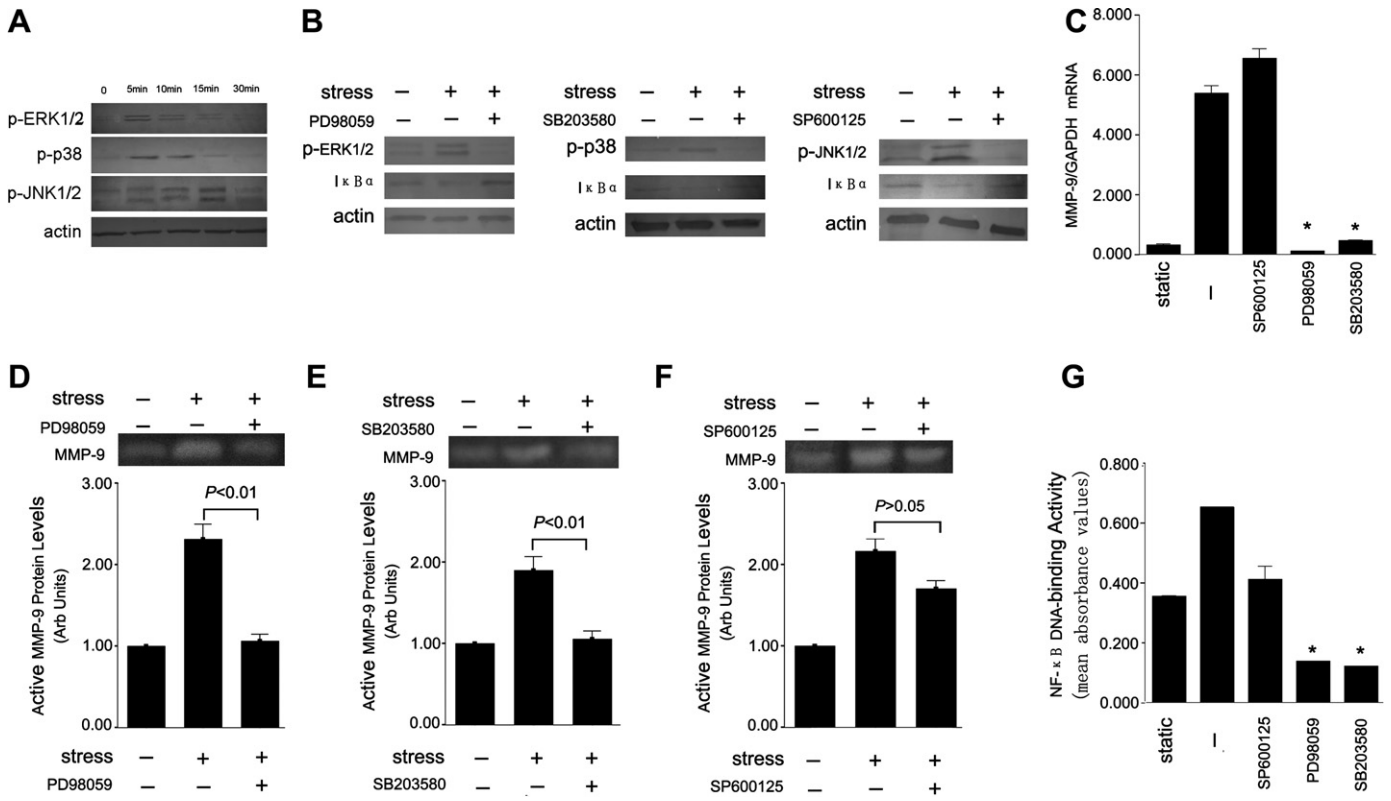

Fig. 3. MAPK pathways are involved in MMP-9 expression. (A) HUVECs were exposed to 4 dyn/cm^2 shear stress at the indicated time points. Phospho-p38 MAPK (p-p38 MAPK), phospho-ERK1/2 (p-ERK1/2) and phospho-JNK1/2 (p-JNK1/2) were measured by Western blotting. Results were normalized to β-actin. (B) Effects of 5 or 15 min 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with SB203580 (5 μM), PD98059 (20 μM) and SP600125 (18 μM) respectively on p-p38 MAPK, p-ERK1/2, p-JNK1/2 activities; Effects of 15 min 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with SB203580 (5 μM), PD98059 (20 μM) and SP600125 (18 μM) on degradation of IκBα in HUVECs. Results were measured by Western blot and normalized to β-actin. (C) Effects of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with kinases inhibitors (PD98059, SB203580, and SP600125, respectively) on MMP-9 mRNA induction in HUVECs. *Significant difference from stressed control without pretreatment, $p < 0.01$. (D) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 20 μM PD98059 on MMP-9 protein activity in HUVECs. (E) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 5 μM SB203580 on MMP-9 protein activity in HUVECs. (F) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 18 μM SP600125 on MMP-9 protein activity in HUVECs. (G) Effects of 1 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with kinases inhibitors on NF-κB DNA-binding activity in HUVECs. *Significant difference from stressed control without pretreatment, $p < 0.01$.

the stimulation of a variety of cell surface receptors [21,22]. We examined whether MAPK was involved in the stress-induced MMP-9 expression. We found that low fluid shear stress were able to activate p38 MAPK, JNK1/2 and ERK1/2. However, only p38 MAPK and ERK1/2 were involved in the induction of MMP-9 because the inhibition of p38 MAPK and ERK1/2 suppressed the induction and the inhibition of JNK1/2 failed. Meanwhile, inhibition of p38 MAPK, and ERK1/2 also inhibited the degradation of IκBα and the activity of NF-κB DNA-binding. These results supported the notion that stress-induced MMP-9 expression was partially dependent on ERK1/2 and p38 MAPK activation.

What upstream signal transducers are involved in mechanical stress induced MMP-9 production? Integrins, as the main receptors that connect the cytoskeleton to the extracellular matrix (ECM) [23,24], have an intimate relationship to mechanical force [25,26]. Some previous studies have implicated that integrins transmitted mechanical stresses across the plasma membrane and played an important role during mechanical force signal transduction [27–29]. Most importantly, they interact and can affect MAPK signaling [30,31]. We further explored whether integrins were involved in the stress-induced MMP-9 expression. We found that GRGDNP decreased stess-induced p38 MAPK, ERK1/2 and JNK1/2 phosphorylations. Inhibition of p38 and ERK1/2 phosphorylation led to suppression of MMP-9 induction. Inhibition of JNK1/2 phosphorylation did not contribute to the suppression of MMP-9 induction. GRGDNP also attenuated degradation of IκBα and NF-κB DNA-binding activity induced by shear stress. These results indicated that integrins were upstream regulators of stress induced-MMP-9 signal pathway in HUVECs.

In conclusion, our results demonstrated that low fluid shear stress induced the release of MMP-9 to extracellular space via ERK1/2 or p38 MAPK-NF-κB signal transduction pathways in HUVECs in vitro. Integrins were

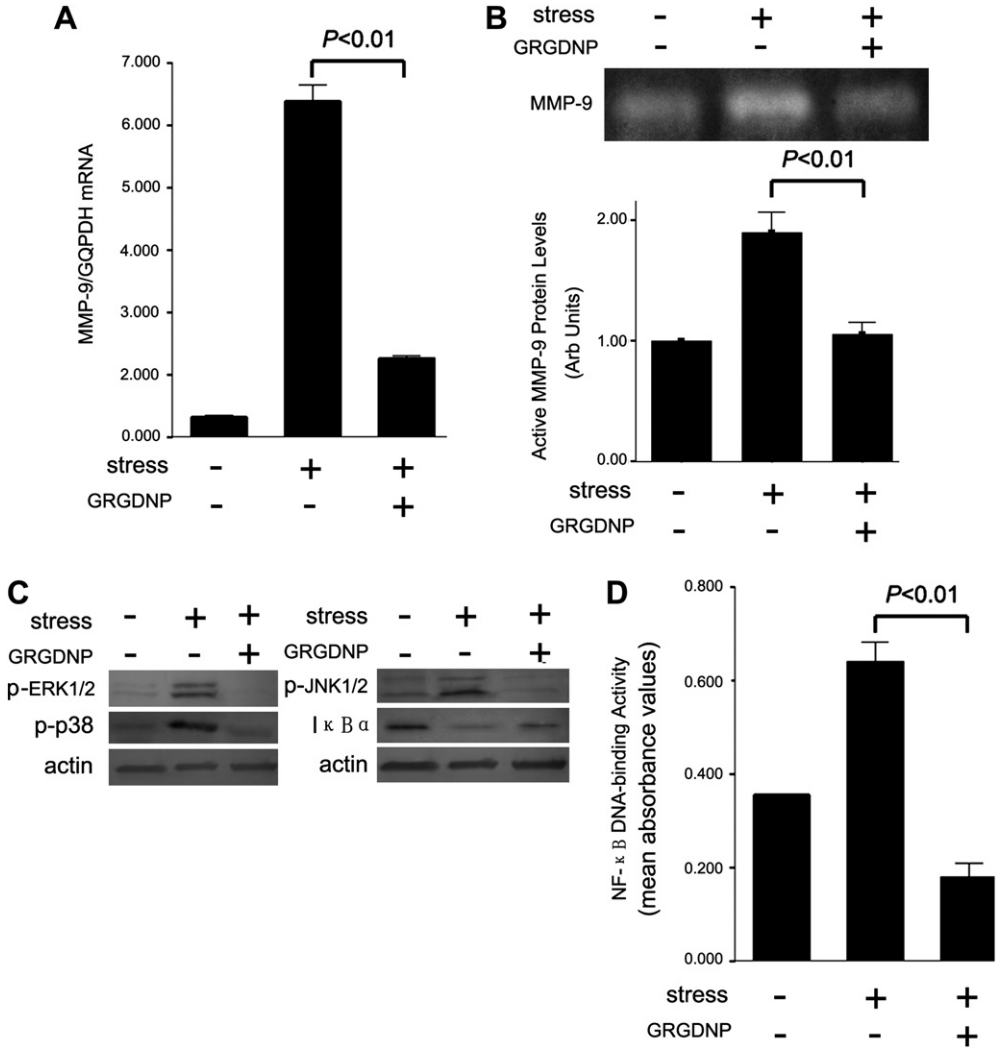

Fig. 4. Integrins mediate induction of MMP-9 via MAPK-NF-κB signaling pathways by low fluid shear stress in HUVECs. (A) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 50 μM GRGDNP on MMP-9 mRNA in HUVECs. (B) Effect of 6 h 4 dyn/cm^2 shear stress exposure following 2 h pretreatment with 50 μM GRGDNP on MMP-9 protein activity in HUVECs. (C) Pretreatment of HUVECs with 50 μM GRGDNP for 2 h markedly inhibited ERK1/2 and p38 MAPK phosphorations after 5 min 4 dyn/cm^2 shear stress exposure in HUVECs. JNK1/2 phosphoration was inhibited and degradation of IκBα was reduced after 15 min. Results were measured by Western blot and normalized to β-actin. (D) Effect of 1 h 4 dyn/CM^2 shear stress exposure following 2 h pretreatment with 50 μM GRGDNP on NF-κB DNA-binding activity in HUVECs.

upstream molecules of this pathway. These findings provided a link between mechanical force and the initiation of atherosclerosis.

Acknowledgments

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