Differential immediate-early gene responses to shear stress and intraluminal pressure in intact human conduit vessels

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Abstract We have previously shown distinct effects of shear stress and pressure on transcription of several potent vascular mediators. In the present study, we tested the hypothesis that c-jun and c-fos are regulated differentially by shear and pressure. Intact human umbilical veins were perfused with various combinations of shear and pressure during 1.5, 3 and 6 h. Protein and gene expressions were assessed by immunofluorescence and real-time reverse transcription PCR, respectively. Shear stress and pressure exert differential temporal effects on c-jun and c-fos gene and protein expression, and these immediate-early gene responses appear to be cell-type specific for endothelial and smooth muscle cells.

Key words: Shear stress; Pressure; C-jun; C-fos; Perfusion; Real-time PCR

1. Introduction

The vascular endothelium is continuously exposed to three major types of fluid dynamic forces: shear stress and compressive and circumferential stretch forces. Besides acute effects, shear stress and intraluminal pressure have been shown to exert sub-acute and chronic modulating effects on cultured vascular endothelial cells [1]. There is increasing evidence that these biomechanical forces have differential modulating effects on endothelial cell functions. We have previously shown that many of the important vascular mediators, e.g. t-PA, are differentially regulated by shear stress and intraluminal pressure at the level of transcription [2,3]. While high shear stress combined with physiological pressure induced an upregulation of t-PA expression, high intraluminal pressure combined with normal shear stress downregulated the t-PA protein and gene expression.

Activator protein-1 (AP-1) can be composed of c-fos/c-jun heterodimers or c-jun/c-jun homodimers, of which the former isoform possesses stronger AP-1 binding activity and thus is more biologically active [4,5]. The AP-1 transcription factor family has been shown to be important in the transcriptional control of numerous genes. Upregulation of monocyte chemotactic protein-1 (MCP-1) has been identified as an AP-1-mediated transcriptional event via interaction with a non-consensus tetra-decanoyl phorbol acetate response element within the promotor of MCP-1 [6]. Downregulation of vascular cell adhesion molecule-1 has been shown to be mediated via AP-1 interaction through two consensus AP-1 binding sites [7]. Using electrophoretic mobility shift assay, AP-1 binding activity has been reported to be upregulated by shear stress [8]. Shear is also known to induce rapid and transient upregulation of immediate-early genes (IEGs), c-jun and c-fos, in cultured endothelial cells [9–11]. Mechanical stretch has been shown to induce c-fos expression in cardiac myocytes [12]. However, it is still poorly understood how physiologically relevant combinations of shear and pressure sub-acutely or chronically regulate the c-jun and c-fos protein and gene expression in intact mammalian vessels.

We have developed a computerized vascular perfusion model, in which intact conduit vessels can be exposed to well-defined combinations of intraluminal pressure and shear stress [13]. In the present study, we investigated possible temporal responses of IEGs, c-fos and c-jun, to complex biomechanical forces. We hypothesized that these endothelial IEGs were differentially regulated by shearing and compressive forces and thus may be involved in the differential transcriptional regulation of many vascular mediators.

2. Materials and methods

2.1. The vascular perfusion model

The perfusion system has recently been described in detail elsewhere [13]. Briefly, perfusion circuits are driven by hydrostatic pressure created by continuous pumping of perfusion medium from a lower to an upper reservoir. Paired vascular segments are mounted in separate perfusion chambers, placed in a 37°C water bath (Tempette Junior TE-8J, Technie Ltd., Cambridge, UK).

The perfusion system is operated by a computerized control and feedback system. Perfusion pressure immediately before and after the vessels and flow rate were recorded by up- and down-stream pressure transducers (DPT-600, pvb medizintechnik gmbh, Kirchseeon, Germany) and an electromagnetic flow meter detector (Toshiba Ultrasmall Electromagnetic Flow Meter Detector model 334, Toshiba, Tokyo, Japan), respectively. The digital signals were processed in a Macintosh Power PC Computer 7600/120 MHz (Apple Computer, Santa Clara, CA, USA), equipped with a custom-assembled program developed by us in LABVIEW 4.0. Shear stress calculation was based on the following formula:

$$\tau = \frac{1}{2}\left(\frac{\Delta P}{L}\right)^{3/4} \cdot \left(\frac{8\eta Q}{\pi}\right)^{1/4},$$

where τ is wall shear stress, ΔP is the pressure drop over the vessel, L is the vessel length, η viscosity of the fluid, and Q is the flow through the vessel. Reynold's number was monitored continuously to ensure a laminar flow profile. Various combinations of shear stress and perfusion pressure/mean intraluminal pressure can be generated by computerized control of height regulators and proportionating solenoid valves (type 2821 with Control electronics type 1094, Bürkert, Ingel-

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fingen, Germany). The pH of the perfusion medium is kept constant by controlled bubbling of a gas mixture of 90% N_2, 5% CO_2 and 5% O_2 gas (AGA Gas AB, Stockholm, Sweden).

2.2. Experimental protocol

2.2.1. Preparation procedure. The studies were approved by the local Ethics Committee of the Göteborg University, and were conducted according to the principles of the Declaration of Helsinki.

Human umbilical cords were collected from the maternity ward of the hospital immediately after vaginal delivery and divided into two 20 cm segments for perfusion in parallel. After an initial 10 min non-recirculating wash-out period, vessels were equilibrated for another 20 min under constant mean intraluminal pressure and flow of 20 mm Hg and 10 ml/min, respectively. The vessel segments were thereafter randomized to perfusion according to different perfusion protocols.

2.2.2. Perfusion protocols. Paired umbilical veins were perfused under high/low shear stress ($25/<4$ dyn/cm^2) under identical mean perfusion pressure of 20 mm Hg or high/low mean perfusion pressure (40/20 mm Hg) at an identical shear stress level of 10 dyn/cm^2. Three series of perfusion experiments were performed for 1.5, 3 and 6 h with each of the perfusion protocol.

2.3. Quantitative immunohistochemistry and immunofluorescence

2.3.1. Sample preparation. At the end of each experiment, vascular segments of approximately 1 cm were fixed in 4% formalin for 24 h. Specimens were embedded in paraffin, sectioned into 5 μm slices and mounted on Superfrost Plusglasses (Menzel, Merck Ltd., Poole, UK). Before immunostaining, sections were deparaffinized in xylene and rehydrated in graded alcohol.

2.3.2. Immunofluorescence. Primary rabbit polyclonal antibodies c-Jun and c-Fos were diluted 1:100 in 0.1 M phosphate-buffered saline, 2.0% NaN_3, 0.2% Triton X-100 containing 0.1% bovine serum albumin (c-Jun (sc-1694), c-Fos (sc-52), Santa Cruz Biotechnology, Inc., CA, USA). Preparations were preincubated with normal goat serum 1:10 for 30 min in a humid chamber. Primary antibodies were applied and sections were incubated for 48 h at room temperature. Sections were thereafter washed in Cadenza buffer (407340, Immunon, Lipshaw, Inc., CA, USA) and incubated with a biotinylated secondary antibody (goat anti-rabbit (111-065-144), 1:400, Jackson Immunoresearch laboratories, Inc., USA) for 1 h. Finally, preparations were incubated with streptavidin conjugate (streptavidin-Cy™3 (016-160-084) 1:400, Jackson), and labeling was visualized in a fluorescence microscope (Olympus BX-60, Olympus Optical Ltd., Tokyo, Japan). To minimize inter-assay variability, preparations from all shear or pressure experiments were stained in one assay run.

2.3.3. Quantitative digital image analysis. Lack of fluorescence labeling in negative controls verified the specificity of the c-Jun and c-Fos antibodies. Quantification of staining intensity was performed according to a previously published method with minor modifications [2]. Briefly, three randomly selected areas of each vessel preparation were digitized with 400 times enlargement through a digital camera (Olympus DP10, Olympus Optical Ltd.). Following grayscale convertion, fluorescence intensity was measured with a digital image analysis routine. Positively stained areas were selected automatically by using a positive staining standard grayscale value. Total fluorescence intensity (TFI) was calculated (TFI = area for positive staining (pixel) × grayscale value). Average fluorescence intensity for each vessel preparation was compared pairwise. Images representing the median values were selected as representative pictures.

2.4. Quantification of c-jun and c-fos gene expression by real-time PCR

2.4.1. Isolation of total RNA and reverse transcription. After perfusion, total cellular RNA was extracted from explanted endothelial cells and reverse-transcribed according to a previously reported protocol [14].

2.4.2. Oligonucleotides for TaqMan® PCR assay. Table 1 shows the nucleotide sequences from the hybridization probes and primers used. These were designed by using Primer Express version 1.0 (Perkin-Elmer Applied Biosystems, Inc.), based on sequences from the GenBank database. Constitutive expressed β-actin was selected as external endogenous control to correct for potential variation in RNA loading, cDNA synthesis or efficiency of the PCR amplification.

2.4.3. 5′ Exonuclease-based PCR assay. A detailed methodological description has been given elsewhere [14]. Briefly, the assay uses the 5′ nuclease activity of *Taq* polymerase to cleave a reporter dye from a non-extendable hybridization probe during the extension phase of the PCR reaction. During the extension phase, the reporter dye is released from the quencher dye and the increase in dye emission is monitored in real-time. The threshold cycle (C_T) is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level (i.e. usually 10 times the standard deviation of the baseline).

PCR assays for c-jun and c-fos were performed in a PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems Inc., Foster City, CA, USA) equipped with a 96-well thermal cycler in the presence of a specific probe for each gene. Typically, PCR was carried out in a 50 μl mix containing: 1:8 of the cDNA templates, 1×TaqMan buffer A, 5 mM $MgCl_2$, 0.2 mM dNTP mix (20 mM dUTP and 10 mM of dATP, dCTP and dGTP), 1.25 U *Taq* Gold polymerase, 0.5 U AmpEraseUNG and 15 pmol of both forward and reverse primers.

Thermal cycling conditions included the following steps: 2 min at 50°C, then the reaction mixture was preheated for 10 min at 95°C before the PCR cycles started. A 50 cycle two-step PCR was performed consisting of 15 s at 95°C and 1 min at 60°C (ABI PRISM® 7700, Perkin-Elmer Applied Biosystems Inc.). All samples were amplified simultaneously in triplicate in one assay run.

2.4.4. Methodological validation. The average amount of extracted RNA from the endothelial cells was approximately 5 μg per 20 cm umbilical vessel. Transcript levels of the endogenous control β-actin were independent of shear or pressure stimulation. No effect of either stimulation conditions was observed when β-actin mRNA levels were expressed relative to GAPDH (data not shown). A standard curve was obtained by performing amplifications of the three target gene cDNAs in a series of 2-fold serial template dilutions of total endothelial cell RNA from 1:1 through 1:32 (Fig. 1). Linear inverse correlation was observed between C_T values (cycles at threshold lines) and the amount of applied cellular RNA ($R^2 = 0.993$, 0.99 and 0.998 for c-fos, β-actin and c-jun, respectively). The normalized amount of target gene is expressed as ratio between the target and control gene cDNA. The amount of target genes and endogenous control was determined from the corresponding standard curves. Log input amount was calculated according to the formula: 10–((cell containing C_T value)$-b$)/m; where $b = y$-intercept of standard curve line, and m = slope of standard curve line (Perkin-Elmer Applied Biosystems, Inc.; User Bulletin no. 2, December 1997).

To further verify the specificity of PCR assays, the TaqMan PCR was performed with non-reverse-transcribed total cellular RNA and samples lacking the DNA template. No amplifications were obtained in any of these samples (data not shown).

Table 1
Oligonucleotide primers and probes used for real-time quantitative PCR

Gene	Oligonucleotide	Sequence	Position
β-Actin	Sense primer	5′-CGT GCT GCT GAC CGA GG-3′	1595–1611
E00829	Antisense primer	5′-GAA GGT CTC AAA CAT GAT CTG-3′	1647–1652
			2095–2111
	Probe	5′-(FAM) CCT GAA CCC CAA GGC CAA CCG (TAMRA)p-3′	1616–1636
c-jun	Sense primer	5′-GCT CGA TGA GTG ACC GCG A-3′	981–999
J04111	Antisense primer	5′-CGC ACT CTT ACT TGT CGA CTC G-3′	1024–1045
	Probe	5′-(FAM) TTT TCA AAG CCG GGT AGC GCG C(TAMRA)p-3′	1001–1022
c-fos	Sense primer	5′-CGA GCC CTT TGA TGA CTT CCT-3′	2984–3004
M16287	Antisense primer	5′-GGA GCG GGC TGT CTC AGA-3′	3033–3050
	Probe	5′-(FAM) TTC CCA GCA TCA TCC AGG CCC A(TAMRA)p-3′	3006–3027

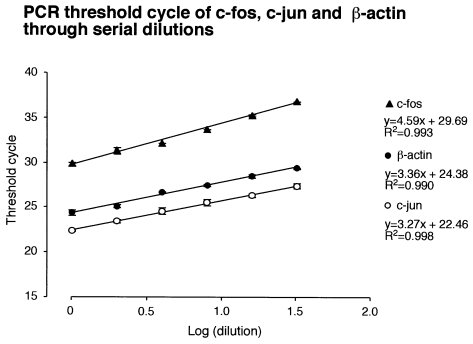

Fig. 1. Validation of amplification efficiency for c-jun, c-fos and β-actin. This diagram shows linear relationship between threshold cycles (C_T value; mean and standard deviation) of target and endogenous control plotted against the relative amount of loaded cDNA in serial dilutions from 1:1 through 1:32. Dilution factors are displayed on *x*-axis after logarithmic transformation.

2.5. Drugs

Unless otherwise stated, all reagents and drugs were purchased from Sigma Chemical Corp., St. Louis, MO, USA. PCR consumables were purchased from Perkin-Elmer Applied Biosystems Inc., Foster City, CA, USA.

2.6. Statistical analysis

Data are expressed as mean and S.E.M. unless otherwise stated. Parametric methods (ANOVA and *t*-test) were used for evaluation of changes in response to different perfusion conditions. For comparison of gene expression during the different stimulation conditions, the ratios of c-jun and c-fos cDNA were evaluated after logarithmic transformation of data. Temporal regulation patterns of target genes were evaluated with ANOVA (time × interaction) and contrast analysis was applied when the over-all ANOVA indicated a significant main effect of treatment or a significant interaction effect. Significance tests were considered significant at $P < 0.05$ (two-tailed test).

3. Results and discussion

3.1. Hemodynamic conditions during 6 h perfusions

During high and low shear stress experiments, average shear stress levels were 25.16 ± 0.91 and 2.73 ± 0.36 dyn/cm^2 in high and low shear systems, respectively (*t*-test, $P < 0.0001$). Average pressure levels were maintained at 19.94 ± 0.03 and 20.02 ± 0.02 mm Hg in respective systems (*t*-test, NS).

During high and low pressure conditions, average pressure levels were 39.92 ± 0.02 and 20.00 ± 0.03 mm Hg, respectively (*t*-test, $P < 0.0001$), while mean shear stress levels were kept at 10.57 ± 0.6 and 9.65 ± 0.36 dyn/cm^2 in respective systems (*t*-test, NS).

3.2. Gene and protein expression of c-fos and c-jun

3.2.1. C-jun gene and protein expression in response to shear and pressure. C-jun gene expression was significantly upregulated by high shear stress with $185 \pm 18\%$ and $156 \pm 22\%$ after 1.5 and 6 h perfusion, respectively (log *t*-test, $P = 0.0005$ and $P = 0.02$, respectively) (Fig. 2a). High pressure induced a gradual increase of endothelial c-jun expression with significant upregulation by $157 \pm 26\%$ and $250 \pm 90\%$ after 3 and 6 h perfusion, respectively (log *t*-test, $P = 0.048$ and $P = 0.03$) (Fig. 2b).

Immunofluorescence revealed distinct endothelial localization of c-jun protein in both sheared and pressurized vessels. Protein expression of c-jun was relatively low after 1.5 h perfusion under both experiment conditions and increased gradually with time. After 6 h perfusion, c-Jun protein expression was markedly upregulated in all high sheared and pressurized vessels (Fig. 3a,b).

These findings are consistent with those of Hsieh et al., who investigated the temporal gene regulation pattern of c-jun in response to shear stress up to 4 h and could report a transient upregulation of c-jun after 1.5 h perfusion [9]. Interestingly, our present data indicate that pressure also exerts significant chronic effects on c-jun gene expression. Increased gene expression was followed by elevated protein synthesis. Significant fluorescence labeling was observed after 3 h of perfusion. By using a modified cone-plate apparatus with incorporated regions of both disturbed and uniform laminar shear stress, Nagel et al. found that the later form of shear stress also induced a significant upregulation of c-jun protein after only 30 min of shear exposure [15]. In the same study, they also detected a high c-jun content in static controls. The temporal discrepancies may be due to different experimental setups, i.e. we used intact vessels exposed to combined laminar shear stress and pressure in serum-free quiescence medium of Tyrode solution.

Upregulation of c-jun gene expression appears to occur exclusively in the endothelium. Lack of immunoreactivity in smooth muscle cells may suggest that shearing force is the primary stimulus for de novo synthesis of c-jun, and compressive/stretching force exert synergistic effects only in the presence of shear stress.

3.2.2. C-fos gene and protein expression in response to shear and pressure. Shear stress induced a significant dynamic regulation pattern of c-fos gene expression (ANOVA,

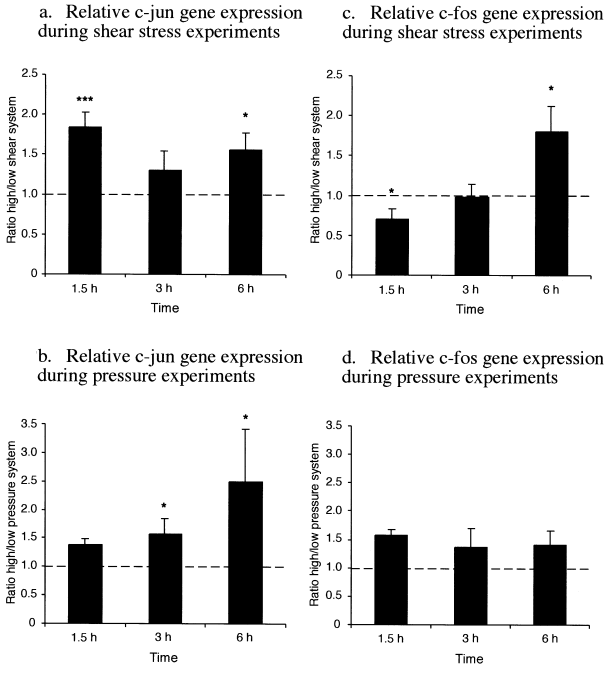

Fig. 2. Bar graphs show relative c-jun gene expression normalized to β-actin in high compared to low shear system (a) and high compared to low pressure (b) system through 1.5, 3 and 6 h perfusion. Corresponding relative c-fos gene expression was shown in c and d for shear and pressure experiments, respectively. Hatched lines represent the ratio of c-jun or c-fos/β-actin expression in low shear and low pressure systems, which was arbitrarily set to one.

time = 0.0036). Following an initial significant downregulation by 31 ± 14% at 1.5 h (log t-test, $P = 0.042$), c-fos gene expression increased to 181 ± 31% of the baseline level after 6 h perfusion (log t-test, $P = 0.038$) (Fig. 2c). High pressure did not induce any significant changes of the c-fos gene expression (Fig. 2d).

Immunofluorescence demonstrated the presence of c-fos protein throughout the vessel wall. C-fos protein expression was markedly increased in both high and low sheared vessels (Fig. 4a). No systematic difference in average fluorescence intensity could be detected between vessels perfused under these two perfusion conditions. Prolonged perfusion decreased c-fos protein content in the vessel wall without significant differences between high and low pressure systems (Fig. 4b).

Gene expression of c-fos has been shown to be transiently upregulated after 30 min shear perfusion and thereafter returns to baseline levels [9]. In our experimental setup, we found a dynamic regulation pattern of c-fos gene expression with an initial downregulation and subsequent profound elevation after 6 h high shear perfusion. Pressure did not seem to influence endothelial c-fos gene expression substantially in a long-term fashion.

At the level of protein, the significant shear-induced transcriptional upregulation was not reflected in the shear experiments. However, the time lag between transcriptional events and de novo protein synthesis in combination with a remarkably dynamic gene regulation pattern may contribute to this discrepancy. A time-dependent gradual increase of TFI was detected during shear experiments independently of shear magnitude. Among vessel preparations perfused for 6 h under different pressure conditions, a greater variation in fluorescence intensity was observed. Allen et al. showed rapid increase of c-fos gene expression in response to elevated pressure per se in isolated mesenteric arteries [16]. The transmural expression of c-fos protein reported here could be a consequence of either that pressure force is transduced across the entire vessel wall or a/several from endothelium secreted factor/s that modulate/s c-fos expression in the smooth muscle cells. The mechanism behind this finding remains to be elucidated.

3.2.3. Differential temporal regulation patterns of c-fos and c-jun gene expression. The temporal regulation patterns of c-fos and c-jun by shear and pressure were significantly different (ANOVA, target gene × treatment × time = 0.0004). Shear and pressure exerted significantly different effects on temporal gene expression patterns of c-fos (ANOVA, treatment × time = 0.039). Temporal gene expression patterns of c-jun and c-fos were differentially regulated by shear stress (ANOVA, target gene × time = 0.001).

By studying gene expression of c-fos and c-jun during subacute mechanical stimulation, we found chronic activation of c-fos and c-jun by shear stress and c-jun by high intraluminal

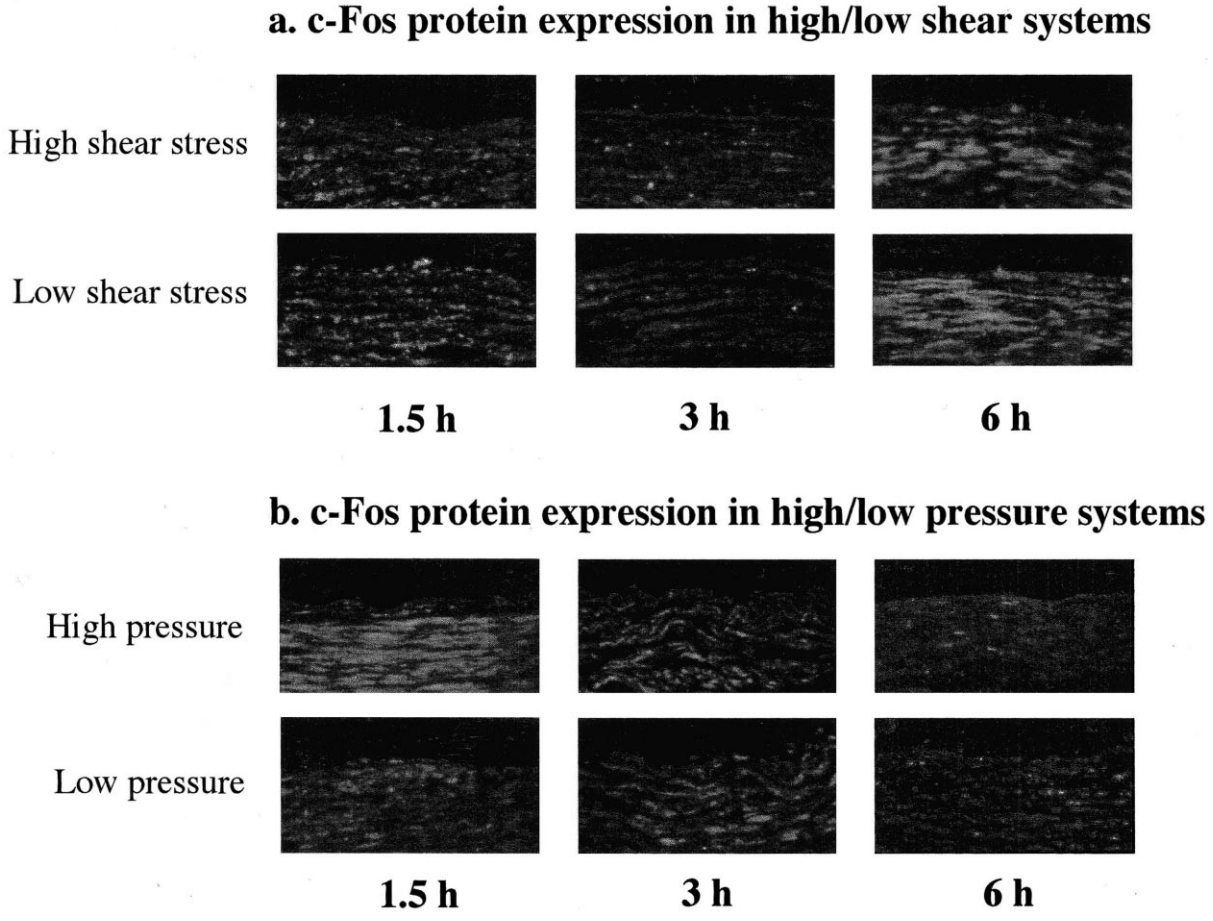

Fig. 3. a: Representative images from paired high/low sheared vessel preparations stained with c-Jun specific antibody. b: Corresponding images from paired high/low pressurized vessel preparations stained with c-Jun specific antibody. The images are representative of at least four independent paired experiments from each of 1.5, 3 and 6 h perfusion series.

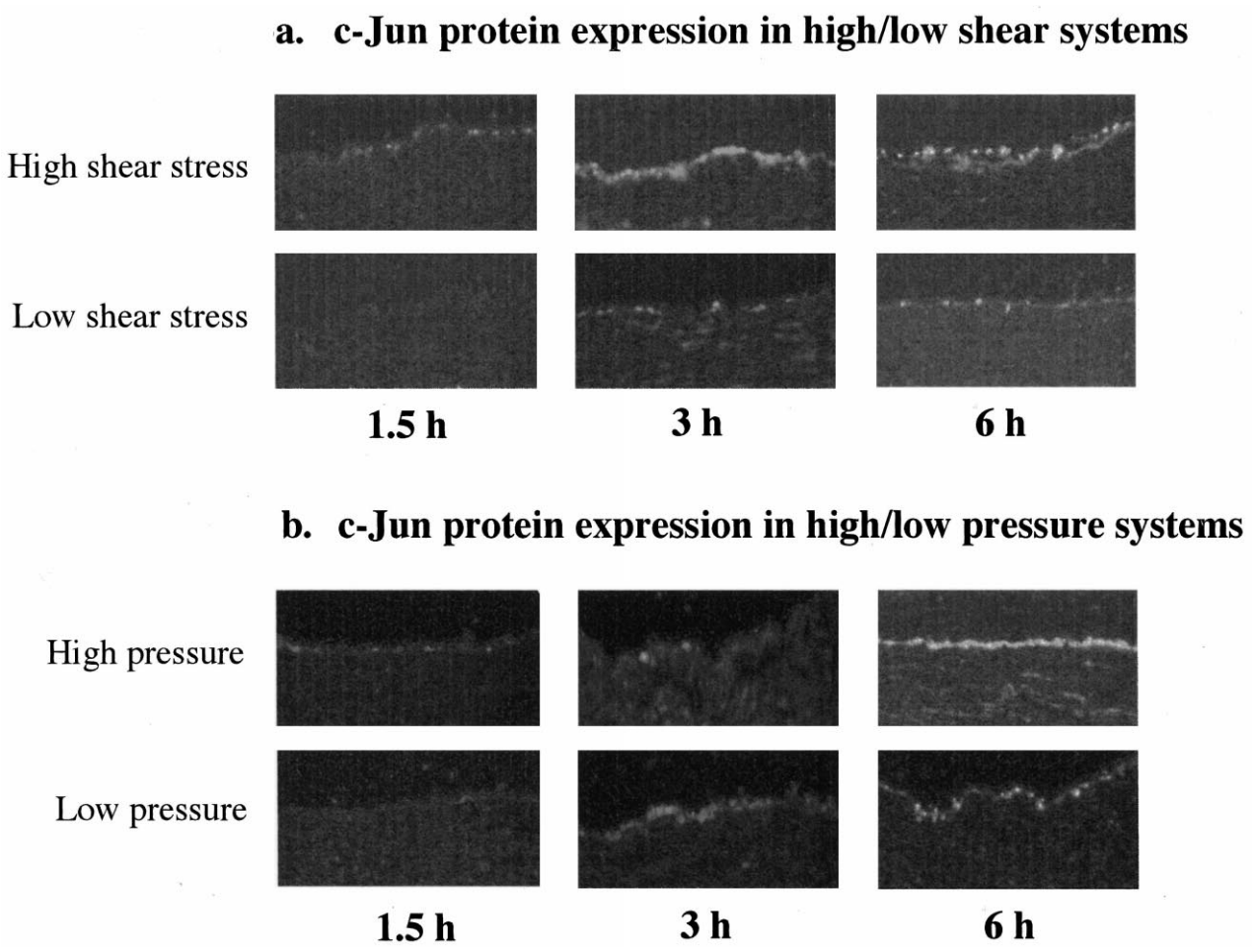

Fig. 4. a: Representative images from paired high/low sheared vessel preparations stained with c-Fos specific antibody. b: Corresponding images from paired high/low pressurized vessel preparations stained with c-Fos specific antibody. The images are representative of at least four independent paired experiments from each of 1.5, 3 and 6 h perfusion series.

pressure. Many of the early transcriptional events in response to extracellular stimuli are induced by rapid activation of pre-existing transcription factors through change in their phosphorylation state [17]. Transcription factors that are encoded by genes induced in the 'first wave' may initiate a 'second wave' induction/suppression of gene expression. Both c-jun and c-fos are known to undergo transcriptional autoregulation. While c-jun has been shown to be positively autoregulated by its product Jun/AP-1 [18], transcription of c-fos is under negative feedback regulation by the fos protein [19]. This complex interplay makes it necessary to study IEGs at both gene and protein levels sub-acutely and chronically for evaluation of possible physiological relevance.

In endothelial cells, c-jun and c-fos are significantly induced by shear after 6 h perfusion. This may contribute to a higher content of Jun/Fos heterodimers, which are capable of modulating transcription of AP-1 constituent genes. High intraluminal pressure induced high c-jun gene and protein expression without significant effect on c-fos. Homodimers of c-jun are known to interact with AP-1 site in promotors of many important genes but with lower binding affinity compared to the c-jun/c-fos heterodimers [4,5,17]. This can be one of the mechanisms underlying the differential transcriptional regulation of t-PA and eNOS by pressure and shear stress.

Following shear stimulation, we recently found that t-PA and eNOS protein expressions were upregulated not only in the vascular endothelium, but also in the smooth muscle cells [2,3]. In the light of our present finding that c-jun is weakly expressed in the smooth muscle cells, upregulation of these endothelial factors is unlikely to be mediated via AP-1 activation. Thus, this present study provides evidence that mechanical stress-induced IEG responses are distinct in endothelial and smooth muscle cells.

4. Concluding remarks

In summary, c-jun and c-fos are dynamically and differentially regulated by shear stress and intraluminal pressure. In addition to the acute regulation at the transcriptional level, chronic shear stimulation induced prolonged responses of c-jun and c-fos via increased de novo protein synthesis. This illustrates how short-lasting extracellular stimuli may modulate endothelial cell function in a long-term fashion. Our data also provide evidence that mechanical stress-induced IEG responses may be cell-type specific. Our findings further emphasize the importance of using a physiologically relevant model system to study these cellular events in intact vessel preparations.

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