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Stress and Vascular Responses: Atheroprotective Effect of Laminar Fluid Shear Stress in Endothelial Cells: Possible Role of Mitogen-Activated Protein Kinases

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Abstract. Atherosclerosis preferentially occurs in areas of turbulent blood flow and low fluid shear stress, whereas laminar blood flow and high shear stress are atheroprotective. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), stimulate expression of endothelial cell (EC) genes that may promote atherosclerosis. Recent findings suggest a steady laminar blood flow decreases EC apoptosis and inhibits TNF-mediated EC activation. EC apoptosis or activation is suggested to be involved in plaque erosion, which may lead to platelet aggregation. TNF-α regulates gene expression in ECs, in part, by stimulating mitogen-activated protein (MAP) kinases, which phosphorylate transcription factors. We hypothesized that steady laminar flow inhibits cytokine-mediated activation of MAP kinases in ECs. To test this hypothesis, we determined the effects of steady laminar flow (shear stress = 12 dynes/cm^2) on TNF-α-stimulated activity of three MAP kinases in human umbilical vein ECs (HUVEC): extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38. TNF-α activated ERK1/2, JNK, and p38 maximally at 15 min in HUVEC. Pre-exposing HUVEC for 10 min to flow inhibited TNF-α activation of JNK, but showed no significant effect on ERK1/2 or p38 activation. Incubation of HUVEC with PD98059, a specific ERK1/2 inhibitor, blocked the flow-mediated inhibition of TNF activation of JNK. Transfection studies with dominant-negative constructs of the protein kinase MEK5 suggested an important role for big mitogen-activated protein kinase 1 (BMK1) in flow-mediated regulation of EC activation by TNF-α. Understanding the mechanisms by which steady laminar flow regulates JNK activation by cytokines may provide insight into the atheroprotective mechanisms induced by laminar blood flow.

Keywords: atherosclerosis, laminar fluid shear stress, mitogen-activated protein kinase, endothelial cell

Introduction

Numerous studies suggest that inflammation plays a key role in the pathogenesis and progression of atherosclerosis (1, 2). Previous findings suggested that inflammatory events contribute at each stage in the development of atherosclerosis. As an example, fatty streak formation is associated with expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (ECs) (3). During the process of plaque formation, the monocytes present in the plaque proliferate, oxidize LDL, and generate multiple cytokines that act as chemoattractants for other inflammatory cells (2). Finally, plaque rupture involves activation of macrophages at the edge of the fibrous cap with subsequent production of matrix proteases (1, 2). Therefore, multiple recurrent inflammatory events contribute to the initiation and progression of atherosclerosis. ECs are the component of vascular wall cells that primarily limit the athero-

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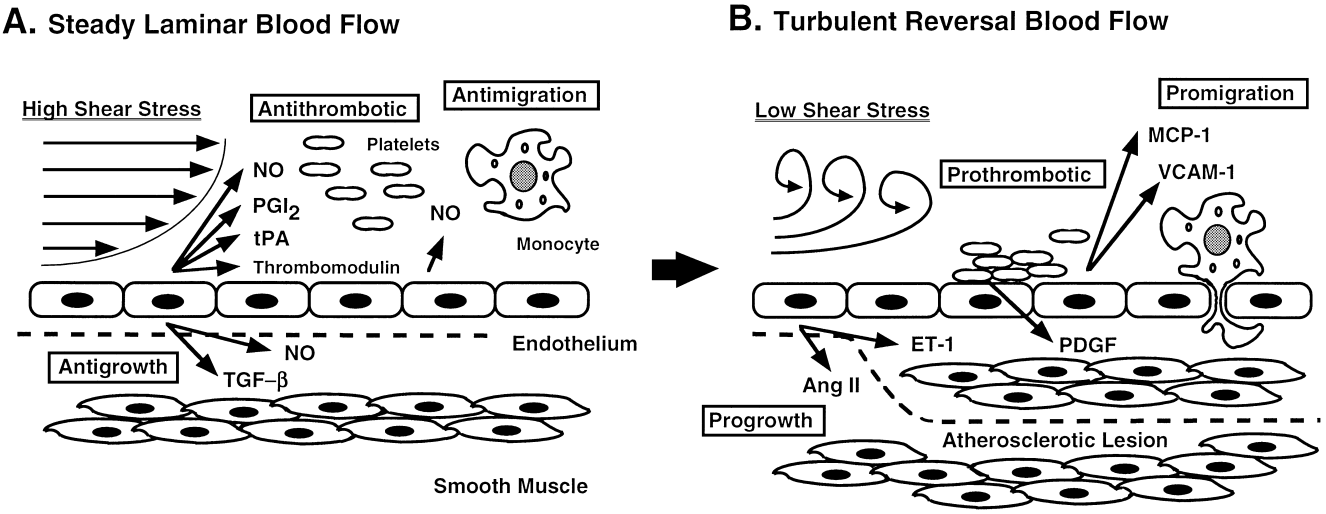

Fig. 1. Endothelial cell biology and shear stress. A: Steady laminar shear stress promotes release of factors from endothelial cells that inhibit coagulation, migration of leukocytes, and smooth muscle proliferation, while simultaneously promoting endothelial cell survival. B: In contrast, low shear stress and turbulent flow shift the profile of secreted factors and expressed surface molecules to one that favors the opposite effects, thereby contributing to the development of atherosclerosis. PGI_2 indicates prostacyclin; tPA, tissue plasminogen activator; TGF-β; transforming growth factor-β; Ang II, angiotensin II; ET-1, endothelin-1; PDGF, platelet-derived growth factor; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

sclerotic processes. ECs produce several molecules (nitric oxide and prostacyclin), hormones (natriuretic peptides), and matrix molecules (heparans and extracellular superoxide dismutase (SOD)) that counteract these inflammatory events. Many of these atheroprotective molecules are produced in response to steady laminar blood flow (4, 5). Conversely, the initiation and progression of atherosclerosis is increased in regions of turbulent blood flow and low fluid shear stress (6, 7). Therefore, our major hypothesis is that steady laminar shear stress prevents atherosclerosis by inhibiting the effects of inflammatory cytokines (Fig. 1).

Among the many approaches to study the nature of the atheroprotective mechanisms of steady laminar flow, we focused on the intracellular signal transduction mechanisms. We and others have shown that flow induces a genetic program of protein expression that should be atheroprotective (4, 5). For example, flow induced gene expressions of nitric oxide synthase (eNOS) and SOD, whereas VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) genes were turned off. Since the activity of transcription factors is controlled by protein phosphorylation, we focused on the protein kinases. We previously found that members of the mitogen-activated protein (MAP) kinase family are activated by flow (8, 9). MAP kinase members phosphorylate and activate transcription factors that include expression of both pro- and anti-inflammatory molecules. In particular, c-Jun N-terminal kinase (JNK) is activated by almost all inflammatory cytokines (e.g., tumor necrosis factor (TNF) and interleukin-1 (IL-1)). Therefore, we propose that understanding the mechanisms by which flow regulates JNK activation by cytokines will provide insight into the atheroprotective mechanisms induced by flow.

TNF-α-mediated JNK activation is inhibited by flow in endothelial cells

TNF-α is one of the major inflammatory cytokines that mediates systemic inflammation and immune responses (10). A major site of action of TNF-α for these effects is the vascular endothelium, where it induces inflammatory responses by enhancing adhesion molecule expression and secretion of inflammatory mediators (11). TNF-α has been shown to stimulate activation of a wide variety of putative second messengers. Some of the known signal transduction pathways induced by TNF-α are coupling to G-proteins (12), activation of phospholipase A_2 (13), calcium mobilization (14), and ceramide generation (15). In the context of ECs activation by TNF-α, involvement of MAP kinase, i.e., extracellular-signal regulated kinase (ERK1/2), JNK, and p38, have been reported (16). Under the above mentioned hypothesis that flow confers an atheroprotective effect via inhibition of specific signal transduction mechanisms, we examined the effect of steady laminar flow on TNF-α-induced MAP kinase activation

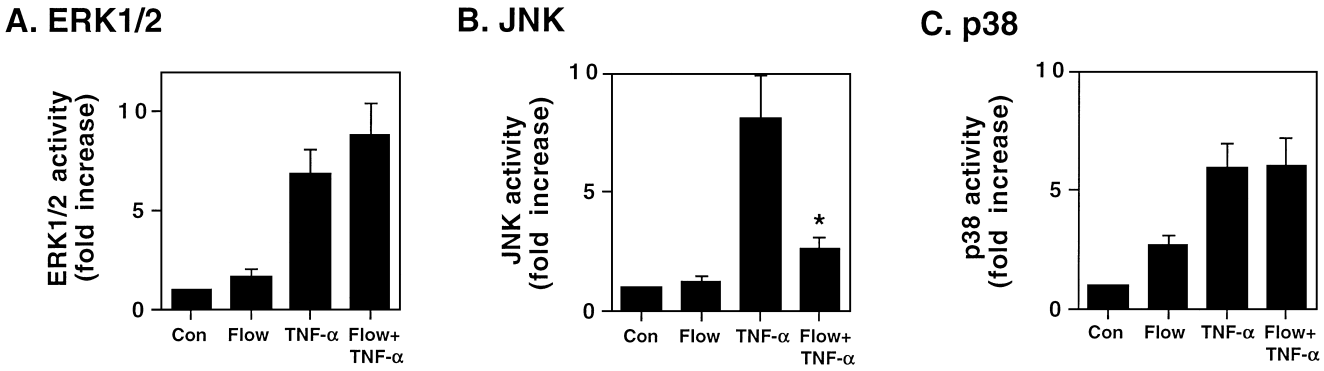

Fig. 2. Flow pre-exposure inhibits TNF-α-mediated JNK activation, but not ERK1/2 and p38 activation, in HUVEC. HUVEC were subjected to the following "preconditioning" protocol: maintained in static conditions for 25 min exposed to flow for 10 min and then held static for 15 min, maintained in static conditions for 10 min followed by TNF-α (10 ng/ml) stimulation for 15 min, or subjected to flow for 10 min followed by TNF-α stimulation for 15 min. Cell lysates were prepared and analyzed for ERK activity (A), JNK activity (B), and p38 activity (C) by Western blotting with phosphospecific antibodies with each MAP kinase. Densitometric analyses of ERK1/2, JNK, and p38 activation are shown. Results were normalized by arbitrarily setting the densitometry of control cells to 1.0 (values are means ± S.D., n = 3). Asterisk represents significant differences compared with the cells stimulated by TNF-α for 15 min (*P<0.05).

in human umbilical vein ECs (HUVEC). We found that steady laminar flow alone does not significantly activate JNK and it inhibits TNF-α-mediated JNK activation (17, 18) (Fig. 2). Consistent with our findings, other studies reported that laminar flow inhibits TNF-α-mediated events in ECs. Dimmeler et al. showed that flow prevented EC apoptosis induced by TNF-α or serum withdrawal (19). Since activation of ERK1/2 has been associated with cell growth and/or survival while activation of JNK and p38 have been associated with apoptosis (20), the findings of Dimmeler suggest that the antiapoptotic effect of flow may be due to selective stimulation of ERK1/2 and inhibition of JNK. It was also reported that flow inhibited TNF-α-mediated increases in proinflammatory events including NF-κB activation, VCAM-1 expression, and monocyte adhesion to ECs (21, 22). These results may support our general hypothesis that flow inhibits inflammatory cytokine-mediated events in ECs.

Flow inhibition of JNK activation by TNF-α depends on activation of the ERK1/2 or big mitogen-activated protein kinase 1 (BMK1) pathway

Since crosstalk among MAP kinase family members has been shown to regulate activity (23 – 25), we examined the possibility that ERK1/2 activation might be responsible for the flow inhibition of JNK activation by TNF-α. To inhibit ERK1/2 activation, cells were pretreated with PD98059, a known MEK1/2 inhibitor. In preliminary experiments, PD98059 completely inhibited TNF-α- and flow-mediated ERK1/2 activation (data not shown), whereas it showed no inhibitory effect on TNF-α-induced JNK activation. Then the effect of PD98059 on flow inhibition of TNF-α-mediated JNK activation was studied. As shown in Fig. 3A, pre-exposure to flow caused significant inhibition of TNF-α-mediated JNK activation. However, in the presence of PD98059, the flow-mediated inhibition of JNK was almost completely prevented. These experiments demonstrate that the inhibition of TNF-α-mediated activation of JNK by flow depends on flow activation of the ERK1/2 signaling pathway. In addition, we investigated the involvement of BMK1, another MAP kinase, which is activated by an upstream MAP kinase kinase, MEK5 (26). Using transfection experiments with the constitutive-active form of MEK5, we found that TNF-α-mediated activation of JNK was inhibited by MEK5 (Fig. 3B). These results suggest that BMK1, as well as ERK1/2, may also be involved in the process of flow inhibition of TNF-α-induced JNK activation.

Concluding remarks

In addition to the effects of ERK1/2 and/or BMK1 on JNK, we reported that 14-3-3 and ASK1 (apoptosis signal-regulating kinase 1) may be involved in flow inhibition of cytokine-induced JNK activation (18). It is likely that flow modulates inflammatory cytokine actions by other mechanisms. These possible mechanisms include the JNK phosphatases and JNK interacting proteins (Fig. 4). Preliminary findings from our laboratory suggest a critical role for SHP-2. The tyrosine phosphatase activity of SHP-2 appears to be required

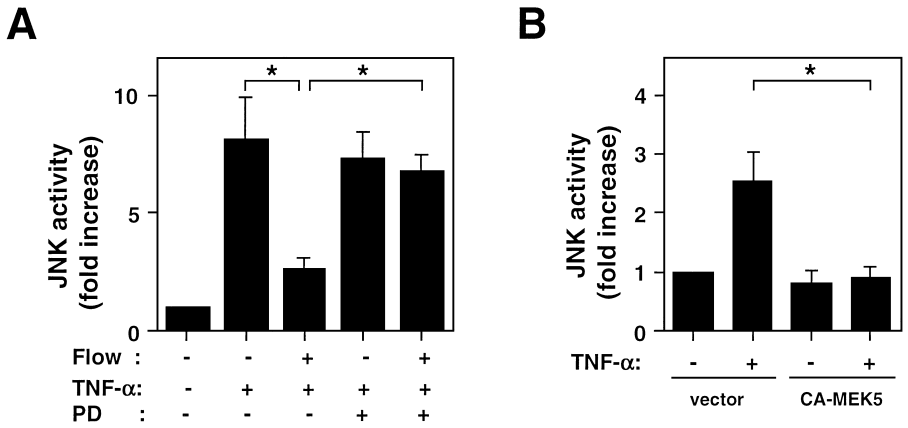

Fig. 3. ERK1/2 and BMK1 are involved in flow inhibition of JNK activation by TNF-α. A: Effect of PD98059 (PD) on TNF-α-mediated JNK activation and its inhibition by flow. HUVEC were pretreated with 30 μM PD98059 for 30 min or vehicle and then subjected to the preconditioning protocol described in Fig. 2. Cells were then stimulated with TNF-α (10 ng/ml) or subjected to flow followed by TNF-α stimulation in the presence or absence of 30 μM PD98059. Cell lysates were prepared and analyzed for JNK activity. B: Effect of constitutively active (CA)-MEK5 on TNF-α activation of JNK. HUVEC were double-transfected with Flag-JNK and pcDNA3.1 vector alone or the constitutive active form of MEK5 (CA-MEK5) and 8 h later, were washed with media and maintained in serum-free medium for 24 h. Cells were treated with TNF-α (10 ng/ml) for 15 min, harvested in lysis buffer, and immunoprecipitation with Flag antibody was performed overnight. Cell lysates were prepared and analyzed for JNK activity by Western blotting with phosphospecific c-Jun antibody. A and B: Densitometric analyses of JNK activation are shown. Results were normalized by arbitrarily setting the densitometry of control cells to 1.0 (values are means ± S.D., n = 3). Asterisk represents significant differences among the indicated groups (*P<0.05).

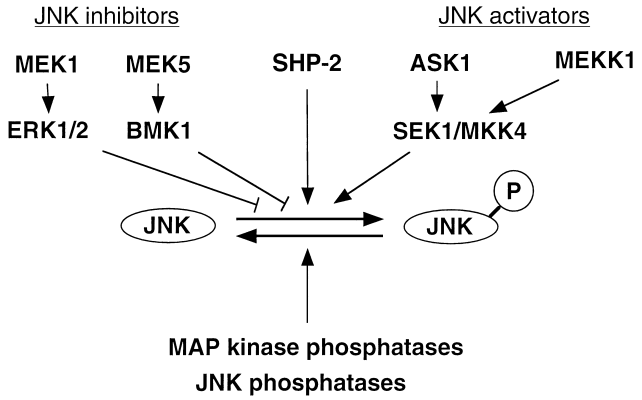

Fig. 4. Multiple mechanisms regulate the activity of JNK. Flow-mediated effects on cytokine signal transduction represent the balance of effects on both activators and inhibitors of JNK. Pathophysiological conditions may modulate the relative potency of these different pathways.

for TNF-α-mediated signal transduction (M. Yoshizumi et al., unpublished observation). Inhibition of the phosphatase activity by flow represents another likely mechanism by which flow modulates cytokine signaling. Pathophysiological conditions may also modulate the relative efficacy of these different pathways. Cardiovascular risk factors, such as smoking, hypercholesterolemia, diabetes, and hypertension, were recently shown to share a common potential pathogenic mechanism in that they all increase reactive oxygen species (ROS) and are associated with endothelial dysfunction (27, 28). An example is that turbulent reversal flow generates higher ROS levels than steady laminar flow (29). It is suggested that an additional manifestation of endothelial dysfunction is a decrease in the ability of ECs to inhibit cytokine-dependent signal transduction. Therefore, a biological hypothesis for endothelial dysfunction is that ERK1/2- and BMK1-dependent pathways activated by steady laminar flow are down-regulated by exposure of ECs to cardiovascular risk factors. Further studies will be required to elucidate the mechanisms by which risk factors alter BMK1 and ERK1/2 activation.

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