Disturbed Blood Flow Induces RelA Expression via c-Jun N-Terminal Kinase 1

A Novel Mode of NF-κB Regulation That Promotes Arterial Inflammation

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Rationale: The nuclear factor (NF)-κB pathway is involved in arterial inflammation. Although the signaling pathways that regulate transcriptional activation of NF-κB are defined, the mechanisms that regulate the expression levels of NF-κB transcription factors are uncertain.

Objective: We studied the signaling mechanisms that regulate RelA NF-κB subunit expression in endothelial cells (ECs) and their role in arterial inflammation.

Methods and Results: Gene silencing and chromatin immunoprecipitation revealed that RelA expression was positively regulated by c-Jun N-terminal kinase (JNK) and the downstream transcription factor ATF2 in ECs. We concluded that this pathway promotes focal arterial inflammation as genetic deletion of JNK1 reduced NF-κB expression and macrophage accumulation at an atherosusceptible site. We hypothesized that JNK signaling to NF-κB may be controlled by mechanical forces because atherosusceptibility is associated with exposure to disturbed blood flow. This was assessed by positron emission tomography imaging of carotid arteries modified with a constrictive cuff, a method that was developed to study the effects of disturbed flow on vascular physiology in vivo. This approach coupled to en face staining revealed that disturbed flow elevates NF-κB expression and inflammation in murine carotid arteries via JNK1.

Conclusions: We demonstrate that disturbed blood flow promotes arterial inflammation by inducing NF-κB expression in endothelial cells via JNK-ATF2 signaling. Thus, our findings illuminate a novel form of JNK–NF-κB crosstalk that may determine the focal nature of arterial inflammation and atherosclerosis. (Circ Res. 2011;108:950-959.)

Key Words: arterial inflammation ▪ blood flow ▪ c-Jun N-terminal kinase ▪ NF-κB

Inflammatory activation of ECs influences atherosclerosis, a chronic inflammatory disease of arteries, by promoting the recruitment of mononuclear cells from the circulation to the vessel wall.1–3 Atherosclerosis occurs at distinct sites of the arterial tree located near branches and bends (eg, inner curvature of aortic arch).4 These regions are susceptible to lesion formation, in part, because ECs at these areas are activated and therefore primed for leukocyte recruitment.4–12 Several lines of evidence suggest that the spatial distribution of lesions is governed by shear stress (the frictional force exerted on ECs by flowing blood), which varies in time, magnitude and direction according to vascular pulsatility and geometry. First, activation of EC and lesion formation occurs preferentially at regions of arteries exposed to low/oscillatory shear stress, whereas regions exposed to high, unidirectional shear are protected.13–15 Second, a causal relationship between shear stress and atherosclerosis was established by Cheng et al16 who used a flow-altering device (constrictive cuff) that reduced shear stress and generated oscillations of the shear stress field in carotid arteries of an atherosusceptible strain of mice. Third, ECs detect shear stress via mechanosignaling complexes17 that transduce biochemical signals that influence proinflammatory activation.9,10,13,18–20

Nuclear factor (NF)-κB transcription factors promote vascular inflammation by inducing adhesion proteins and other proinflammatory molecules in vascular ECs.21,22 The most abundant form of NF-κB in ECs, the RelA/p50 heterodimer, is sequestered in the cytoplasm of unstimulated cells through

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binding to inhibitory IκB molecules. Signaling to NF-κB proceeds via distinct pathways that converge to destabilize IκB,\textsuperscript{21,22} thus releasing NF-κB for nuclear translocation and transcriptional activation. Despite numerous detailed studies of the signaling pathways that promote nuclear translocation and activation of NF-κB,\textsuperscript{21} the factors that regulate the expression levels of NF-κB subunits and the physiological importance of differential NF-κB expression have received little attention. Here we describe the first example of a signaling pathway that controls RelA NF-κB subunit expression. Specifically, RelA is positively regulated by JNK1, a mitogen-activated protein (MAP) kinase,\textsuperscript{23} and the downstream transcription factor ATF2. This pathway was stimulated in ECs by low or low/oscillatory shear stress and it enhanced expression of RelA and promoted inflammation at atherosusceptible sites. Thus we suggest that crosstalk between the JNK and NF-κB signaling pathways may influence the spatial distribution of atherosclerotic lesions.

Methods

Cuff Placement

Constrictive cuffs with an internal diameter of 500 μm tapering to 250 μm over 1 mm or nonconstrictive cuffs (internal diameter 600 μm) were placed on the right carotid artery of isoflurane-anesthetized mice following published methods.\textsuperscript{16}

Transfection

Cell cultures that were 80% to 90% confluent were transfected with small interfering (si)RNAs (or with nontargeting scrambled controls) by microporation (Digital BioTechnology, Seoul, Korea) following the instructions of the manufacturer.

Comparative Real-Time PCR

Transcript levels were quantified by comparative real-time PCR as described previously.\textsuperscript{18}

Western Blotting

Total cell lysates were prepared using a kit (Active Motif). Western blotting was carried out using specific primary antibodies, horse radish peroxidase-conjugated secondary antibodies and chemiluminescent detection.

Assay of NF-κB DNA Binding

Binding of RelA NF-κB subunits to consensus oligonucleotides was assessed by DNA-binding ELISA (Active Motif) using total cell lysates that were prepared using a kit (Active Motif). Chromatin immunoprecipitation was carried out using either anti-ATF2 antibodies or isotype-matched IgG control antibodies as described previously.\textsuperscript{24} Precipitation of specific genomic DNA fragments was assessed by real-time PCR.

Studies of Murine Arteries

The expression levels of specific proteins were assessed in ECs at regions of the inner curvature (susceptible site) and outer curvature (protected site) of murine aortae by en face staining as described.\textsuperscript{2,9–11} In vivo 18F-fluorodeoxyglucose positron emission tomography/computed tomography imaging was carried out using a combined PET/CT preclinical scanner as described.\textsuperscript{25} CT, PET and CT angiography data were coregistered and standardized uptake values normalized to body weight were calculated for regions of interest.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>FDG</td>
<td>fluoroexyglucose</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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Statistics

Differences between samples were analyzed using an unpaired or paired Student t test or 1-way ANOVA with Bonferroni adjustment.

Results

JNK1-ATF2 Signaling Positively Regulates RelA Expression

We previously identified transcriptional programs regulated by JNK using a microarray-based approach to study human umbilical vein ECs (HUVECs) treated with a pharmacological JNK inhibitor (CT536706).\textsuperscript{11} Further analysis of these data revealed that treatment with CT536706 reduced the basal expression of components of the NF-κB signaling pathway including RelA (downregulated 1.6 fold). We validated this observation by independent experiments which confirmed that pharmacological inhibition of JNK using either CT536706 or SP600125 led to reduced basal expression of RelA at both mRNA (Figure 1A) and protein (Figure 1B) levels. Further validation was obtained using JNK1-specific siRNA which suppressed the expression of JNK1 (without altering ATF2 expression) and significantly reduced the expression of RelA, whereas a scrambled, nontargeting control had no effect (Figure 1C).

We subsequently examined whether JNK influences RelA expression by activating the downstream transcription factors ATF2 or c-Jun. Immunofluorescent staining revealed that activation of ATF2 by phosphorylation occurred constitutively in unstimulated cells (Figure 1E, top). Silencing of ATF2 led to a significant reduction of RelA at both mRNA (Figure 1D) and protein (Figure 1E, bottom) levels, whereas silencing of c-Jun did not influence RelA expression (Figure 1, available in the Online Data Supplement at http://circres.ahajournals.org). Our data therefore indicate that RelA expression in ECs is positively regulated by JNK-ATF2 signaling, thus revealing crosstalk between the MAP kinase and NF-κB signaling pathways.

We hypothesized that ATF2 may directly regulate the expression of RelA by binding to its promoter. To address this, we firstly carried out sequence alignment of RelA genes in multiple species to identify conserved sequences in the 5′ untranslated region that may promote RelA expression. This
approach revealed two distinct regions of homology located upstream of the transcription start site which are likely to correspond to the RelA gene promoter (Online Figure II, A). Further analysis revealed the human RelA gene promoter contained five putative ATF2-binding sites located within 400bp of the transcriptional start site and that four of these sites were conserved between human and mouse (Online Figure II, B). The ability of ATF2 to target the RelA gene in HUVECs was assessed experimentally by chromatin immunoprecipitation (ChIP) using anti-ATF2 antibodies followed by quantitative PCR to measure coprecipitation of a portion of the RelA promoter that contained putative ATF2 binding sites (sequence shown in Online Figure II, B). Our studies revealed that the RelA promoter sequence coprecipitated with anti-ATF2 antibodies but not with an isotype-matched irrelevant IgG (Figure 1F, compare bars 1 and 2), whereas an irrelevant DNA sequence (GAPDH coding region) was not enriched by anti-ATF2 antibodies (Figure 1F, compare bars 1 and 3). Thus we conclude that ATF2 binds to specific DNA sequences within the RelA promoter. We next examined whether the observed effect of CT536706 on RelA expression is attributable to the inhibition of ATF2 binding to the RelA gene promoter. Treatment of HUVECs with CT536706 significantly reduced the enrichment of the RelA promoter sequence by anti-ATF2 antibodies. Cell nuclei were identified using ToPro3 (purple). Representative images are shown. RelA protein levels were quantified using image analysis software and are presented as mean values (±SEM).
bodies (Figure 1G), indicating that JNK positively regulates RelA expression by promoting binding of ATF2 to the RelA promoter.

**JNK Primes ECs for Inflammatory Activation by Enhancing RelA Expression**

Previous studies revealed that JNK acts as a positive regulator of inflammatory activation in cultured ECs. To assess whether JNK can prime cells for enhanced NF-κB activation, we examined whether inhibition of JNK can influence the capacity of NF-κB to bind consensus DNA sequences. Analysis of total lysates revealed that RelA-DNA binding in response to TNFα was reduced in cells pretreated with CT536706 (Figure 2) or SP600125 (data not shown) compared to cells pretreated with vehicle alone.

**JNK Enhances NF-κB Expression and Promotes Inflammation at an Atherosusceptible Site in the Murine Aorta**

We previously demonstrated that JNK1 is activated constitutively in ECs at a region of the murine aorta that is susceptible to inflammation and atherosclerosis (ie, the inner curvature of the aortic arch), whereas JNK1 activation is suppressed at a region that is resistant to atherosclerosis (ie, the outer curvature of the arch). Given our observation that RelA is positively regulated by JNK1, we predicted that RelA and its target gene VCAM-1 will be expressed preferentially at atherosusceptible sites via a JNK1-dependent mechanism.

Consistent with this hypothesis, en face staining studies of wild-type mice revealed that ECs at the susceptible site express higher levels of RelA protein (Figure 3, compare images 1 and 2) and mRNA (Online Figure III) compared to cells pretreated with vehicle alone.
ECs in the protected site. Moreover, the proportion of RelA that localized to the nucleus (an indicator of NF-κB activation) was greater in ECs at the susceptible site compared to the protected site (Online Figure IV, A). Elevated expression and nuclear localization of RelA at the susceptible site in wild-type mice correlated with expression of VCAM-1 and the accumulation of CD68-positive macrophages, whereas VCAM-1 expression was lower and macrophages were not observed at the atheroprotected site (Figure 3, compare images 4 and 7 with images 5 and 8). Genetic deletion of JNK1 reduced NF-κB and VCAM-1 expression (Figure 3, compare images 2 and 5 with images 3 and 6) and suppressed the accumulation of macrophages at the susceptible site (compare images 8 and 9), indicating that JNK1 positively regulates EC expression of RelA and inflammation at this region.

Low and Low/Oscillatory Shear Stress Enhances RelA Expression by Activating JNK

Atherosusceptible regions of arteries are associated with disturbed patterns of blood flow which exert low and/or oscillatory shear stress fields on ECs. Given that JNK1 activation9 and RelA expression (Figure 3) were observed preferentially at an atherosusceptible site, it is plausible that RelA expression may be regulated by shear stress via a JNK1-dependent mechanism. To test this hypothesis, we altered flow in the murine carotid artery by placing a constrictive cuff which causes tapering of the vessel lumen to generate high shear stress at the stenosis, a low shear environment upstream of the cuff and a low/oscillatory shear stress field downstream.16

We observed using wild-type mice that exposure of ECs to low or low/oscillatory shear stress for 7 days led to enhanced activation of JNK (Figure 4, images 1 and 3) and ATF2 (Online Figure V, images 1 and 3) and increased expression of RelA (Figure 4, images 4 and 6) compared to exposure of ECs to high shear stress. Genetic deletion of JNK1 reduced ATF2 activation and RelA expression in ECs exposed to low or low/oscillatory shear stress for 7 days (Online Figure V, compare images 1 and 3 with images 4 and 6; Figure 4, compare images 4 and 6 with images 7 and 9), indicating that
shear stress influences ATF2 activity and RelA expression via a JNK1-dependent mechanism. Interestingly, although both flow patterns induced RelA to a similar extent, the application of low/oscillatory shear stress induced nuclear localization of RelA (a measure of NF-κB activity; Online Figure IV, B) and VCAM-1 expression (Online Figure VI) to a greater extent than the application of low shear stress. These data suggest that a low/oscillatory shear stress environment may exert particularly proinflammatory effects on ECs by promoting JNK1-dependent expression and subsequent activation of NF-κB. To assess whether vascular physiological changes occurred as a direct response to the altered mechanical environment we studied endothelial cells following the application of low or low/oscillatory shear stress for a relatively short period. We observed that cuff placement for 2 days enhanced the expression of RelA (Online Figure VII) and VCAM-1 (Online Figure VIII) through a JNK-1 dependent mechanism which is consistent with a direct response to altered shear stress.

Low/Oscillatory Shear Stress Promotes Inflammation by Activating JNK1

We examined whether induction and activation of RelA by placement of a constrictive cuff can initiate inflammation in carotid arteries using PET-imaging of 18F-fluorodeoxyglucose (FDG) uptake, an indicator of metabolic activity that correlates with vascular inflammation.28,29 The role of JNK1 was determined by comparing FDG uptake in wild-type and JNK1−/− animals. The potential effects of surgery and cuff placement per se were assessed by measuring FDG uptake in arteries modified with nonconstrictive cuffs that did not influence blood flow. FDG uptake from the contralateral side (control side opposite to the cuff) was similar for all groups (0.79±0.02; 1.07±0.21 and 0.99±0.01 g/mL for wild type with constrictive cuff, JNK1−/− with constrictive cuff and wild type with nonconstrictive cuff respectively). Therefore, we expressed the data as the ratio between ipsi (cuffed artery) and contralateral uptake.

We observed that FDG uptake from the ipsilateral side was significantly higher at the site exposed to low/oscillatory shear stress (distal to the constrictive cuff) compared to sites exposed to either low (proximal to the constrictive cuff) or high shear stress (Figure 5, top left images). We concluded that FDG uptake was elevated as a response to altered hemodynamics and not as a consequence of the surgical procedure per se because application of a control, nonconstrictive cuff did not induce a similar relative increase in uptake in the distal region (Figure 5, lower left images). En face immunostaining revealed that exposure of arteries to low/oscillatory shear stress promoted the accumulation of intimal CD68-positive macrophages, which were observed at a relatively low frequency following 2 days exposure (Online Figure VII) and at a significantly higher frequency following 7 days (Figure 6; P<0.05). By contrast, CD68-positive cells were not observed at sites exposed to low or high shear for either 2 or 14 days (Online Figure VII; Figure 6). We observed by PET and immunostaining that genetic deletion of JNK1 reduced FDG uptake (Figure 5, top right images) and accumulation of macrophages (Figure 6, compare images 1 and 4) in the region exposed to low/oscillatory shear stress, indicating that JNK1 is required for inflammation at this site. Collectively, our in vivo studies demonstrate that JNK1 positively regulates endothelial NF-κB expression and inflammation at regions of arteries exposed to disturbed flow.

Discussion

The NF-κB and MAP kinase signaling pathways are regulated through multiple mechanisms that involve crosstalk at
several different levels. For example, NF-κB induces several negative regulators of MAP kinases which regulate the kinetics of JNK and p38 activation. Here we identify a novel mode of cross talk between these pathways where JNK pathway activation leads to elevated expression of RelA. Although it is established that c-Rel NF-κB subunits are regulated at the expression level, few studies have suggested that RelA expression can be altered by physiological stimuli. Thus our observations may be of fundamental importance because they indicate that RelA is regulated at the level of expression as well as at post-translational levels.

We suggest that the JNK-ATF2-NF-κB signaling pathway that we described promotes inflammatory activation. This novel observation is consistent with previous studies demonstrating that VCAM-1 expression is positively regulated by both NF-κB and AP-1 family transcription factors. Thus JNK-ATF2 signaling may regulate VCAM-1 expression through two parallel pathways – a direct route involving ATF2 interaction with the VCAM-1 promoter and an indirect route involving ATF2-dependent induction of RelA for subsequent VCAM-1 promoter interaction. Aside from its importance in regulating inflammatory activation, JNK-ATF2 signaling may also prime cells for expression of other NF-κB target genes that regulate diverse physiological processes. Although we focused on JNK1 because previous studies revealed that this isoform positively regulates endothelial activation and apoptosis, we cannot rule out a potential role for other JNK isoforms in the regulation of NF-κB.

We validated our in vitro findings by demonstrating that JNK positively regulates NF-κB expression and promotes inflammation in murine arteries. Previous studies have revealed that the morphological and physiological properties of...
ECs differ greatly between atheroprotected and atherosusceptible sites.\textsuperscript{4–11,40} ECs at atherosusceptible sites express relatively high levels of NF-κB proteins and are primed for inflammation, whereas ECs at protected sites express low amounts of NF-κB and are resistant to inflammation.\textsuperscript{5–7,12} Here we reveal that the molecular mechanism underlying the distinct spatial distribution of NF-κB expression involves JNK1 signaling which enhances NF-κB expression at atherosusceptible sites but not protected sites.

Spatial variation in EC morphology and physiology has been attributed to spatial variation in hemodynamics. Atheroprotected sites are exposed to high rates of unidirectional flow whereas atherosusceptible sites are exposed to lower rates of disturbed patterns of flow.\textsuperscript{5,13–15,41} Here we investigated the influence of blood flow on EC physiology by applying a flow-altering constrictive cuff which generates two different forms of atherosusceptible blood flow – a low shear environment and a low/oscillatory shear environment.\textsuperscript{16} Our studies of cuffed arteries revealed that both low/oscillatory and low shear stress induced RelA expression via JNK1. It is unlikely that the underlying mechanism involves the recruitment of CD68-positive inflammatory cells as they were not observed at the low shear region and were infrequent at the low/oscillatory shear site (2 days following cuff placement). It is plausible that atherosusceptible blood flow induces RelA expression via shear stress-mediated activation of mechanosignaling complexes, however we cannot rule out other possibilities eg, alterations in mass transport.\textsuperscript{41} Interestingly, we observed that activation of NF-κB and expression of VCAM-1 was enhanced at the low/oscillatory shear region compared to the low shear site, and that inflammation occurred exclusively at the low/oscillatory shear region. These data suggest that low and oscillatory forces have differential effects on EC activation and vascular inflammation. Specifically, low shear may prime ECs for inflammatory activation by inducing NF-κB expression, whereas oscillatory shear promoted both expression and nuclear localization (activation) of NF-κB which subsequently promotes inflammation. At a broader level, our observation that different forms of atherosusceptible blood flow have specific effects on EC physiology reinforces the concept that heterogeneity in EC phenotypes may arise because of local differences in the magnitude or direction of shear forces that they are exposed to.\textsuperscript{5,40}

Our observation that low/oscillatory shear can promote vascular inflammation in normocholesterolemic conditions is consistent with previous reports of low-grade inflammation at atherosusceptible sites in wild-type mice.\textsuperscript{12} It suggests that low/oscillatory shear stress may promote early atherogenesis by enhancing the accumulation of macrophages/dendritic cells at branches and bends.\textsuperscript{22,42} However, a previous study revealed that low/oscillatory shear stress promoted the formation of stable type lesions that contained few inflammatory cells in hypercholesterolemic ApoE\textsuperscript{−/−} mice,\textsuperscript{16} suggesting that low/oscillatory shear stress may have differential effects on vascular inflammation during plaque initiation and progression.

PET imaging of cuffed arteries allowed us to assess the effects of altered blood flow on vascular function in living animals. In preliminary studies, we attempted to assess FDG-PET uptake in regions of the murine aorta exposed to disturbed (inner curvature of arch) or unidirectional flow (outer curvature), however this approach was unsuccessful because PET did not have sufficient spatial resolution to distinguish between these two sites (data not shown). By contrast, modification of carotid arteries with a constrictive cuff (1 mm length) generated distinct flow fields with sufficient spatial separation for PET. Using this approach, we observed that low/oscillatory shear stress enhanced FDG uptake in the arterial wall. We suggest that disturbed flow influences FDG uptake by promoting the recruitment of CD68-positive cells, which were shown by en face staining to accumulate at the low/oscillatory region. Although this concept is consistent with previous observations that macrophages in atherosclerotic lesions display high FDG uptake,\textsuperscript{58,29} we cannot rule out the possible contribution of other cell types. At a broader level, we envisage that molecular imaging of cuffed arteries may be used to assess the influence of biomechanical forces on diverse aspects of vascular physiology including remodeling and plaque progression.\textsuperscript{43}

Our observation that JNK1 promotes vascular inflammation is consistent with previous reports that JNK isoforms play important roles in cardiovascular injury and disease. Animal studies have revealed that JNK can be activated in arteries in response to injury\textsuperscript{44–46} and during the development of aneurysms,\textsuperscript{47} or atherosclerotic lesions.\textsuperscript{48,49} Moreover, it was concluded that JNK is involved in the pathophysiology of atherosclerosis because gene transfer of a dominant negative form of JNK1 reduced neointimal formation in injured arteries\textsuperscript{44} and genetic deletion of JNK2 reduced foam cell formation and EC dysfunction in hypercholesterolaemia.\textsuperscript{49,50}

Thus, we suggest that therapeutic targeting of JNK may reduce vascular inflammation.

In summary, our findings illuminate a novel level of crosstalk between the NF-κB and JNK signaling pathways that is regulated by shear stress. This pathway may influence the spatial distribution of atherosclerotic lesions by promoting inflammation at atherosusceptible sites.

Sources of Funding

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Disclosures

None.

References

5. Passerini AG, Polacek DC, Shi CZ, Francesco NM, Manduchi E, Grant GR, Pritchard WF, Powell S, Chang GY, Stocek CF, Davies PF.


Novelty and Significance

**What Is Known?**

- The transcription factor nuclear factor (NF)-κB promotes inflammation of arteries by inducing adhesion molecules and other proinflammatory transcripts in vascular endothelial cells.
- Vascular inflammation and atherosclerosis occur preferentially at branches and bends of the arterial tree that are exposed to disturbed patterns of blood flow.

**What New Information Does This Article Contribute?**

- Studies using cultured endothelial cells revealed that NF-κB expression is enhanced by the transcription factor ATF2 via a signaling pathway that involves c-jun N-terminal kinase (JNK)1, a member of the mitogen-activated protein (MAP) kinase family.
- Analysis of aortae and carotid arteries modified with a flow-altering device demonstrated that disturbed blood flow enhances ATF2 activation and NF-κB expression in endothelial cells and promotes vascular inflammation.
- Studies of mice that lacked the JNK1 gene indicated that disturbed flow promotes inflammation through a JNK1-ATF2-NF-κB signaling pathway.
- The work identifies a novel form of crosstalk between the MAP kinase and NF-κB signaling pathways that influences the spatial localization of arterial inflammation.

Vascular inflammation occurs predominantly at branches and bends of arteries that are exposed to disturbed patterns of blood flow. NF-κB transcription factors promote vascular inflammation by activating proinflammatory genes in endothelial cells. Although the signaling pathways that activate NF-κB are relatively well-defined, the regulation and physiological significance of NF-κB expression has received little attention. This was addressed through studies of cultured endothelial cells which revealed that the expression of RelA NF-κB subunits is positively regulated by the MAP kinase JNK1 and the transcription factor ATF2. Subsequent experimentation demonstrated that disturbed blood flow promotes arterial inflammation by inducing NF-κB expression in endothelial cells via JNK-ATF2 signaling, indicating that crosstalk between the MAP kinase and NF-κB signaling pathways may influence the spatial localization of vascular inflammation. The work sheds new light on the biomechanical mechanisms that regulate arterial inflammation and suggests that therapeutic targeting of JNK1 or ATF2 may reduce inflammatory activation of endothelial cells. An additional novel element of the study is the development of a molecular imaging method that couples positron emission tomography with the generation of disturbed flow fields in murine carotid arteries, enabling the influence of biomechanical forces on vascular physiology to be studied in living animals.
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SUPPLEMENTAL MATERIAL

Detailed Methods

**Animals** Male C57BL/6 mice between 2 and 3 months of age were used. The JNK1 knockout mouse strain (JNK1\(^{-/-}\) (C57BL/6)) was generously supplied by Prof Roger Davis, University of Massachusetts Medical School, USA\(^1\). Constrictive cuffs with an internal diameter of 500\(\mu\)m tapering to 250\(\mu\)m over 1mm or non-constrictive cuffs (internal diameter 600\(\mu\)m) were manufactured from PTFE. They were placed on the right carotid artery of isoflurane-anaesthetized mice following published methods\(^2,3\). All experiments were performed within guidelines set out by the Federation of European Laboratory Animal Science Associations.

**Reagents and antibodies** Human recombinant TNF\(\alpha\) and anti-RelA (Santa Cruz), anti-tubulin (Sigma Aldrich), anti-phosphorylated-ATF2 T71 (Cell Signalling Technology), anti-phosphorylated-JNK T183/Y185 (Cell Signalling Technology), anti-CD31-FITC (BD Biosciences Pharmingen), anti-CD68 (Serotec) and anti-murine VCAM-1 (MK2.7 hybridoma line obtained from the American Type Culture Collection (Manassas, USA) antibodies were obtained commercially. CT536706, a pharmacological inhibitor of JNK\(^4,5\), was generously supplied by Dr Jeremy Davies (UCB Celltech, Slough, UK) and dissolved in DMSO. An alternative JNK inhibitor (SP600125) was obtained from Calbiochem and dissolved in DMSO. Other reagents were purchased from Sigma Aldrich unless otherwise stated.

**Endothelial cell culture** Human umbilical vein endothelial cells (HUVEC) were collected using collagenase and cultured as described previously\(^6\). HUVEC were passaged no more than four times and confluent cultures were used for experimentation.

**Gene silencing** RNA interference was carried out using small interfering (si)RNAs that specifically target JNK1 (s11153, Applied Biosystems), ATF2 (sc-2905, Santa Cruz Biotechnology) or c-Jun (s7658, Applied Biosystems)\(^5\). Alternatively, cells were treated with non-targeting scrambled controls (non-targeting siRNA no.1; Dharmacon). Cell cultures that were 80% to 90% confluent were transfected with siRNA (1-5 \(\mu\)M final concentration) by microporation (Digital BioTechnology, Seoul, Korea) following the manufacturer’s instructions and then incubated in growth medium without antibiotics for 48h before analysis.

**Transfection** Cell cultures that were 80% to 90% confluent were transfected with plasmids by microporation (Digital BioTechnology, Seoul, Korea) following the manufacturer’s instructions and then incubated in growth medium without antibiotics for 48h before analysis.

**Comparative real time PCR** Transcript levels were quantified by comparative real-time PCR using the following gene-specific primers: JNK1 (sense, 5’-GAAGCTCCACCACAAAGAT-3’; antisense, 5’-GGTTCTCTCCTCAGTCCA-3’), RelA (sense, 5’-TCAAGATCTGCCGAGTGAAC-3’; antisense, 5’-TGTCCTCTTCTGCACCTTG-3’), ATF2 (sense, 5’-GTACCATTGGCACAAGT-G-3’; antisense, 5’-GTGAAGGTACTGCCTGCTGA-3’),
c-Jun (sense, 5’-CAGCCCACTGAGAAGTCAAA-3’; antisense, 5’-CACCAATTCCTGCTTTGAGA-3’)) and β-actin (sense, 5’-CTGGAACGGTGAGGTGACA-3’; antisense, 5’-AAGGGACTTCTGTAAACATGCA-3’). Extraction and reverse transcription of total RNA and real-time PCR were carried out as described previously\(^6\). Reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product. Adjustment for the amount of input RNA was achieved by measuring β-actin mRNA levels as we described previously\(^6\).

**Western blotting** Total cell lysates were prepared using a kit (Active Motif). Western blotting was carried out using specific primary antibodies, horse radish peroxidase-conjugated secondary antibodies and chemiluminescent detection.

**Immunofluorescent staining of HUVEC** The expression of phosphorylated ATF2 and RelA in cultured HUVEC was assessed by staining of paraformaldehyde-fixed cells using rabbit anti-phosphorylated ATF2 antibodies and Alexafluor 568-conjugated goat anti-rabbit secondary antibodies followed by co-staining using mouse anti-RelA antibodies conjugated to Alexafluor488. Nuclei were identified using the DNA-binding probe ToPro3. Fluorescence was assessed by laser-scanning confocal microscopy (LSM 510 META; Zeiss). Image analysis was performed using Zeiss LSM 510 META software to calculate average fluorescence values after subtracting background fluorescence values from cells stained with secondary antibody alone. Control experiments revealed that the antibodies did not cross-react (data not shown).

**Assay of NF-κB DNA binding** Binding of RelA NF-κB sub-units to consensus oligonucleotides was assessed by DNA-binding ELISA (Active Motif) using total cell lysates that were prepared using a kit (Active Motif).

**Chromatin immunoprecipitation** A total of 9 x 10\(^6\) HUVECs were fixed by adding formaldehyde (1% final concentration for 10 minutes). Nuclear extracts were subjected to sonication for 8 minutes (30 secs on/30 secs off cycle Bioruptor UCD-200, Diagenode). For immunoprecipitation reaction, the nuclear lysates were incubated overnight at 4°C with protein G magnetic beads (Dynabeads Invitrogen) coated with either anti-ATF2 antibodies (sc-187x Santa Cruz Biotechnology) or isotype-matched IgG control. Immunocomplexes were washed, and co-precipitating DNA fragments were purified as described previously\(^7\). Precipitation of specific genomic DNA fragments was assessed by real-time PCR using the following primers: RelA (sense 5’-CTTAAGGAAAATTGAGGGAGGGCAGCGC-3’; antisense 5’-TGACTCAGTTTCCCTCCACACCCTCC-3’; see Supplemental Fig. II), GAPDH (sense 5’-TGATGACATCAAGAAGGTGGAAG-3’; antisense 5’-TCCTTGAGGCCCATGTGGGCCAT-3’).

**En face immunostaining** The expression levels of specific proteins were assessed in EC at regions of the inner curvature (susceptible site) and outer curvature (protected site) of murine
aortae by en face staining as we described previously. Animals were killed by CO₂ inhalation. Aortae were perfused in situ with PBS (at a pressure of approximately 100 mm Hg) and then perfusion-fixed with 2% formalin prior to harvesting. Fixed aortae were tested by immunostaining using specific primary antibodies and Alexafluor568-conjugated secondary antibodies (red). EC were identified by co-staining using anti-CD31 antibodies conjugated to the fluorophore FITC (green). Nuclei were identified using a DNA-binding probe with far-red emission (To-Pro-3; Invitrogen). Stained vessels were mounted prior to visualization of endothelial surfaces en face using confocal laser-scanning microscopy (Zeiss LSM 510 META). Isotype-matched monoclonal antibodies raised against irrelevant antigens or pre-immune rabbit sera were used as experimental controls for specific staining (data not shown). The expression of particular proteins at each site was assessed by quantification of fluorescence intensity for multiple cells (at least 50 per site) using LSM 510 software (Zeiss) and calculation of mean fluorescence intensities with standard error of the mean.

**En face in situ hybridization** The expression levels of RelA transcripts were assessed in EC at regions of the inner curvature (susceptible site) and outer curvature (protected site) of murine aortae using a Cy3-conjugated single-stranded DNA probe with sequence identity to murine RelA (5’- GGTCGGTGAATACCTAATG-3’). Animals were killed by CO₂ inhalation and aortae were perfusion-fixed with 2% formalin prior to harvesting as above. They were then incubated with 1nM PE-RelA probe overnight at 55°C and then washed thoroughly with PBS. EC were identified by co-staining using anti-CD31 antibodies conjugated to the fluorophore FITC (green). Stained vessels were mounted prior to visualization of endothelial surfaces en face using confocal laser-scanning microscopy (Zeiss LSM 510 META). A Cy3-conjugated probe with irrelevant sequence was used to control for specific staining (data not shown). The expression of RelA transcripts at each site was assessed by quantification of fluorescence intensity using LSM 510 software (Zeiss) and calculation of mean (+/- SE) fluorescence intensities.

**In vivo FDG PET/CT imaging** A combined PET/CT preclinical scanner was used (Inveon, Siemens Preclinical Solutions, Inc). A CT scan was first acquired to correct PET data for photon attenuation with the power of the X-ray source set to 80 kVp and 500 μA, 220 projections and an exposure time of 200 ms. Three bed positions were acquired to match the full PET field of view with a final isotropic resolution of 103 microns. A one hour PET acquisition started simultaneously to intravenous injection of 18F-FDG (8.9 ± 3.6 MBq) with an energy window of 350-650 keV and a coincidence timing window of 3.4 ns. PET data were then histogramed into 3 frames of 20 min and reconstructed using OSEM-MAP algorithm with 2 OSEM3D iterations and 16 subsets, followed by 18 MAP iterations. The smoothing factor β in MAP reconstructions was set to 0.551. A matrix of 256 x 256 and a zoom of 2 were applied to all data during reconstruction providing a final resolution of 0.215x0.215x0.796 mm. Finally, the animal bed was moved back to the center of the CT field of view and a CT angiography acquired using the following parameters: ECG and respiration gating, X-ray source set to 80 kVp and 500 μA, 220 projections over 220 degrees and an exposure time of 200 ms. During CT acquisition, Ultravist
370 (Bayer Healthcare Pharmaceuticals) was infused intravenously at a speed of 6ml/hr using a syringe pump (PHD 22/2000, Harvard Apparatus). Analysis was performed in IRW 3.0 (Siemens Preclinical Solutions, Inc). Briefly, CT, PET and CTA data were loaded simultaneously and co-registered. Then regions of interest localized around the right and left carotids were identified on the CTA (Supplemental Figure IX) and overlayed with PET data for quantification. Standardized uptake values (SUV) normalized to body weight were calculated providing a comparable quantification (g/ml) for each animal.

**Statistics** Differences between samples were analysed using an unpaired or paired Student’s t-test or one-way ANOVA with Bonferroni adjustment.
Supplemental References


Supplemental Fig. I c-Jun does not influence RelA expression. HUVEC were treated with c-Jun-specific siRNA or with a scrambled non-targeting sequence and were incubated for 48h. c-Jun and RelA transcript levels were quantified by real-time PCR. Mean values (+/- SEM) were calculated from data pooled from five independent experiments.
Supplemental Fig. II Identification of putative AP-1 binding sites in the RelA gene promoter. (A) Multiple sequence alignment was used to identify regions of mouse, rat, dog and macaque RelA genes that have sequence similarity to the human RelA gene. Regions of homology appear as peaks and are plotted in relation to the human RelA gene structure (top; blue rectangles represent exons; arrows indicate direction of transcription). A region of sequence homology positioned immediately upstream of the transcriptional start site was identified as a putative promoter of transcription. (B) The sequence of a 402 base pair portion of the 5' untranslated region (positioned immediately upstream of the transcription start site; see box in A) was analysed. Putative binding sites for ATF2 transcription factors were identified using VISTA software (http://genome.lbl.gov/vista/index.shtml) and the JASPAR database (jaspar.cgb.ki.se/) and their positions are indicated by boxes (asterisks indicate putative binding sites that are conserved between human and murine sequences). Red text indicates the sequence that was amplified in ATF2 ChIP experiments. Brown text indicates transcribed sequence.
Supplemental Fig. III Expression of RelA transcript levels is enhanced at an atherosusceptible site. RelA mRNA levels in EC were assessed by *en face in situ* hybridization of susceptible and protected regions of the aorta in wild-type mice (red; n=7 per group). EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Representative images and quantitation of RelA mRNA expression (mean +/- SEM) are shown. MFI, mean fluorescence intensity.
Supplemental Fig. IV  Low/oscillatory shear stress activates RelA in murine arterial EC

(A) The intracellular localization of RelA in EC was assessed at susceptible (S) or protected (P) regions of the aorta in C57BL/6 mice (n=3) using anti-RelA antibodies and fluorescent secondary antibodies. EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Cell nuclei were identified using ToPro3 (purple). The proportion of RelA localized to the nucleus was calculated by dividing nuclear fluorescence by total fluorescence for multiple cells and mean values (+/- SEM) are shown. (B) Flow-altering constrictive cuffs were placed on the right carotid arteries of C57BL/6 mice. Right carotid arteries were harvested after 2 or 7 days and intracellular localization of RelA in EC was assessed by en face staining as above. The proportion of RelA localized to the nucleus was calculated as above and mean values (+/- SEM) are shown. Data are representative of two experiments (using 3 or 4 mice per group) that gave closely similar results.
Supplemental Fig. V  Low shear stress enhances levels of phosphorylated ATF2 in murine arterial EC. Flow-altering constrictive cuffs were placed on the right carotid arteries of wild-type (n=4) or JNK1−/− mice (n=3). Right carotid arteries were harvested after 7 days. Levels of phosphorylated ATF2 were measured in EC by en face staining of regions exposed to low (L), high (H) or low/oscillatory (L/O) shear stress (red). EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Cell nuclei were identified using ToPro3 (purple). Representative images and quantitation of ATF2 phosphorylation (mean +/- SEM) are shown. MFI, mean fluorescence intensity.
Supplemental Fig. VI  Low shear stress enhances VCAM-1 expression in murine arterial EC.
Flow-altering constrictive cuffs were placed on the right carotid arteries of wild-type mice (n=4). Right carotid arteries were harvested after 7 days. VCAM-1 expression on EC was assessed by en face staining of regions exposed to low (L), high (H) or low/oscillatory (L/O) shear stress (red). EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Cell nuclei were identified using ToPro3 (purple). Representative images and quantitation of VCAM-1 expression (mean +/- SEM) are shown. MFI, mean fluorescence intensity.
Supplemental Fig. VII  Exposure of EC to low shear stress for two days enhances RelA expression via JNK1 in the absence of macrophages. Flow-altering, constrictive cuffs were placed on the right carotid arteries of wild-type or JNK1−/− mice (n=3 per group). Right carotid arteries were harvested after 2 days. Levels of RelA in EC and the accumulation of CD68-positive cells was assessed by en face staining of regions exposed to low (L), high (H) or low/oscillatory (L/O) shear stress (red). EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Cell nuclei were identified using ToPro3 (purple). Representative images and quantitation of RelA expression and CD68-positive cells (mean +/- SEM) are shown. MFI, mean fluorescence intensity.
Supplemental Fig. VIII  Exposure of EC to low/oscillatory shear stress for two days enhances VCAM-1 expression via JNK1. Flow-altering, constrictive cuffs were placed on the right carotid arteries of wild-type or JNK1−/− mice (n=3 per group). Right carotid arteries were harvested after 2 days. Levels of VCAM-1 in EC were assessed by en face staining of regions exposed to low (L), high (H) or low/oscillatory (L/O) shear stress (red). EC were identified by co-staining with anti-CD31 antibodies conjugated to FITC (green). Cell nuclei were identified using ToPro3 (purple). Representative images and quantitation of VCAM-1 expression (mean +/- SEM) are shown. MFI, mean fluorescence intensity.
Supplemental Fig. IX Detection of an external carotid artery cuff by CT. Flow-altering, constrictive cuffs were placed on the right carotid arteries of wild-type mice (n=5 per group). After 14 days, the anatomical location of the cuff was visualized by CT angiography (arrows) using iodine based contrast agent. Representative images are shown.