High Pressure Promotes Monocyte Adhesion to the Vascular Wall

Stéphanie Riou, Barend Mees, Bruno Esposito, Régine Merval, Jose Vilar, Dominique Stengel, Ewa Ninio, Rien van Haperen, Rini de Crom, Alain Tedgui, Stéphanie Lehoux

Abstract—Hypertension is a known risk factor for the development of atherosclerosis. To assess how mechanical factors contribute to this process, mouse carotid arteries were maintained in organ culture at normal (80 mm Hg) or high (150 mm Hg) intraluminal pressure for 1, 6, 12, or 24 hours. Thereafter, fluorescent human monocytic cells (U937) were injected intraluminally and allowed to adhere for 30 minutes before washout. U937 adhesion was increased in vessels kept at 150 mm Hg 12 hours (23.5±5.7 versus 9.9±2.2 cells/mm at 80 mm Hg; P<0.05) or 24 hours (26.7±5.7 versus 8.8±1.5 cells/mm; P<0.05). At 24 hours, high pressure was associated with increased mRNA expression of monocyte chemoattractant protein-1, interleukin-6, keratinocyte-derived chemokine, and vascular cell adhesion molecule-1 (6.9±2.1, 4.4±0.1, 9.8±2.8, and 2.4±0.1-fold respectively; P<0.05), as assessed by quantitative RT-PCR and corroborated by immunohistochemistry, which also revealed an increase in intracellular adhesion molecule-1 expression. Nuclear factor κB inhibition using SN50 peptide abolished the overexpression of chemokines and adhesion molecules and reduced U937 adhesion in vessels at 150 mm Hg. Moreover, treatment of vessels and cells with specific neutralizing antibodies established that monocyte chemoattractant protein-1, interleukin-6, and keratinocyte-derived chemokine released from vessels at 150 mm Hg primed the monocyes, increasing their adhesion to vascular cell adhesion molecule-1 but not intracellular adhesion molecule-1 via αβ integrins. The additive effect of chemokines on the adhesion of U937 cells to vascular cell adhesion molecule-1 was confirmed by in vitro assay. Finally, pressure-dependent U937 adhesion was blunted in arteries from mice overexpressing endothelial NO synthase. Hence, high intraluminal pressure induces cytokine and adhesion molecule expression via nuclear factor κB, leading to monocytes cell adhesion. These results indicate that hypertension may directly contribute to the development of atherosclerosis through nuclear factor κB induction. (Circ Res. 2007;100:1226-1233.)

Key Words: hypertension ■ atherosclerosis ■ cytokines ■ NF-κB ■ VCAM-1

Atherosclerosis is an inflammatory disease characterized by an accumulation of leukocytes, lipids, and fibrous tissue in the intima of arteries. In the early phases of atherosclerotic plaque development, activated endothelial cells express elevated amounts of adhesion molecules such as selectins (P-selectin and E-selectin) and intracellular (ICAM-1), vascular (VCAM-1), and platelet endothelial cell (PECAM-1) adhesion molecules at their surface. Cytokines and chemokines are also secreted in excess by activated vascular cells. These conditions favor the recruitment and the accumulation of monocytes and lymphocytes in the intima of vessels.1

It is well known that the zones of the vascular tree where blood flow is disturbed or oscillatory are predisposed to the formation of atherosclerotic lesions, whereas vessels exposed to laminar shear stress remain relatively plaque-free,2 mostly credited to local release of NO.3 However, blood pressure also influences plaque formation, arterial hypertension being an independent risk factor for atherosclerosis.4 In hypertensive patients, high concentrations of circulating ICAM-1, VCAM-1, and E-selectin,5 as well as monocyte chemoattractant protein (MCP-1),6 have been reported. Experimental animal models have also linked hypertension with cytokine and adhesion molecule expression, as well as the propensity for atherosclerotic plaque development. The expression of MCP-1 is more prevalent in the aorta of hypertensive SHR rats than that of control Wistar rats.7 Moreover, chronic hypertension caused by endothelial NO synthase (eNOS) deficiency8 or induced by clamping renal arteries9 or by aortic constriction10 exacerbates atherosclerosis in ApoE−/− mice. However, the direct role of arterial pressure in the development of atherosclerotic plaques has not yet been clearly demonstrated.

Original received August 29, 2006; resubmission received February 27, 2007; accepted March 15, 2007.

From INSERM U689 (S.R., B.E., R.M., J.V., A.T., S.L.), Centre de Recherche Cardiovasculaire Inserm Lariboisière, Paris, France; Departments of Cell Biology & Genetics (B.M., R.v.H., R.d.C.) and Vascular Surgery (B.M., R.d.C.), Erasmus University Medical Center, Rotterdam, The Netherlands; and INSERM U525 (D.S., E.N.), Université Pierre et Marie Curie 6, Faculté de Médecine Pierre et Marie Curie, Paris, France.

Correspondence to Dr Stéphanie Lehoux, Centre de Recherche Cardiovasculaire Inserm Lariboisière, INSERM U689, 41 Boulevard de la Chapelle, 75010 Paris, France. E-mail lehoux@larib.inserm.fr

© 2007 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000265231.59354.2c

1226
A recent study performed in our laboratory showed that stretch of the arterial wall caused by an increase in the intraluminal pressure induces the activation and nuclear translocation of the transcriptional factor nuclear factor \( \kappa B \) (NF-\( \kappa B \)).\(^{11}\) This factor intervenes in the transcription of a large number of inflammatory genes coding for cytokines, chemokines, and adhesion molecules.\(^{12}\) Consequently, we hypothesized that high arterial pressure could contribute to the development of atherosclerotic lesions directly by inducing monocyte adhesion via NF-\( \kappa B \). Using an in vitro organ culture model of whole vessel, we assessed monocyte adhesion to the vascular endothelium of arteries kept at normal or high pressure. We also evaluated which adhesion molecules and chemokines might be implicated in this process and verified their induction by NF-\( \kappa B \).

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Organ Culture**

Mouse left and right carotid arteries were isolated, cannulated at both extremities, and immersed in an organ culture bath filled with DMEM (Gibco BRL) supplemented with 5% FCS as described previously.\(^{11}\) Each arterial segment was connected to a closed perfusion circuit consisting of a 3-port reservoir, a peristaltic pump (Alitea), and a pressure chamber allowing for the application of a controlled intraluminal hydrostatic pressure. Organ culture of carotid segments was performed under sterile conditions in an incubator containing 5% \( \text{CO}_2 \) at 37°C. The flow was set at 1.38 mL/min, allowing for renewal of the medium within the intraluminal space while creating minimal shear forces (0.5 dyne/cm²). Likewise, to avoid the potentially confounding effect of cyclic stretch vessels were exposed to steady, continuous stretch, although we have previously shown that NF-\( \kappa B \) is not induced in pulsatile vessels.\(^{13}\)

Arterial segments were maintained at an intraluminal pressure of 80 mm Hg for 1 hour for stabilization after surgery. Thereafter, vessels were exposed to a pressure of 80 mm Hg or 150 mm Hg for 1, 6, 12, or 24 hours. High pressure imposed a stretch corresponding to a 20±3% increase in diameter.

Most experiments were undertaken using vessels from C57BL/6 mice. However, arteries from mice overexpressing (eNOS-\( \times \)) and underexpressing (eNOS-\( \times \)) eNOS described previously,\(^{13}\) or their wild-type littermates were also used where indicated. In 1 set of experiments, vessels maintained for 24 hours at 80 mm Hg were treated with lipopolysaccharide (LPS) (10 \( \mu \)g/mL). Some arteries kept at 80 or 150 mm Hg for 24 hours were incubated with the NF-\( \kappa B \) inhibitor peptide SN50 (AAVALLPAVLLALLAP-VQKRKRKQLMP, 50 \( \mu \)g/mL; Upstate Biotechnology), with the pharmacological inhibitor of NF-\( \kappa B \) ammonium pyrrolidine dithiocarbamate (10 \( \mu \)mol/L; Sigma), or with the eNOS inhibitor \( N \)-nitro-l-arginine methyl ester (L-NAME) (10 \( \mu \)mol/L, Sigma) added to the culture medium at the onset of the equilibration period.

**Fluorescent Cell Preparation**

Cells of the human monocytic cell line U937 were cultivated in RPMI medium 1640 (GIBCO BRL) containing penicillin (100 U/L) and supplemented with 5% FCS (Boehringer-Mannheim). U937 cells were labeled with 0.5 \( \mu \)mol/L, fluorescent dye (CellTracker Orange CMTMR; Molecular Probes). Briefly, the cells were incubated with the fluorescent dye for 30 minutes and then resuspended in culture medium.

To circumvent a potential direct effect of pressure on U937 adhesion, the intraluminal pressure of all vessels was reset to 80 mm Hg 30 minutes before intraluminal cell injection. The fluorescent U937 cells were injected in the lumen of cultured vessels by the distal end and allowed to interact for 30 minutes (5×10⁶ cells/mL). After a 10-minute washout at low-flow shear stress (0.5 dyne/cm²), vessels were fixed in 4% paraformaldehyde for 15 minutes. Adherent cells were counted under a fluorescence microscope. In some experiments, blocking antibodies targeting VCAM-1 (25 \( \mu \)g/mL; AF643), ICAM-1 (5 \( \mu \)g/mL; AF796), MCP-1 (100 \( \mu \)g/mL; AB479), interleukin (IL)-6 (1 \( \mu \)g/mL; AF406), or keratinocyte-derived chemokine (KC) (10 \( \mu \)g/mL; AF453) (R&D Systems) were added to the intraluminal compartment 30 minutes before U937 injection. Alternatively, U937 cells were incubated with a blocking anti-\( \alpha_4 \) (5 \( \mu \)g/mL; BBA37; R&D Systems) or anti-\( \beta_2 \) integrin antibody (5 \( \mu \)g/mL; 553715; BD PharMingen), or with a nonblocking anti-\( \alpha_4 \) antibody (5 \( \mu \)g/mL; 9C10; Research Diagnostics), before being injected in vessels.

**Immunohistochemical Analysis and Quantitative RT-PCR**

Details regarding immunohistochemical analysis and quantitative RT-PCR appear in the online supplement. Primary goat polyclonal antibodies used for immunohistochemistry and targeting VCAM-1, ICAM-1, E-selectin, MCP-1, IL-6, and KC, were obtained from Santa Cruz Biotechnology. All primers were designed using Primer Express 2 Software and are reported in the Table.

**Cell Adhesion Assay**

Cell culture plates were coated with recombinant mouse VCAM-1/Fc chimera (2 \( \mu \)g/mL; R&D Systems) at 4°C overnight. After washout, saturation with 2% BSA was performed for 1 hour. Fluorescent U937 cells (10⁶/well) were made to adhere untreated or in presence of recombinant mouse IL-6 (0.06 ng/mL), MCP-1 (10 ng/mL), and/or KC (5 ng/mL; R&D Systems) for 1 to 30 minutes. In another set of experiments, fluorescent U937 were made to adhere for 30 minutes in the presence of a blocking anti-\( \alpha_4 \) (5 \( \mu \)g/mL; R&D Systems) or anti-\( \beta_2 \) (5 \( \mu \)g/mL; PharMingen) integrin antibody. After washout, adherent U937 cells were counted under a fluorescence microscope.

**Statistics**

The data are presented as mean±SEM. Data were analyzed by ANOVA, and when results were found to be significant, comparisons were performed by Bonferroni test or Student’s paired \( t \) test (to
Results

Pressure Induces Monocytic Cell Adhesion via NF-κB

The effects of high intraluminal pressure on monocytic cell adhesion were first assessed in carotid arteries maintained in culture for 1, 6, 12, or 24 hours at 80 or 150 mm Hg (Figure 1A). The number of adherent U937 cells did not vary significantly between vessels maintained at 80 or 150 mm Hg for 1 hour (2.15±1.32-fold increase in cell adhesion at 150 mm Hg) or 6 hours (2.19±0.66-fold). However, high intraluminal pressure led to a significant increase in primary mouse monocytic cell adhesion at 150 mm Hg (1.6±0.3 cells/mm at 80 mm Hg versus 10.2±0.5 cells/mm at 150 mm Hg). The dotted line represents the expression of adhesion molecules and chemokines (70% of the normal group). The data are means±SEM of n=6 experiments evaluated in duplicate. 

Pressure-Dependent Monocytic Cell Adhesion Is Equivalent to LPS Stimulation

To compare the effects of the stretch stimulus to a more traditional inflammatory mediator, vessels were cultured for 24 hours at 80 mm Hg and treated with or without LPS, a known activator of endothelial cells. We found that the number of adherent U937 cells in arteries incubated with LPS (18.7±3.5 cells/mm) was almost 3-fold greater than that in arteries cultured without LPS (5.9±1.5 cells/mm; P<0.001) (Figure 1B), equivalent to that induced by high pressure.

Pressure-Dependent Expression of Adhesion Molecules and Chemokines

We then studied the expression of adhesion molecules and chemokines potentially involved in regulating monocytic cell adhesion to the vascular wall. Quantitative RT-PCR of genes encoding VCAM-1, ICAM-1, E-selectin, MCP-1, IL-6, and KC was undertaken in vessels maintained for 24 hours at normal or high intraluminal pressure (Figure 2). On the one hand, the expression of ICAM-1 and E-selectin was not different in vessels maintained at 80 or 150 mm Hg. On the
other hand, the expression of VCAM-1, MCP-1, KC, and IL-6 was, respectively, 2.0±0.1-, 8.1±2.0-, 9.8±2.8-, and 5.8±0.1-fold greater at 150 mm Hg than at 80 mm Hg (P<0.05).

To verify whether pressure-dependent changes in mRNA expression corresponded to modified protein content, immunohistologic staining for cytokines and adhesion molecules was evaluated. High pressure did not alter the expression of E-selectin in vessels (Figure 3A), whereas staining for ICAM-1 and VCAM-1 was significantly enhanced in vessels incubated for 24 hours at 150 mm Hg compared with arteries kept at 80 mm Hg (Figure 3A). Quantification of relative vessel wall surface staining revealed greater expression of ICAM-1 in the endothelium (6.9±0.9% versus 1.8±0.5%, P<0.01) and more VCAM-1 in the endothelium and the media (25.8±3.5% versus 9.8±1.6%, P<0.001) of arteries at 150 mm Hg compared with 80 mm Hg (Figure 3B). The expression of MCP-1, IL-6, and...
between the 2 remained significant (150 mm Hg (24.3.8 cells/mm), such that the difference of adhesion molecules and chemokines via the NF-

minal pressure induces an increase in the vascular expression

These immunohistologic studies indicated that high intralu-

minal pressure prevents stretch-induced adhesion, either completely (VCAM-1, MCP-1) or partially (IL-6, KC). The data are means±SEM of n=4 to 6 experiments. *P<0.05, **P<0.01, ***P<0.001 vs 80 mm Hg; §§§P<0.001 vs 150 mm Hg untreated.

KC was also increased in the endothelium and the media of vessels maintained for 24 hours at 150 mm Hg versus 80 mm Hg (17.4±1.1% versus 10.8±0.9%, P<0.001; 18.3±1.9% versus 6.8±1.5%, P<0.05; and 15.9±1.6% versus 8.4±3.3%, P<0.05, respectively) (Figure 3A and 3B). In all cases, incubation of vessels with the inhibitor of NF-κB reduced the intensity of immunostains such that protein expression of the cytokines and adhesion molecules no longer differed between 80 and 150 mm Hg (Figure 3A and 3B). These immunohistologic studies indicated that high intraluminal pressure induces an increase in the vascular expression of adhesion molecules and chemokines via the NF-κB pathway.

Adhesion Molecules and Chemokines Contribute to Pressure-Dependent Monocytic Cell Adhesion

To determine the relative importance of adhesion molecules and cytokines in pressure-dependent U937 adhesion, vessels maintained for 24 hours at 80 or 150 mm Hg were treated with blocking antibodies directed against these proteins for 1 hour before the intraluminal monocytic cell injection. The anti–ICAM-1 antibody did not affect U937 adhesion at all in vessels cultured at 80 mm Hg (10.9±2.5 cells/mm) or 150 mm Hg (24±3.8 cells/mm), such that the difference between the 2 remained significant (P<0.01) (Figure 4). In comparison, there was complete inhibition of pressure-dependent U937 adhesion in vessels treated with antibodies directed against VCAM-1 or MCP-1 (7.0±0.4 cells/mm and 6.8±1.3 cells/mm at 150 mm Hg, respectively; P<0.001 versus 150 mm Hg untreated). Blocking IL-6 and KC strongly reduced the number of adherent monocytic cells associated with high pressure (P<0.001), although a small increment in U937 adhesion was still distinguishable in arteries kept at 150 mm Hg compared with 80 mm Hg (P<0.05).

The direct effect of chemokines on monocytic cell adhesion was verified in an in vitro cell adhesion assay, which was performed using fluorescent U937 cells made to adhere to VCAM-1–coated plates. U937 adhesion was bolstered by cytokine treatment (MCP-1, IL-6 and KC), reaching 223.0±9.8 cells/well at 30 minutes compared with 102.5±18.5 cells/well for untreated monocytes (Figure 5A). Cell adhesion on plastic was negligible. The independent effect of MCP-1, IL-6, and KC on monocytic cell adhesion was evaluated at 30 minutes (Figure 5B). All 3 cytokines induced a significant increase in U937 adhesion to VCAM-1 compared with control (119.8±16.7 cells/well), rising to 169.8±17.4 (MCP-1), 162.8±15.0 (IL-6), and 167.0±15.7 cells/well (KC) (P<0.05), and they exerted an additive effect on U937 adhesion to VCAM-1 (244.7±26.0 cells/well; P<0.01).

Role of α4 and β1 Integrins in Pressure-Dependent Monocytic Cell Adhesion

To establish that monocytic αβ, known to interact with endothelial VCAM-1, was implicated in pressure-dependent monocytic adhesion, U937 cells were treated with blocking antibodies directed against α4 or β1, integrin for 1 hour before
intraluminal injection in vessels. The blockade of either α4 or β1 strongly decreased U937 adhesion in vessels maintained at 150 mm Hg for 24 hours compared with untreated monocytic cells (4.9 ± 0.6 and 7.2 ± 1.7, respectively, versus 16.9 ± 0.5; \( P < 0.001 \)) (Figure 6A). Similarly, pretreatment of U937 cells with the blocking anti-α4 or anti-β1 antibodies significantly blunted their adhesion on VCAM-1–coated monocytes on VCAM-1–coated plates at 30 minutes. The data are means ± SEM of \( n = 4 \). ***\( P < 0.001 \) vs untreated control, §§§\( P < 0.001 \) vs monocytes treated with MCP-1 + IL-6 + KC without antibody incubation.

Enhanced NO Production Prevents Pressure-Induced Monocyte Adhesion

The protective effect of shear stress is mostly attributable to the local synthesis and release of NO via activation of eNOS. To verify whether the protective effects of NO may counterbalance the proatherosclerotic effects of high pressure, we verified stretch-induced U937 adhesion in vessels obtained from mice overexpressing (eNOS-tg) or underexpressing (eNOS\(^{-/-}\)) endothelial NO synthase, or from wild-type littersmates. As demonstrated in Figure 7, lack of NO synthase in eNOS\(^{-/-}\) vessels did not affect high-pressure-induced monocyte adhesion, but overexpression of eNOS was accompanied by a marked reduction of U937 binding in vessels maintained at 150 mm Hg, such that cell adhesion levels no longer varied between arteries exposed to normal or high pressure. Treatment of vessels with L-NAME restored the stretch-dependent increment in monocyte adhesion in eNOS-tg vessels but did

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Key role of α4 and β1 integrins in monocytic cell adhesion. A, Increased U937 adhesion in vessels maintained for 24 hours at 150 mm Hg was blunted by incubating the cells with blocking anti-α4 or anti-β1 antibodies 1 hour before their intraluminal injection. The data are means ± SEM of \( n = 6 \) experiments. ***\( P < 0.001 \) vs 80 mm Hg; §§§\( P < 0.001 \) vs 150 mm Hg untreated. B, Similarly, the blocking anti-α4 or anti-β1 antibodies reduced the adhesion of chemokine-stimulated monocytic cells on VCAM-1–coated plates at 30 minutes. The data are means ± SEM of \( n = 4 \). ***\( P < 0.001 \) vs untreated control, §§§\( P < 0.001 \) vs monocytes treated with MCP-1 + IL-6 + KC without antibody incubation.

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Overexpression of eNOS counters enhanced monocytic cell adhesion associated with high intraluminal pressure. Carotid arteries from eNOS-overexpressing (eNOS-tg), eNOS knockout, and wild-type littersmates were maintained for 24 hours at 80 or 150 mm Hg, with or without L-NAME treatment, before U937 cell injection. Monocytic cell adhesion was similarly increased in wild-type and eNOS\(^{-/-}\) carotids exposed to high intraluminal pressure. This response was blunted in arteries of eNOS-tg mice but was restored by treatment with L-NAME. The data are means ± SEM of \( n = 6 \) to 8 experiments. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) vs 80 mm Hg.
not affect U937 binding in vessels from eNOS−/− mice or wild-type littermates.

Discussion

The present study reveals that exposing arteries to high intraluminal pressure induces the expression of adhesion molecules and cytokines by endothelial and smooth muscle cells, leading to increased monocyte adhesion to the vascular wall. Moreover, we show that NF-κB plays a central role in this mechanosensitive process. To the best of our knowledge, this is the first demonstration of a direct proatherogenic effect of pressure alone, independent of hormonal conditions associated with hypertension.

Many studies in human subjects and animal models have described an association between hypertension and increased atherosclerotic plaque formation. However, it was recently demonstrated in a rat model of aortic coarctation that enhanced expression of adhesion molecules occurred only in aortic segments exposed to a high pressure. Similarly, in a rabbit model of experimental atherosclerosis with aortic stenosis, monocyte adhesion and expression of VCAM-1 were more prevalent in the proximal aorta, where pressure is elevated than the normotensive distal aorta. These reports showed that high pressure is necessary to stimulate monocyte adhesion. Nevertheless, increased levels of angiotensin II, which characterize these models, may very well have acted synergistically with the applied stretch to produce proatherosclerotic effects. Indeed, angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers prevent adhesion molecule overexpression as well as monocyte infiltration in hypertensive transgenic rats and are associated with reduced plaque size in hypertensive ApoE−/−, eNOS−/− mice. However, our study clearly demonstrates that the hypertensive mechanical environment is sufficient to elicit chemokine and adhesion molecule expression leading to monocyte adhesion to the vascular wall, in the absence of confounding hormonal factors. Moreover, even adding angiotensin II to the culture medium did not enhance U937 adhesion to the vessel wall, either at normal or high pressure, indicating that stretch is a more potent stimulus of monocyte cell adhesion than angiotensin II in whole arteries, at least in the ex vivo setting.

Arteries are exposed to a complex mechanical environment, including both cyclic strain and shear stress. Some studies have shown that exposing smooth muscle cells to cyclic stretch stimulates the expression of IL-6 and IL-8 via the activation of c-Jun N-terminal kinase and NF-κB pathways, whereas cyclic stretching of endothelial cells is associated with increased expression of ICAM-1 and E-selectin, along with greater monocyte adhesion. Reproducing the tonic component of vessel stretch, continuous stretch also stimulated the expression of IL-6 via NF-κB in cultured endothelial cells. Nevertheless, the phenotype of vascular cells is deeply altered in vitro, such that the responses to mechanical stimuli differ significantly from in vivo conditions, where cells are exposed to a complex, tensile and 3D matrrial environment. Indeed, unlike what is reported in vascular cells in vitro, cyclic stretch did not activate NF-κB in whole vessels. Moreover, exposing arteries to a normotensive degree of stretch (80 mm Hg), sufficient to maintain smooth muscle cell phenotype, did not activate NF-κB or induce monocyte adhesion in the present study. High intraluminal pressure alone, reminiscent of the hypertensive state, stimulated NF-κB-dependent expression of chemokines and adhesion molecules, allowing for monocytic cell adhesion. On the other hand, overexpression of eNOS, associated with enhanced NO release, blunted these proatherosclerotic effects of high pressure in vessels from eNOS-tg mice. Hence, hypertensive mechanical conditions facilitate atherosclerotic plaque formation in vascular regions where blood flow is low or oscillatory, whereas the protective effect NO release prevails in vessels exposed to laminar shear stress.

It has been shown that monocytes preincubated with MCP-1 and IL-8 adhere on endothelial cells. Likewise KC, the murine homologue of IL-8, but not MCP-1, triggered monocyte arrest on early atherosclerotic endothelium in a reconstituted flow chamber system, and blockade of αβ1 integrins or VCAM-1, but not ICAM-1, inhibited this process. Moreover, VCAM-1 and its ligand αβ1 are critical for monocyte rolling and adhesion in early atherosclerotic lesions, and formation of lesions is markedly reduced in atherosclerosis-prone mice after peptide perfusion to block αβ1, compared with unperfused mice. In the present study, we found that high pressure increases the endothelial expression of ICAM-1 and induces a strong overexpression of VCAM-1, MCP-1, IL-6, and KC in all vascular cells, through induction of NF-κB. In agreement with the previous studies cited above, we demonstrated that monocyte adhesion occurs via the interaction of αβ1 integrins with VCAM-1 rather than ICAM-1. However, blockade of MCP-1, IL-6, or KC led to a strong decrease in monocyte adhesion to the vascular wall, and our in vitro studies confirmed that all 3 cytokines are necessary to prime monocyte adhesion. This contrasts with a previous report establishing that KC plays a predominant role in mediating monocyte adhesion in vessels from ApoE−/− mice fed a Western-type diet; disparities between that study and our present findings indicate that the nature of the proatherosclerotic stimulus may influence which chemokines participate in monocyte recruitment. Regardless, beyond the initial endothelial cell adhesion step, the enhanced VCAM-1 and chemokine expression observed in smooth muscle cells of arteries at high intraluminal pressure could facilitate inflammatory cell infiltration in the vascular wall. Finally, the fact that all of the outcomes of high pressure described here could be reversed by blocking NF-κB highlights the key role of this transcription factor in the deleterious, proatherosclerotic effects associated with hypertensive conditions.

In summary, our results demonstrate that high intraluminal pressure alone, in the absence of external hormonal factors, is sufficient to induce chemokine and adhesion molecule expression and to trigger monocyte adhesion to the vascular wall. More importantly, our work indicates that high blood pressure may directly contribute to the development of atherosclerosis though induction of NF-κB, suggesting that this pathway may provide an interesting therapeutic target to counter adverse effects of hypertension.
Sources of Funding

This work was supported by the European Vascular Genomics Network, a Network of Excellence supported by the European Community’s sixth Framework Programme for Research Priority 1 Life Sciences, Genomics and Biotechnology for Health (contract no. LSHM-CT-2003–503254 to S.L. and A.T.); and by research grants from the Societe Francaise d’Hypertension Arterielle (to S.L. and E.N.), The Netherlands Organization for Health Research and Development (Agikko stipend 920–03-291 to B.M.), and The Professor Michael-van Vloten Fund (to B.M.).

Disclosures

None.

References

14. van Vloten Fund (to B.M.).
High Pressure Promotes Monocyte Adhesion to the Vascular Wall

Stéphanie Riou, Barend Mees, Bruno Esposito, Régine Merval, Jose Vilar, Dominique Stengel, Ewa Ninio, Rien van Haperen, Rini de Crom, Alain Tedgui and Stéphanie Lehoux

Circ Res. 2007;100:1226-1233; originally published online March 29, 2007;
doi: 10.1161/01.RES.0000265231.59354.2c

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/100/8/1226

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/03/29/01.RES.0000265231.59354.2c.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
MATERIAL AND METHODS

Organ culture

Experiments were performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and were approved by the local ethics committee. Male between 8 and 10 weeks of age were sacrificed by a lethal injection of sodium pentobarbital (50 mg/kg IP). Carotid arteries were immersed in an organ culture bath filled with Dulbecco’s modified Eagle’s medium containing antibiotics (100UI/L penicillin, 100 mg/L streptomycin and 10 µg/L fungizone) and supplemented with 5% fetal calf serum (Boehringer-Mannheim).

Primary cell adhesion *ex vivo and in vitro*

Primary mouse low density mononuclear cells were harvested from the bone marrow by flushing the tibias and femurs, and isolated by density-gradient centrifugation with Ficoll (1). The cells were then labeled by 30 minute incubation with 0.5 µM fluorescent dye (CellTracker Orange CMTMR ; Molecular Probes) and resuspended in culture medium. The fluorescent cells were injected in the lumen of vessels cultured 24 hours at 80 or 150 mmHg and allowed to interact 30 minutes (10⁶ cells/mL). After washout and fixation, adherent cells were counted under a fluorescence microscope. Alternatively, the mouse primary monocytic cells (2x10²/well), treated or not with IL-6, MCP-1 and KC, were allowed to adhere on VCAM-1-coated plates for 30 minutes before washout and counting.
Effects of angiotensin II on monocytic cell adhesion

Mouse carotid arteries were maintained during 24 hours at 80 or 150 mmHg, with or without concurrent treatment with angiotensin II (Hypertensin, $10^{-6}$ mol/L, Ciba). At the end of the 24 hour period, 2mL of the culture medium was used to stimulate ERK1/2 activity in smooth muscle cells in culture to confirm that hypertensin remained active (data not shown). The fluorescent cells were injected in the lumen of vessels and allowed to interact 30 minutes ($5\times10^6$ cells/mL). After washout and fixation, adherent cells were counted under a fluorescence microscope.

Immunohistochemical analysis

Arterial segments were vertically embedded in OCT compound (Tissue-tek, Sakura) and serial 10 µm sections were cut. The adhesion molecules and chemokines were detected using their respective antibodies (Santa Cruz). The phosphorylated form of the p65 subunit of NF-κB was detected using polyclonal anti-phospho-p65-NF-κB (Ser529; Rockland). Immunostains were revealed with the use of avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit, Vector Laboratories) and revealed by AEC (3-amino-9-ethylcarbazol). Semi-quantitative assessment of staining intensity was done independently by two researchers using Histolab software: the medial and endothelial layers of 3 sections of each stained vascular segment were outlined and the surface area of positive staining detected by the software within this contour was quantified as a percentage of total surface area.

Quantitative RT-PCR

The mRNA from vessels incubated during 24h at 80 or 150 mmHg was isolated using the Chomczynski method (2). Briefly, the tissue was minced and homogenized in a solution
containing 4M guanidinium thiocyanate, 25mM sodium citrate (pH=7), 0.5% sarcosyl and 0.1M β-mercaptoethanol. Sodium acetate (2M, pH=4) and phenol-chloroform (5 : 1) were successively added. After centrifugation, isopropanol was added in order to extract the mRNA. Reverse transcription was performed with superscript II (Invitrogen) at 42°C for 2 hours. The cDNA obtained was quantified using Packard Fusion apparatus with oligreen ssDNA Quantification kit and dilutions were performed in order to obtain equal amounts of cDNA in each sample. Real-time quantitative PCR (Q-PCR) was performed in a MX 4000 apparatus (Strata gene) using a SYBRGreen detection kit (Sigma). In all assays, cDNA was amplified using a standardized program (2 minutes JumpStartTM Taq Polymerase activation step at 95°C ; 40 cycles of 30 seconds at 95°C and 1 minute at 60°C ; a 1 minute step at 95°C and a dissociation step : 36 cycles of 30 seconds between 60°C-95°C). All Q-PCR assays were performed in a final volume of 20µL using 5ng cDNA and mouse primers at final concentration of 0.33µM. Each experiment was performed in duplicate and each Q-PCR determination was realized twice. The quantification of target gene expression was performed using a mathematical model of normalization by geometric averaging of internal control genes (3). Two housekeeping genes were used : HPRT and α-actin. Normalization and calculations were done using GenNorm Software (Excel version).

**Western blot**

For western blot, vessel segments were ground in ice-cold lysis buffer A containing 20mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L EDTA, 0.2% NP-40, 10% glycerol and protease inhibitors. Samples were centrifuged, and the nuclear pellets were extracted in 40 µL of buffer B containing 20 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L EDTA, 0.35 mmol/L NaCl, 10% glycerol and protease inhibitor. Nuclear lysates containing 20 µg of protein were electrophoresed on polyacrylamide gels and transferred to
nitrocellulose membranes (Amersham ECL). Membranes were incubated overnight with anti-p65-NF-κB or anti-tubulin antibodies (Santa Cruz). An enhanced chemiluminescence system was used as the detection method (ECL+, Amersham).

**Statistics**

Data were analyzed by ANOVA, and when results were found to be significant comparisons were performed by Bonferroni’s test or Student’s paired t-test (to compare arteries from the same animal). Statistical significance was accepted for p<0.05.

**FIGURE LEGENDS**

**Online Figure 1.** High intraluminal pressure leads to an increase in mouse primary mononuclear cell adhesion via NF-κB. A: Vessels were incubated at 80 or 150 mmHg during 24 hours; fluorescent mononuclear cells were then injected in the intraluminal space, allowed to adhere during 30 minutes, and counted after washout. Cell adhesion was enhanced in vessels exposed to high pressure. Data are mean±SEM of n=8 experiments. ***p<0.001 vs 80 mmHg B: Untreated or chemokine-stimulated primary mononuclear cells were made to adhere for 30 min on VCAM-1-coated plates. Results show that combined treatment with MCP-1, IL-6 and KC enhanced cell adhesion compared with untreated controls. Data are mean±SEM of n=5 experiments. ***p<0.001 vs untreated control.

**Online Figure 2.** A: High intraluminal pressure is associated with enhanced phosphorylation of NF-κB throughout the vascular wall, as demonstrated by IHC. This effect is abolished in carotid arteries treated with SN50. Representative of n=4 separate experiments. B: In parallel, degradation of the NF-κB inhibitor IκBα is observed in untreated arteries maintained at 150
mmHg, but this effect is no longer observed in SN50-treated vessels. Data are mean±SEM of n=6. *p<0.05 vs 80 mmHg.

**Online Figure 3.** Treatment with angiotensin II (Ang II) does not further enhance monocyte adhesion in vessels. Arteries were maintained during 24h at 80 or 150 mmHg, with or without concurrent Ang II treatment (Hypertensin, 10^{-6} mol/L, Ciba). Monocyte adhesion to the vascular wall was equivalent in Ang II-treated and untreated arteries kept at equal pressure. Data are mean±SEM of n=3-4. *p<0.05 vs 80 mmHg.

**REFERENCE**


**A**

Cells/mm

- 80 mmHg
- 150 mmHg

---

**B**

Cells/well

- control
- MCP-1 +IL-6+KC

---

***
Online Figure 2

A

Positive staining (% surface)

Pressure
SN50
80
150
150
mmHg
-
-
+

***

§

B

p65

Tubulin

Nuclear p65 (AU)

SN50
-
+
+

80 mmHg
150 mmHg

*
Online Figure 3

AngII

Cells/mm

80 mmHg

150 mmHg

0 4 8 12 16

AngII - - + +

Circulation Research Manuscript #CIRCRESAHA/2007/151308 Original Contribution