

Inhibition of c-Jun N-Terminal Kinase Attenuates Low Shear Stress–Induced Atherogenesis in Apolipoprotein E–Deficient Mice

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Atherosclerosis begins as local inflammation of arterial walls at sites of disturbed flow, such as vessel curvatures and bifurcations with low shear stress. c-Jun NH₂-terminal kinase (JNK) is a major regulator of flow-dependent gene expression in endothelial cells in atherosclerosis. However, little is known about the *in vivo* role of JNK in low shear stress in atherosclerosis. We aimed to observe the effect of JNK on low shear stress–induced atherogenesis in apolipoprotein E-deficient (ApoE^{-/-}) mice and investigate the potential mechanism in human umbilical vein endothelial cells (HUVECs). We divided 84 male ApoE^{-/-} mice into two groups for treatment with normal saline (NS) (n = 42) and JNK inhibitor SP600125 (JNK-I) (n = 42). Perivascular shear stress modifiers were placed around the right carotid arteries, and plaque formation was studied at low shear stress regions. The left carotid arteries without modifiers represented undisturbed shear stress as a control. The NS group showed atherosclerotic lesions in arterial regions with low shear stress, whereas the JNK-I group showed almost no atherosclerotic lesions. Corresponding to the expression of proatherogenic vascular cell adhesion molecule 1 (VCAM-1), phospho-JNK (p-JNK) level was higher in low shear stress regions with NS than with JNK-I inhibitor. In HUVECs under low shear stress, siRNA knockdown and SP600125 inhibition of JNK attenuated nuclear factor (NF)-κB activity and VCAM-1 expression. Furthermore, siRNA knockdown of platelet endothelial cell adhesion molecule 1 (PECAM-1) (CD31) reduced p-JNK and VCAM-1 levels after low shear stress stimulation. JNK may play a critical role in low shear stress–induced atherogenesis by a PECAM-1–dependent mechanosensory pathway and modulating NF-κB activity and VCAM-1 expression.

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INTRODUCTION

Atherosclerotic lesions form preferentially at distinct sites in the arterial tree, especially at or near branch points, bifurcations and inner curvatures, where there is low or oscillatory blood flow (that is, displaying directional change and boundary layer separation). In contrast, straight regions of the vasculature exhibit uniform laminar shear stress, which is atheroprotective. Shear stress is critically important in regulating the

vascular physiology and pathobiology of the vessel wall by modulating endothelial cell (EC) function (1–4). The pathogenic feature of early atherosclerosis is an inflammatory process in which the endothelium is activated by proinflammatory cytokines (5). Previous investigators showed that the expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte binding were increased in rabbit carotids chronically exposed to low

shear stress compared with normal shear stress (6).

However, the relation between shear stress and atherosclerosis is based almost exclusively on clinical observations in humans or *in vitro* experiments (4,7–9). Cheng *et al.* (10) developed a perivascular shear stress modifier (called a cast) that could induce changes in shear stress patterns in a straight vessel and used the model to assess the effect of shear stress alterations on the development of atherosclerosis in apolipoprotein E deficient (ApoE^{-/-}) mice. The authors found that atherosclerotic lesions developed under low and oscillatory shear stress but not increased shear stress.

Shear stress and stretch could modulate EC functions by activating mechanosensors and signaling pathways. The Jun

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N-terminal kinases (JNKs) are mitogen-activated protein kinases (MAPKs) traditionally considered stress-activated protein kinases. JNK1 and JNK2 are ubiquitously expressed, and JNK3 is expressed mainly in the heart, brain and testes (11). JNKs are widely activated by inflammatory cytokines and environmental stresses, including osmotic stress and mechanical stress, and are involved in regulation of proinflammatory mediators of ECs (12,13). JNK is thought to be among the major regulators of flow-dependent inflammatory gene expression in ECs in atherosclerosis (14). The JNK1/2 inhibitor SP600125 and genetic deletion of JNK2 decreased atherosclerotic plaque formation in ApoE^{-/-} mice by regulating the scavenger receptor (15).

EC surfaces are equipped with numerous mechanosensors responding to shear stress (16). PECAM-1 (CD31) has recently been shown to form an essential element of a mechanosensory complex that mediates endothelial responses to fluid shear stress. CD31 plays a crucial role in the activation of the nuclear factor (NF)- κ B and Akt pathways and inflammatory cell accumulation during vascular remodeling (17,18). In addition, CD31 contributes to atherosclerotic lesion formation in regions of disturbed flow by regulating NF- κ B-mediated gene expression *in vivo* and *in vitro* (19).

Because of these links between fluid shear stress, JNK expression, proinflammatory cytokine induction and atherosclerosis, we investigated the activation of JNK in the context of shear stress, VCAM-1 expression and atherosclerosis by using the shear stress model in ApoE^{-/-} mice. To test whether CD31 as a sensor and NF- κ B were involved in this signaling pathway, we further validated their actions in low shear stress-stimulated human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Animals

Male ApoE^{-/-} mice (n = 84), 8 wks old (25–30 g), were obtained from The Jack-

son Laboratory (Bar Harbor, ME, USA), housed at a constant temperature (24°C) and given a normal diet with free access to water. All experiments were performed in compliance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985) and Shandong University.

Shear Stress Modifier Placement and Grouping

To induce standardized changes of shear stress *in vivo*, we used a cast, which imposes a fixed geometry on the carotid vessel wall, thereby causing gradual stenosis resulting in decreased blood flow upstream from the cast (low shear stress [low]) (10). On the left side, a straight segment of the carotid artery without the cast had undisturbed shear stress (undisturbed) as a control. To validate the shear stress modifier, we performed microultrasound imaging to measure velocity of the carotid artery [Vmax]) and end-systolic diameters (Ds) before and after surgery (3 d) to calculate changes in shear stress (SS) with use of the formula: $SS = 4 \mu V_{max}/Ds$ (20,21).

One week after surgery, 84 mice were given a high-fat Western-type diet containing 0.25% cholesterol and were divided into two groups (n = 42 each) for intraperitoneal injection with 0.2 mL saline (normal saline [NS] with an equal volume of dimethyl sulfoxide [DMSO]) or 0.2 mg/kg/d (22) of the JNK-I inhibitor SP600125 (Invitrogen, Carlsbad, CA, USA).

Blood samples were taken before and after injection to monitor the levels of total cholesterol and low-density lipoprotein (LDL) cholesterol by use of an automatic biochemistry analyzer (Hitachi, Tokyo, Japan).

Tissue Harvesting and Preparation for Morphological Analysis

To compare the effect of low shear stress on lesion formation in the two groups, 15 mice in each group were humanely euthanized at 10 wks after surgery. Tissues were harvested from mice

anesthetized by use of sodium pentobarbital (210 mg/kg). Vessels were perfused with phosphate-buffered saline at a constant physiological pressure of 100 mmHg and then constant pressure perfusion *in situ* with 4% polyformaldehyde. Bilateral common carotid arteries were carefully removed and fixed in 4% polyformaldehyde overnight. Serial cryosections (6 μ m) embedded in OCT compound were classified by low and undisturbed regions for the two groups. The serial cryosections were stained with hematoxylin and eosin (both Sigma-Aldrich, St. Louis, MO, USA) to observe the morphologic transmutation of the carotid arteries. The intima-media ratio was measured by use of an automated image analysis system (Image-Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA) attached to a color charged-coupled device video camera.

Cell Culture and Flow Experiment, and siRNA Transfection *In Vitro*

HUVECs purchased from the American Type Cell Collection (Manassas, VA, USA) were cultured in EBM-2 medium (Lonza, Walkersville, MD, USA) containing 5% fetal bovine serum. Cells were cultured up to the fourth passage for experiments. A parallel-plate flow system was used to impose low shear stress (4 dynamics (dyn)/cm²) on HUVECs (23). To determine the mechanism of low shear stress-inducing VCAM-1 expression in HUVECs, we transfected cells separately with 330 pmol control or human CD31 or human JNK1/2 siRNA (both GenePharma, Shanghai, China) in 3 mL Opti-mem Medium (Invitrogen) mixed with Lipofectamine 2000 (Invitrogen) for 6 h and 10 μ mol/L SP600125 (Invitrogen) for 15 min before low shear stress.

RT-PCR Analysis

Six carotid artery specimens for low shear stress and three for undisturbed regions were pooled for each treatment group for RNA isolation with TriZol (Invitrogen). HUVECs stimulated with low shear stress or kept under static condi-

tions were pretreated with siRNA or inhibitor. Purified RNA (1 µg) was treated with DNase and reverse transcribed (RevertAid M-MuLV Reverse Transcriptase; Fermentas UAB, Mainz, Germany) following the manufacturer's protocol. Real-time PCR involved use of the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Four technical replicates were run for each gene in each sample. Primers were for *VCAM-1* (forward) 5'-ATGACATGCTTGAGCCAGG-3' and (reverse) 5'-GTGTCCTCTCTTTGACACT-3', and *β-actin* (forward) 5'-TGGACATCCGCAAAGAC-3' and (reverse) 5'-GAAAGGGTGTAAACCGAACTA-3' as a control. PCR amplification was at 95°C for 5 min, 36 cycles at 95°C for 10 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s. *VCAM-1* mRNA expression was normalized to that of *β-actin*.

Western Blot Analysis

Cytoplasm and nuclear proteins were extracted from three and six contralateral carotid artery specimens after undisturbed and low shear stress treatment, respectively, for each group. HUVECs for each group were collected for protein extraction. Equal amounts of protein (2 mg/mL) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After being blocked with 5% nonfat milk, the blots were washed in Tris-buffered saline with Tween (TBS-T) 3× for 10 min and incubated at 4°C overnight with an appropriate primary antibody: rabbit anti-β-actin (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-total-JNK (t-JNK) or rabbit anti-p-JNK (both 1:1,000 dilution; Cell Signaling Technology, Boston, MA, USA); rat anti-CD31 (1:500 dilution; Becton Dickinson, Franklin Lakes, NJ, USA); or goat anti-VCAM-1 (1:200 dilution; Santa Cruz Biotechnology). The blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary anti-

body (1:5,000 dilution; Jingmei Biotech, Shanghai, China) for 2 h at room temperature. After three washes in TBS-T, the membrane was visualized by enhanced chemiluminescence plus reagents (Millipore, Plano, TX, USA).

Immunocytochemistry and Immunohistochemistry

HUVECs were fixed in 4% paraformaldehyde and permeabilized in phosphate-buffered saline containing 0.5% Triton X-100. After being blocked with normal serum, cells and cryosections were incubated with rabbit anti-p-JNK (1:1,000 dilution); rabbit anti-p-NFκB p65 antibody or goat anti-VCAM-1 (both 1:200 dilution; Santa Cruz Biotechnology); or rabbit anti-von Willebrand factor (vWF) (1:500 dilution; Abcam, Boston, MA, USA) overnight at 4°C. Alexa 488-conjugated donkey anti-rabbit IgG (1:2,000) and Alexa 568-conjugated donkey anti-goat IgG (1:2,000; both Invitrogen) were secondary antibodies. A drop of Prolong Gold antifade reagent with diaminido phenyl indol (DAPI) (Invitrogen) was used to seal coverslips. Images were acquired by laser scanning confocal microscopy (LSM710; Carl Zeiss, Oberkochen, Germany) and analyzed by use of Image Pro Plus 6.0 (Media Cybernetics).

Statistical Analysis

Data are expressed as mean ± SD. SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA), was used for statistical analysis. Differences between groups in low shear stress-induced plaque formation were analyzed by the χ² test. Correlations between NF-κB activity and VCAM-1 level in HUVECs after low shear stress were determined by Spearman rank correlation test. Differences for two groups were compared by Student *t* test and for more than two groups by analysis of variance. *P* < 0.05 was considered statistically significant.

RESULTS

During the experiments, three mice in the NS group and two in the JNK-I group died, so data for 39 and 40 mice,

Table 1. Changes of shear stress in low and undisturbed regions between two groups detected by microultrasound imaging measurement before and after surgery (3 d).

	Undist (n = 8)	Low (n = 8)
Vmax (cm/s)	28.76 ± 7.12	3.84 ± 0.47
Ds (cm)	0.42 ± 0.11	0.45 ± 0.09
SS (dyn/cm ²)	36.61 ± 9.37	4.56 ± 1.03 ^o

Vmax, maximum velocity of carotid artery; Ds, end-systolic diameters of carotid artery; SS, shear stress of carotid artery, SS = 4 µVmax/Ds; µ, blood viscosity of mice; undist, undisturbed shear stress; low, low shear stress. Data are means ± SD. ^o*P* < 0.05 comparing low with undisturbed by paired Student *t* test.

respectively, remained for analysis. The mice were in good health, and SP600125 was well tolerated. The values of shear stress after cast implantation were markedly lower in low regions than undisturbed regions (Table 1, *P* < 0.05). Levels of total and LDL cholesterol did not differ between the groups before or after injection (Table 2, *P* > 0.05). Therefore, SP600125 had no significant effect on lipid levels in the circulation system, and diet or lipid levels did not contribute to the observed atherosclerotic lesion differences between two groups.

Inhibition of JNK Activity Reduced Low Shear Stress-Induced Atherosclerotic Plaque Formation

In the NS group, the incidence of plaque in low regions was 100% and 0% in undisturbed regions. However, of

Table 2. Levels of total cholesterol and LDL cholesterol after injection.

	NS group (n = 39)	JNK-I group (n = 40)
Total cholesterol (mmol/L)	5.97 ± 1.62	5.79 ± 1.07
LDL cholesterol (mmol/L)	3.92 ± 1.01	3.47 ± 0.78

JNK-I, SP600125 group. Data are means ± SD. *P* > 0.05 comparing two groups by paired Student *t* test.

Table 3. Incidence of atherosclerotic plaque in two groups induced by low and undisturbed shear stress.

	Shear stress regions			
	NS group (n = 15)		JNK-I group (n = 15)	
	Low	Undist	Low	Undist
Atherosclerotic plaque	15	0	2	0
Percent within group	100	0	13.33 ^a	0

Undist, undisturbed shear stress; low, low shear stress. Data are means \pm SD.

^a $P < 0.05$ comparing the JNK-I group with the NS group in low regions by χ^2 test.

the 15 JNK-I mice, only 2 (13.33%) showed plaque in low regions, and the incidence was markedly lower than that for NS mice (Table 3, $P < 0.05$). Photomicrographs of NS mice (Figure 1A) showed low regions with atherosclerotic lesions, and the plaque size was significantly larger than in undisturbed regions (intima-media ratio 1.21 ± 0.23 versus 0.03 ± 0.005 ; $P < 0.05$; Figure 1B). In contrast, the JNK-I mice showed no atherosclerotic lesion formation in low and undisturbed regions (see Figure 1A), and the intima-media ratio was signifi-

cantly lower in low regions than in such regions in NS mice (0.07 ± 0.003 versus 1.21 ± 0.23 ; $P < 0.05$; see Figure 1B).

To determine the inhibition efficiency of SP600125 on JNK, we further detected the expression of t-JNK and p-JNK in different regions by Western blot analysis (Figure 1C). The ratio of p-JNK to t-JNK protein expression was lower in JNK-I mice than in NS mice. The inhibition efficiency was 63.37% and 25.35% that of the control in low and undisturbed regions, respectively (0.38 ± 0.05 versus 1.04 ± 0.09 in low regions; 0.37 ± 0.04 versus

0.48 ± 0.03 in undisturbed regions, $P < 0.05$; Figures 1C, D).

Inhibition of JNK Activity Reduced Low Shear Stress-Induced VCAM-1 Expression

Because of the key role of proinflammatory cytokines in early atherosclerosis, we assessed VCAM-1 expression in different regions. Immunofluorescence analysis revealed VCAM-1 protein expression upregulated 2.60 ± 0.12 -fold in low regions compared with undisturbed regions in NS mice ($P < 0.05$; Figures 2A, B). Western blot analysis revealed VCAM-1 protein level upregulated 2.21 ± 0.11 -fold in low regions compared with undisturbed regions in NS mice (1.42 ± 0.12 versus 0.64 ± 0.08 relative to β -actin; $P < 0.05$; Figures 2C, D). Inhibition of JNK significantly reduced VCAM-1 protein levels in low regions compared with NS mice by immunofluorescence (0.68 ± 0.09 versus 2.60 ± 0.12 , normalized to undisturbed regions in the NS group; $P < 0.05$; see Figures 2A, B) and Western blot analysis (0.64 ± 0.09 versus 2.21 ± 0.12 relative to β -actin; $P < 0.05$; see Figures 2C, D).

These *in vivo* findings were consistent with inhibition of JNK activity, reducing low shear stress-induced upregulation of proatherogenic inflammatory mediators (VCAM-1) in atherosclerotic lesion formation.

Low Shear Stress-Induced Time-Dependent Upregulation of VCAM-1 in HUVECs

To further investigate the mechanism of low shear stress in atherosclerosis, we tested the levels of VCAM-1 exposure to low shear stress (4 dyn/cm^2) at various times (0, 6, 12, 18 and 24 h) in HUVECs. The elevation of VCAM-1 mRNA was detected within a short time—4.93-fold that of control at 6 h ($P < 0.05$; Figure 3A). The expression peaked at 12 h (17.58-fold versus control) and then decreased at 24 h ($P < 0.05$; see Figure 3A). The protein detection by Western blot analysis showed the same pattern ($P < 0.05$; Figure 3B, C). Therefore, we stimulated HUVECs

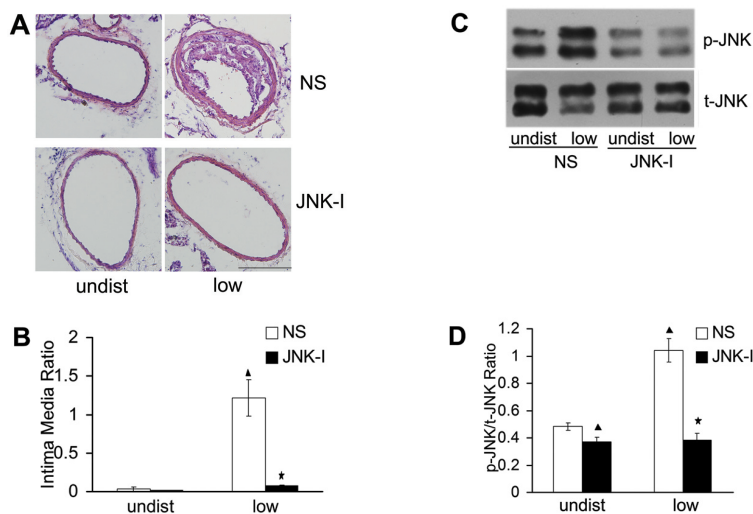


Figure 1. Morphology of carotid artery arteriosclerotic plaque and the efficiency of SP600125 to inhibit JNK at 10 wks after cast placement in ApoE^{-/-} mice. (A) Representative images from low and undisturbed flow regions in two groups (hematoxylin and eosin staining; 200 \times). Bar = 200 μ m. (B) Quantification of the intima-media ratio obtained by measuring the area confined by the external elastic lamina of the instrumented carotid artery in NS and JNK-I mice. (C) Western blot analysis of JNK protein expression (including p-JNK and t-JNK) for two groups of mice. (D) The activity of JNK (ratio of p-JNK to t-JNK) in different groups. All data are mean \pm SD of sections from 15 different animals. undist, undisturbed shear stress; low, low shear stress. $\blacktriangle P < 0.05$ versus undist regions in NS group. $\ast P < 0.05$ versus low regions in NS group.

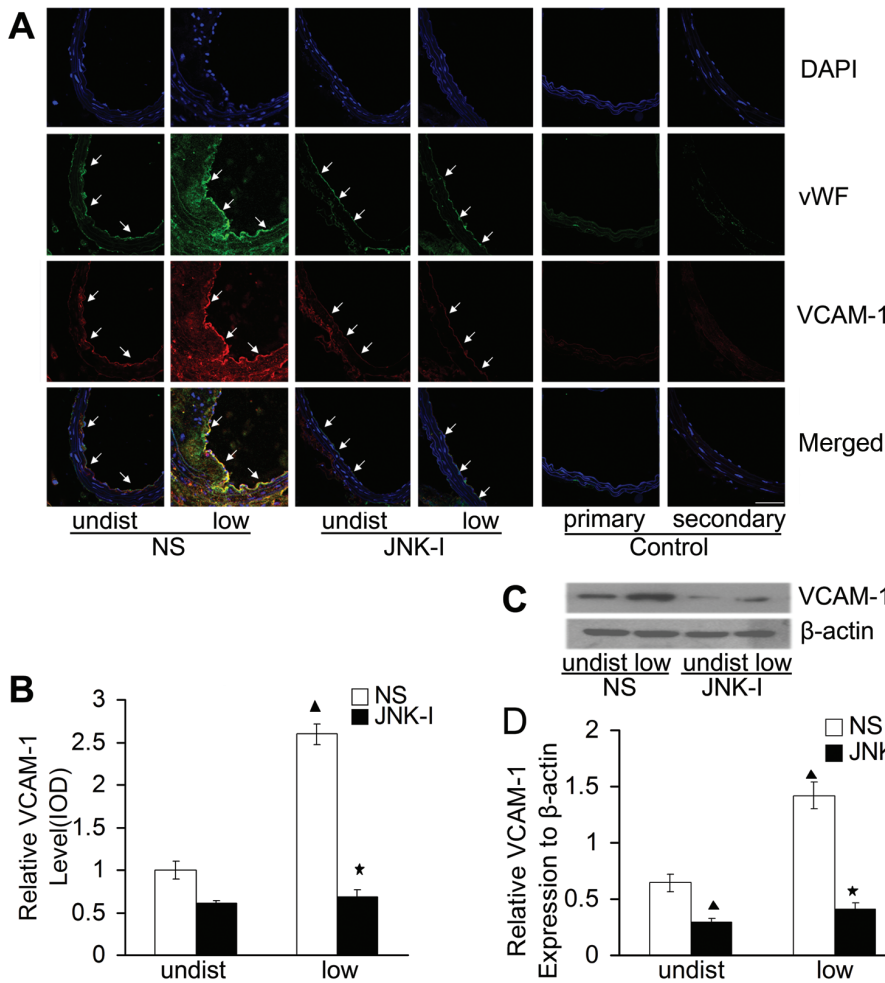


Figure 2. Relative VCAM-1 protein expression in different carotid artery regions. (A) Immunofluorescence analyses of VCAM-1 expression within vascular endothelium including primary and secondary antibody controls. Representative images acquired by laser scanning confocal microscopy (400×) showed vWF staining (green) of intact endothelium, VCAM-1 staining (red) and DAPI staining (blue) for cell nuclei. Bar = 100 μm; arrows denote the ECs. (B) Relative quantification of VCAM-1 expression in low and undisturbed (undist) regions by measuring the integrated optical density (IOD) of positive red area normalized to undisturbed flow region of the NS group. Western blot analysis of VCAM-1 in different regions (C) and quantification of protein expression of VCAM-1 relative to β-actin (D). Data are mean ± SD from six animals from low flow regions from experiments performed in triplicate. **P* < 0.05 versus low regions in NS the group. ▲*P* < 0.05 versus undisturbed regions in the NS group.

with low shear stress for 12 h in the following experiments.

ACTIVITIES OF JNK AND NF-κB ENHANCED WITH UPREGULATION OF VCAM-1 INDUCED BY LOW SHEAR STRESS IN HUVECS

With VCAM-1 elevation stimulated by low shear stress for 12 h, we compared the activity of JNK (p-JNK) and NF-κB

(p-p65) in HUVECs by Western blot analysis and immunofluorescence. The activity of JNK (p-JNK/t-JNK) was enhanced 4.34-fold (*P* < 0.05; Figures 4A, C) or 3.14-fold (*P* < 0.05; Figures 4E, G), respectively, by low shear stress compared with the static control group. Simultaneously, after low shear stress for 12 h, the activity of NF-κB (p-p65) was enhanced 1.89-fold (*P* < 0.05; Figures 4B, D) or

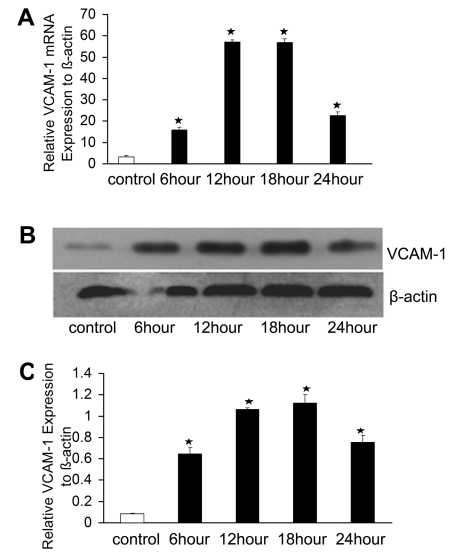


Figure 3. Relative levels of VCAM-1 with low shear stress (4 dyn/cm²) at various times in HUVECs. (A) RT-PCR results of VCAM-1 mRNA expression relative to β-actin. (B) Western blot analysis of VCAM-1 and β-actin protein expression in different groups. (C) Quantification of VCAM-1 protein expression. Control represents static (0 h). **P* < 0.05 versus control.

1.86-fold (*P* < 0.05; Figures 4F, H), respectively.

Downregulation of CD31 Depressed the Enhanced Activity of JNK Stimulated by Low Shear Stress in HUVECs

To determine whether CD31 was involved in JNK and VCAM-1 upregulation induced by low shear stress, we determined the activity of JNK with or without human siRNA for CD31 (si-CD31). Before low shear stress treatment, HUVECs were transfected with si-CD31 and negative siRNA oligo CD31 (si-Neg), a control of si-CD31. The efficiency of siRNA on knocking down the CD31 expression was detected by Western blot analysis (Figure 5A). In static and low groups, the relative quantitation of CD31 with si-CD31 pretreatment was significantly downregulated 66.50% and 69.51%, respectively, compared with the respective control (0.35 ± 0.04 versus 1.00 ± 0.10 in the static group; 0.30 ± 0.02 versus 1.54 ±

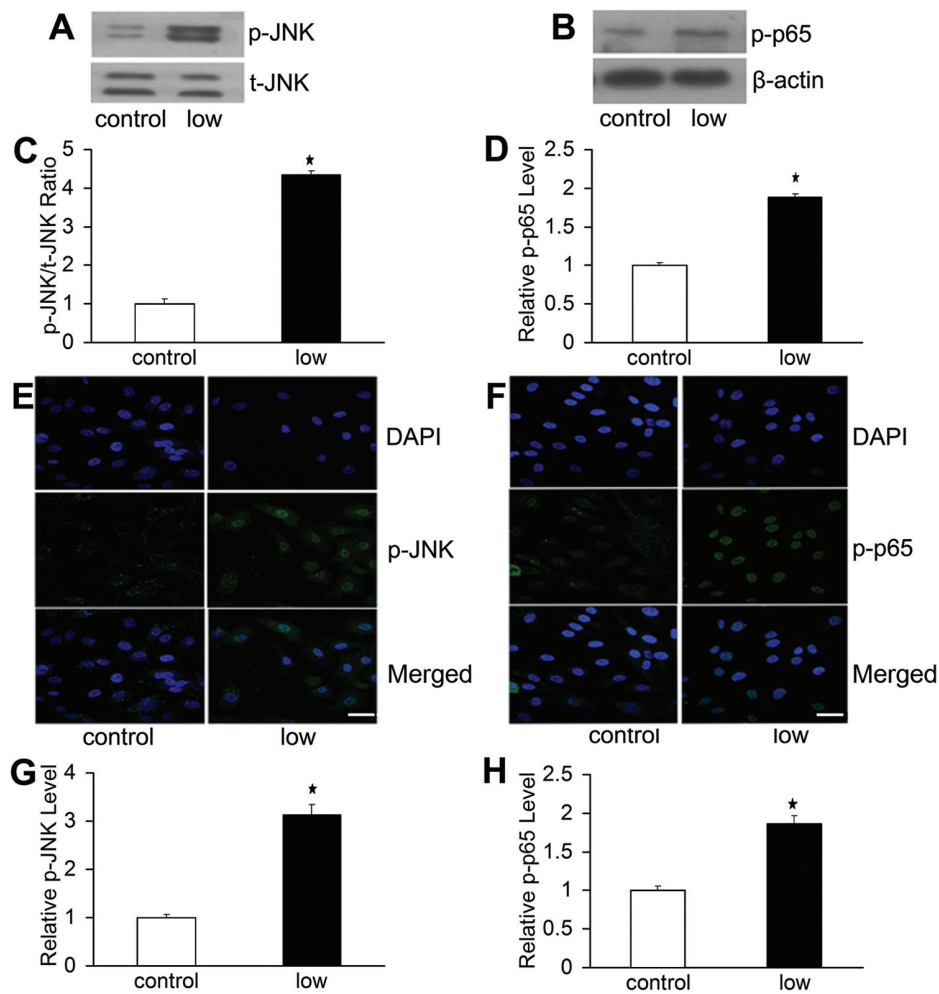


Figure 4. Western blot and immunofluorescence analysis of the active JNK (p-JNK) and NF- κ B (p-p65) expression induced by low shear stress for 12 h in HUVECs. (A) Western blot analysis of JNK protein expression (p-JNK and t-JNK) and (B) active NF- κ B (p-p65) of control and low shear stress groups. (C) Quantification of activity of JNK (p-JNK/t-JNK) before and after low shear stress stimulation. (D) Quantification of p-p65 relative to β -actin before and after low shear stress stimulation. (E) Representative immunofluorescence images by laser scanning microscopy showing p-JNK staining (green) of HUVECs before and after low shear stress for 12 h. (F) Representative immunofluorescence images showing p-p65 staining (green) of HUVECs. DAPI blue staining of cell nuclei is shown. Bar = 20 μ m. (G) Quantification of relative p-JNK level by measuring the positive green area of two groups. (H) Quantification of p-p65 relative level to control. All relative expressions were normalized to the control group. * $P < 0.05$ versus control.

0.14 in the low groups; $P < 0.05$; Figure 5B). With the valid knockdown of CD31, the activity of JNK in different groups was detected by Western blot analysis and immunofluorescence (Figures 6A, B). Western blot results showed that low shear stress could increase JNK activity compared with static control (2.37-fold and 2.74-fold of low control and low si-

Neg versus static control, $P < 0.05$; Figures 6A, C), with no significant difference in JNK level with si-Neg treatment from the static control ($P > 0.05$, see Figures 6A, C). With si-CD31 pretreatment, the activity of JNK (p-JNK/t-JNK) was decreased 24.68% and 31.16% compared with the static and low control, respectively (0.13 ± 0.01 versus 0.54 ± 0.03 and 0.39 ± 0.03 ver-

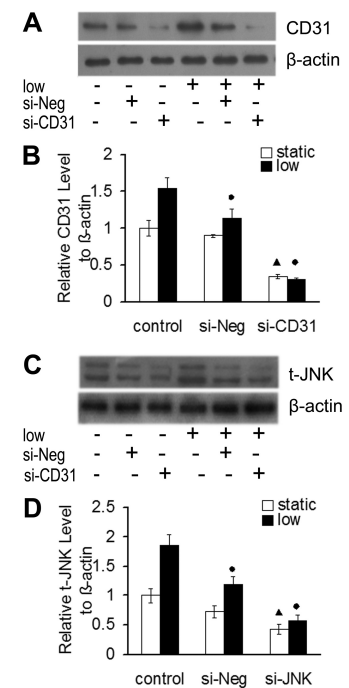


Figure 5. The efficiency of siRNA in knockdown of CD31 and JNK in HUVECs. (A) Western blot image of CD31 expression and β -actin with or without si-CD31 or low shear stress pretreatment. (B) Relative quantitation of CD31 normalized to control of static group in different groups. (C) Western blot image of t-JNK expression after si-CD31 or low shear stress treatments. (D) Quantitation of relative JNK level normalized to control of static group. Each data point was obtained from triplicate experiments. $\blacktriangle P < 0.05$ versus static control. $\bullet P < 0.05$ versus low shear stress control.

sus 1.27 ± 0.06 ; $P < 0.05$; see Figure 6C). The expression of p-JNK by immunofluorescence showed a similar pattern ($P < 0.05$; Figures 6B, D), except that the degree of p-JNK reduction by si-CD31 was not statistically significant by the static control group ($P > 0.05$; see Figures 6B, D).

Inhibition of JNK Attenuated the Increase of NF- κ B Activity and VCAM-1 Level Induced by Low Shear Stress in HUVECs

To validate the role of JNK in low shear stress altering NF- κ B activity (p-p65) and VCAM-1 levels in HUVECs, we used human JNK1/2 siRNA (si-JNK)

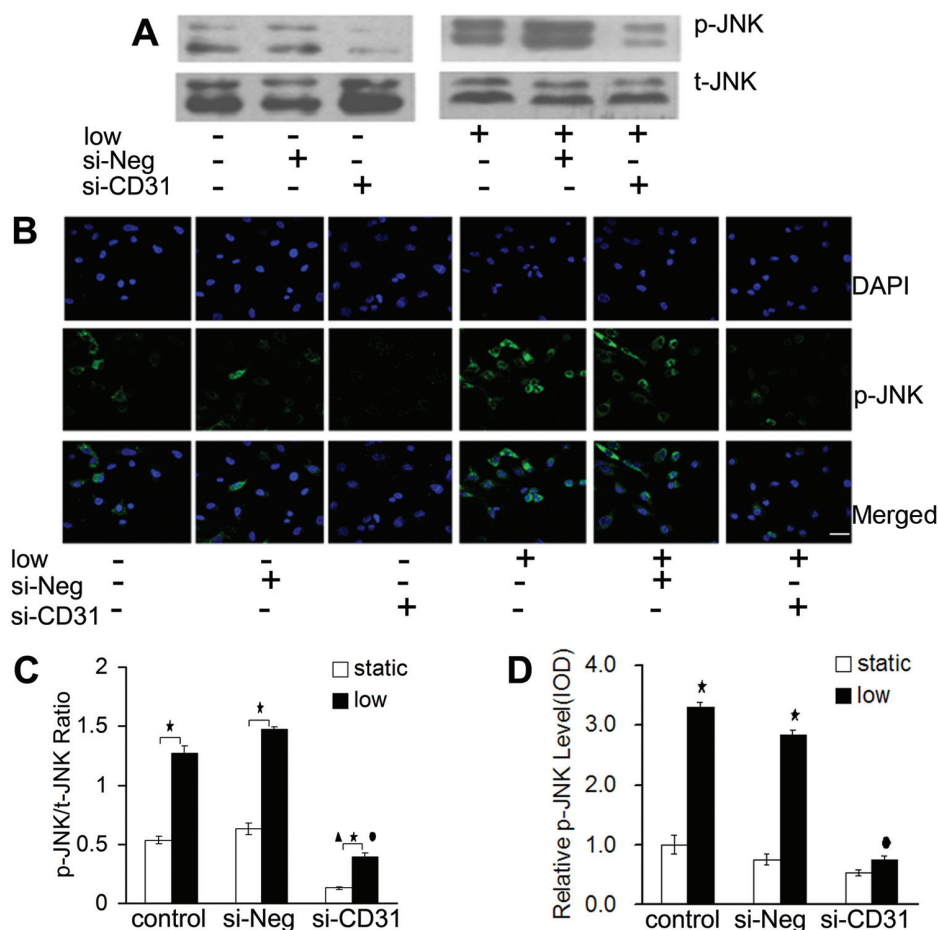


Figure 6. Activities of JNK (p-JNK) detected by Western blot and immunofluorescence analysis in HUVECs transfected with or without si-CD31 and si-Neg before and after low shear stress treatment. (A) Western blot analysis of JNK expression (p-JNK and t-JNK) in HUVECs with different stimulations. (B) Representative immunofluorescence images showing p-JNK staining (green). DAPI blue staining of cell nuclei is shown. Bar = 20 μ m. (C) Quantitation of JNK (p-JNK/t-JNK) in different groups by Western blot analysis. (D) Quantitation of relative p-JNK level (IOD) normalized to control of static group. * $P < 0.05$ versus static group. $\blacktriangle P < 0.05$ versus control of static group. $\bullet P < 0.05$ versus control of low shear stress group.

and SP600125 to downregulate JNK level. Negative siRNA oligo JNK1/2 (si-Neg) and no treatment were considered controls in the low and static groups. The efficiency of siRNA on knocking down JNK1/2 protein expression was also detected by Western blot (Figure 5C). The inhibition efficiency of si-JNK was significantly downregulated, to 66.99% and 69.39% of the respective control (0.43 ± 0.09 versus 1.00 ± 0.12 in the static group; 0.57 ± 0.13 versus 1.86 ± 0.11 in low groups; $P < 0.05$; Figure 5D). Immunofluorescence detection of the p-p65 expres-

sion was significantly increased by low shear stress without interference of JNK (si-JNK or SP600125) (2.67 ± 0.08 - and 2.47 ± 0.02 -fold in the low control and si-Neg groups versus static control, $P < 0.05$; Figures 7A, C). However, with low shear stress stimulation with si-JNK pretreatment or SP600125 inhibition, the activity of p-p65 was decreased, to 39.28% and 43.54%, respectively, compared with the low control (1.05 ± 0.06 and 1.16 ± 0.05 versus 2.27 ± 0.08 ; $P < 0.05$; Figures 6A, B). Western blot analysis revealed a similar pattern with alteration of p-p65

($P < 0.05$; Figures 7C, D). Meanwhile, VCAM-1 expression showed a similar pattern ($P < 0.05$; Figures 7C, E). Changes of p-p65 and VCAM-1 level were significantly correlated ($r^2 = 0.992$, $P < 0.01$).

DISCUSSION

Our study aimed to investigate the effect and mechanism of JNK on low shear stress-induced atherogenesis. We confirmed that low shear stress was essential in plaque formation, and inhibition of JNK markedly reduced low shear stress-induced plaque formation in ApoE^{-/-} mice. The expression of active JNK (p-JNK) was related to the expression of a proatherogenic inflammatory mediator (VCAM-1). Therefore, JNK may take part in atheroprone low shear stress-induced adhesion molecule (VCAM-1) expression in promoting plaque formation. Finally, *in vitro* experiments with si-CD31-based knockdown reduced endothelial p-JNK and VCAM-1 levels in HUVECs. Downregulation of JNK by si-JNK or SP600125 inhibition both attenuated NF- κ B activity and VCAM-1 expression induced by low shear stress in HUVECs. JNK may play a critical role in low shear stress-induced atherogenesis, at least in part by CD31-dependent mechanical sensation and modulating NF- κ B activity and VCAM-1 expression.

The relationship among shear stress, JNK signaling and atherogenesis (24) has been documented. Arterial regions exposed to high shear stress are protected against endothelial activation, inflammation and atherosclerosis (25), whereas regions exposed to low/oscillatory shear stress are susceptible (26). The risk of atherosclerosis is lower in vasculature exposed to steady laminar flow than disturbed flow with low shear stress (27,28). However, most of the previous research was on the basis of rabbit aortas (29), pigs (30), HUVECs or human arterial ECs (25,26,31,32), so we further investigated the mechanism of different patterns of shear stress in atherogenesis in ApoE^{-/-} mice.

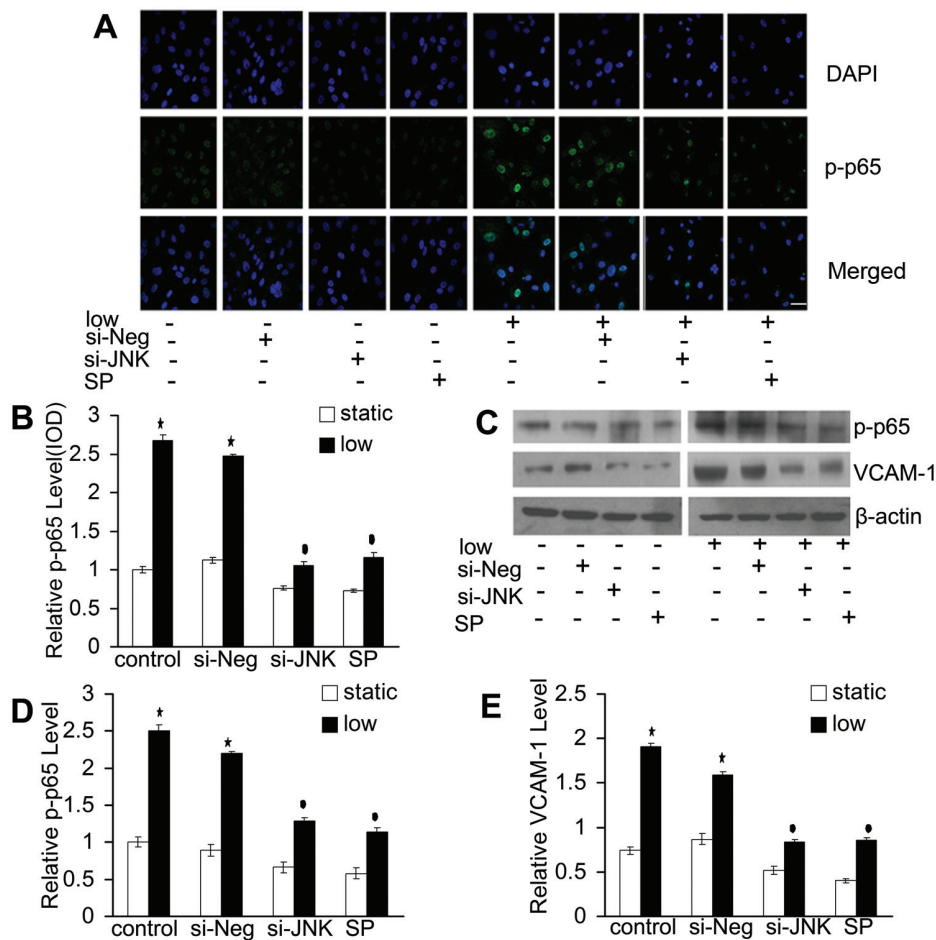


Figure 7. Activity of NF- κ B (p-p65) and VCAM-1 expression detected by immunofluorescence and Western blot analysis with or without downregulation of JNK before and after low shear stress treatment. (A) Representative immunofluorescence images of p-p65 expression staining (green) in HUVECs with different stimulations. DAPI blue staining of cell nuclei. Bar = 20 μ m. (B) Activity of p-p65 in different groups detected by immunofluorescence (IOD) was normalized to control of static group. (C) Western blot analysis of p-p65 and VCAM-1 expression of different groups. Quantitation of p-p65 level (D) and VCAM-1 level relative to β -actin (E). SP, SP600125; si-Neg was control for si-JNK. * $P < 0.05$ versus static group. • $P < 0.05$ versus control within low group.

Previous research reported the use of a shear stress modifier, a cast, in ApoE^{-/-} mice (10). We used the same model and calculated the shear stress by microultrasound imaging measurement. As a control, placing no constrictive cast did not induce atherosclerosis in all regions, which indicated the validity of the model. Images acquired by laser scanning confocal microscopy revealed that the endothelium was continuous in all shear stress regions by vWF staining, which suggested the responsiveness of

the shear stress because the cast placement did not elicit a nonspecific atherogenic response.

In our preliminary experiment, we found low shear stress highly proatherogenic, as did other studies (20,33,34). The plaques in the low region contained more lipids and less collagen than those in oscillatory regions (downstream of the cast), which are characteristic features of vulnerable plaques. Undisturbed regions as controls were protected against atherosclerotic lesion formation. As well, spatiotemporal

oscillations of shear stress downstream were measured in a rabbit model rather than in mice (10,35). Moreover, we focused on the role of JNK in disturbed shear stress-induced vessel pathology (11). Therefore, we further studied the changes in JNK inhibition in low shear stress in the proatherogenic process.

Inhibition of JNK by SP600125 could reduce low shear stress-induced pathological changes, including plaque incidence and size, but had no influence in undisturbed regions, which agrees with the accepted notion that plaques do not develop under relatively normal conditions. Because chronic inflammatory disease of arteries is one feature of atherosclerosis (36) and the key role in expression of cytokines and adhesion molecules (26,37), we tested the expression of a proinflammatory mediator, VCAM-1, in different regions regulated by JNK. Because of the low expression of VCAM-1 in the physiological condition (undisturbed regions in NS group), the reduced expression of VCAM-1 with SP600125 seemed lower in undisturbed regions than in low regions. JNK inhibition simultaneously reduced VCAM-1 expression in all regions with the reduction in atherosclerosis.

Many studies have revealed that low shear stress potentiated proinflammatory activation of ECs, and regions of the vasculature exposed to low shear stress might be susceptible to inflammation because of constitutive activation of activator protein 1 (AP-1) and NF- κ B pathways and increased expression of adhesion molecules (VCAM-1 and intracellular adhesion molecule 1 [ICAM-1]) (38). Our results confirmed those findings *in vivo*. In HUVECs, the enhanced NF- κ B activity and VCAM-1 expression by low shear stress for 12 hours could be attenuated by JNK knockdown with siRNA or SP600125. Although the acute shear experiments may not necessarily reflect exposure of ECs to chronic flow *in vivo*, these data indicate that JNK might take part in atheroprone shear stress-induced atherosclerosis by activating downstream NF- κ B and up-regulating VCAM-1.

To determine why we found no plaques in the JNK-I group, we learned that blood flow influences atherosclerosis by exerting disturbed shear stress on the inner surface of arteries and altering endothelial physiology (6,39). Atherosclerosis is one of the chronic inflammatory artery diseases (40) triggered by proinflammatory mediators, especially adhesion molecules (for example, VCAM-1) (41) in ECs activated by p38 and JNK MAPKs (42,43). Cuhlmann *et al.* (44) recently demonstrated that disturbed blood flow promotes arterial inflammation by inducing NF-κB expression in ECs via JNK-ATF2 signaling. Therefore, SP600125 might inhibit the low shear stress-induced atherosclerosis by abating the signaling pathway of NF-κB-VCAM-1.

Some investigators showed that PECAM-1 knockout mice did not show activated NF-κB and downstream inflammatory genes in regions of disturbed flow (18,45). However, the mechanosensing pathway was discordant, because MAPKs (ERK1/2, p38) and AKT could be phosphorylated by shear stress independently of CD31 in vascular ECs (46). We wondered whether CD31 directly transmitted a mechanical force to intracellular signaling pathway MAPKs (JNK) and downstream targets. By using knockdown of CD31, we illustrated *in vitro* that CD31 may function as one of the major mechanoreceptors for activation of JNK followed by downstream NF-κB and VCAM-1 in HUVECs treated with low shear stress.

In summary, our study identified JNK as an essential factor in atheroprone shear-stress-induced atherosclerotic lesion formation in ApoE^{-/-} mice that involves CD31 sensing and activating NF-κB. We elucidated the protective mechanism of SP600125 in atherosclerosis in reducing the expression of VCAM-1. These findings might be helpful in designing novel therapeutic strategies for protecting arteries against disturbed shear stress-induced inflammation in atherosclerosis.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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