Biomechanical and biochemical regulation of cathepsin K expression in endothelial cells converge at AP-1 and NF-κB

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Abstract

Cathepsins K and V are powerful elastases elevated in endothelial cells by tumor necrosis factor-α (TNFα) stimulation and disturbed blood flow both of which contribute to inflammation-mediated arterial remodeling. However, mechanisms behind endothelial cell integration of biochemical and biomechanical cues to regulate cathepsin production are not known. To distinguish these mechanisms, human aortic endothelial cells (HAECs) were stimulated with TNFα and exposed to pro-remodeling or vasoprotective shear stress profiles. TNFα upregulated cathepsin K via JNK/c-jun activation, but vasoprotective shear stress inhibited TNFα-stimulated cathepsin K expression. JNK/c-jun were still phosphorylated, but cathepsin K mRNA levels were significantly reduced to almost null indicating separate biomechanical regulation of cathepsin K by shear stress separate from biochemical stimulation. Treatment with Bay 11–7082, an inhibitor of IκBα phosphorylation, was sufficient to block induction of cathepsin K by both pro-remodeling shear stress and TNFα, implicating NF-κB as the biomechanical regulator, and its protein levels were reduced in HAECs by vasoprotective shear stress. In conclusion, NF-κB and AP-1 activation were necessary to activate cathepsin K expression in endothelial cells, highlighting integration of biochemical and biomechanical stimuli to control cathepsins K and V, powerful elastases implicated for arterial remodeling due to chronic inflammation and disturbed blood flow.

Keywords
proteases; shear stress; sickle cell disease; TNF-α

Introduction

Cathepsin K has become of particular interest for arterial remodeling as a powerful collagenase and elastase, implicated in the progression of cardiovascular disease (Chapman et al., 1997; Li et al., 2004; Lutgens et al., 2006; Platt et al., 2007). It is well established that regions of arteries can be exposed to a vasoprotective shear stress which is high in magnitude and unidirectional, shown to protect arteries from atherosclerotic plaque

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formation. On the other hand, regions of arteries at branches, bifurcations, or sharp curves are exposed to pro-remodeling shear stress, which has been defined as low or oscillatory and occurs at regions where atherosclerotic plaques preferentially form, preceded by elastic lamina degradation and neointimal thickening (Glagov et al., 1961; Ku, 1997; Ku and Giddens, 1987; Zarins et al., 1983). Mechanisms that control endothelial cell expression of cathepsin K include shear stress, the dragging force of blood across the endothelial cells lining the artery wall, with pro-remodeling shear stress stimulating endothelial cell expression and activation of cathepsin K while vasoprotective, unidirectional shear stress inhibits its expression in endothelial cells (Platt et al., 2007).

Cytokines such as TNFα also stimulate endothelial cells during inflammation associated with atherosclerotic plaque formation, and TNFα also induces endothelial cell expression and activation of cathepsins K and V (Keegan et al., 2012a). However, the interplay between TNFα and shear stress regulation of cathepsin activity in large artery endothelial cells remains unclear. These concurrent stimuli are present during atherosclerotic progression, and also in the unique situation of sickle cell disease where the accelerated elastic lamina degradation predisposes children with this genetic disease to strokes (Boros et al., 1976; Merkel et al., 1978; Stockman et al., 1972; Switzer et al., 2006). Higher circulating plasma levels of TNFα are associated with sickle cell disease (Malavé et al., 1993), and it has recently been implicated in upregulation of cathepsins K and V in endothelial cells (Keegan et al., 2012b). Combinatorial studies of TNFα stimulation with either vasoprotective or pro-remodeling shear stress are described here to determine mechanisms by which endothelial cells integrate biochemical and biomechanical cues to regulate cathepsin expression and activity in disease contexts.

Results

Vasoprotective shear stress is sufficient to reduce active cathepsin K levels in endothelial cells in the presence of TNFα stimulation

To determine cathepsin K regulation in response to physiological shear stress with TNFα stimulation, human aortic endothelial cells (HAECs) were grown to confluence, stimulated with TNFα, and cultured in a cone-and-plate viscometer to actuate a vasoprotective shear stress profile, pro-remodeling shear stress profile, or static conditions; shear stress profiles over one cardiac cycle are shown (Figure 1A). After 24 hours of shear stress in the presence or absence of TNFα, HAECs were lysed, and equal amounts of protein were loaded for cathepsin zymography with cleared white bands indicative of active cathepsins K and V (37 and 35 kDa, respectively). These bands do appear close to each other by this assay but we have verified each of their identities in HAECs in a previous study (Li et al., 2010; Wilder et al., 2011).

Either TNFα or pro-remodeling shear stress were individually sufficient to turn on cathepsin K (Figure 1B,C). Co-stimulation with both pro-remodeling shear stress and TNFα stimulation induced the highest amount of active cathepsin K. Vasoprotective shear stress, however, significantly reduced TNFα-stimulated cathepsin K by 50% and was even lower than TNFα stimulated conditions under static conditions (Figure 1B; n=4, p<0.05). For cathepsin V, there was no significant difference by shear stress alone. TNFα.

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treatment upregulated cathepsin V significantly, but not under vasoprotective shear stress (n=4, p<0.05). Cathepsin K was being regulated differentially by shear stress and by the biochemical stimuli, and the alternate mechanisms behind this were the focus of the following studies.

Immunocytochemistry staining for cathepsin K indicated that TNFα-stimulation and pro-remodeling shear stress both increased cathepsin K protein expression as indicated by the brighter fluorescence. Conversely, vasoprotective shear stress HAECs had reduced staining intensity of cathepsin K, even when they were stimulated by TNFα (Figure 2A). These images corroborate the zymography data from Figure 1.

Cathepsin K mRNA levels were quantified by real-time PCR to determine if vasoprotective shear stress was regulating cathepsin K at the transcriptional level. TNFα increased cathepsin K mRNA in HAECs cultured under pro-remodeling shear stress or static conditions, but vasoprotective shear stress inhibited TNFα-mediated induction of cathepsin K significantly (Figure 2B). Although the magnitude was smaller, pro-remodeling shear stress in the absence of TNFα still significantly increased cathepsin K mRNA compared to static conditions. Together the mRNA data supports the zymography and immunostaining data.

JNK/c-jun signaling, shear stress, and cathepsin K regulation

With shear stress and TNFα differentially regulating cathepsin K at the mRNA level, intracellular kinase-signaling cascades that integrate biochemical and biomechanical stimuli were next investigated. Previously, our group established that TNFα-dependent activation of cathepsin K activity in large artery endothelial cells is dependent on the JNK/c-jun signaling axis (Keegan et al., 2012a), but these studies were conducted under static conditions. We tested the hypothesis that vasoprotective shear stress would modulate TNFα-induced cathepsin K expression by reducing JNK or c-Jun phosphorylation. Immunoblotting was performed on HAEC lysate collected from cells stimulated as described earlier. TNFα alone resulted in a 3-fold increase in JNK phosphorylation, and a 3-fold increase in c-Jun phosphorylation compared to vehicle controls (Figure 3; n=3, p<0.05) under all shear stress conditions, even vasoprotective shear stress. There was no reduction in JNK or c-Jun phosphorylation under vasoprotective shear stress as expected, and pro-remodeling shear stress alone did not significantly increase JNK or c-jun phosphorylation, although there was increased cathepsin K mRNA and active enzyme.

Phosphorylation of c-jun activates it to then dimerize and form the transcription factor AP-1, which has binding sites in the promoter region of the cathepsin K gene (Pang et al., 2007). With phosphorylation of c-jun still occurring even under vasoprotective shear stress even though cathepsin K was not active, we next tested if shear stress altered nuclear localization of phosphorylated c-Jun. Immunostaining with an anti-phospho-c-jun antibody was completed after 24 hours of shear stress and TNFα stimulation. TNFα induced a substantial increase in phosphorylated c-Jun in the nucleus of HAECs compared to vehicle, under all shear conditions (Figure 4A). Interestingly, pro-remodeling shear stress alone induced translocation of phosphorylated c-Jun, though to a lesser extent than that of TNFα stimulation (Figure 4A). Consistent with the Western blot results, vasoprotective shear stress
did not reduce TNFα-mediated phosphorylation or nuclear translocation of c-Jun (Figure 4A).

Chromatin immunoprecipitation (ChIP) analyses were used to determine if the phosphorylated c-jun/AP-1 in the nucleus under vasoprotective shear stress was actually binding to the cathepsin K promoter. Under static conditions, TNFα alone increased c-jun/AP-1 binding by 2.7-fold compared to vehicle (Figure 4B, white bars), and vasoprotective shear stress did not reduce AP-1 binding as hypothesized. In fact, vasoprotective shear stress alone increased c-Jun binding by 8.3-fold over static conditions, and TNFα co-stimulation with vasoprotective shear stress increased c-Jun binding to the cathepsin K promoter region by 28-fold (Figure 3A; n=4, *p<0.05). These data indicated that vasoprotective shear stress was not inhibiting AP-1 binding to cathepsin K promoter region, and that while JNK/c-Jun activation is involved in TNFα induction of cathepsin K, the shear stress-mediated regulation was occurring by another mechanism.

**Biomechanical shear stress regulates cathepsin K via NF-κB**

To elucidate the multi-pathway cross talk with JNK/c-jun in the synergistic regulation of proteolysis by biomechanical and inflammatory stimuli, alternative downstream kinase pathways were investigated. TNFα-stimulation is upstream of both JNK and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation and transcriptional regulation. There is evidence to suggest that NF-κB is shear stress regulated, and has been implicated in arterial remodeling in vivo (Gareus et al., 2008). Therefore, NF-κB functioning as the shear stress responsive element in endothelial cell cathepsin K activation was tested. Immunoblotting for total NF-κB expression indicated that vasoprotective shear stress significantly decreased whole-cell levels of NF-κB compared to pro-remodeling shear stress, regardless of TNFα stimulation (Figure 5A). To test for nuclear localization of NF-κB in response to shear stress or TNFα stimulation, immunocytochemistry was performed using antibodies for NF-κB. Co-staining for nucleus was done with DAPI, and F-actin was used to identify endothelial cell borders. These images were captured under the same imaging settings and it corroborates the Western blots that indicate a loss of NF-κB in HAECs after culture under vasoprotective shear stress conditions and with that, a loss of nuclear localization of NF-κB (Figure 5B).

To establish a link between cathepsin K and NF-κB, we next tested the hypothesis that loss of NF-κB signaling, such as the loss of bioavailable NF-κB in HAECs cultured under vasoprotective shear stress, would inhibit activation of cathepsin K under pro-remodeling shear stress. Bay 11–7082 is a small molecule that inhibits IκB activation, and therefore blocking the release of NF-κB and its downstream actions. Cathepsin K was stimulated by TNFα and by pro-remodeling shear stress in vehicle treated controls, but inhibition of NF-κB activation with Bay11–7082 significantly reduced the amount of active cathepsin K under pro-remodeling shear stress by almost 40% in the absence of TNFα, and by 50% with TNFα treatment as seen in the zymogram (Figure 6A), and quantified by densitometry (Figure 6C; n=4, p<0.05). The findings from this data implicate shear-regulated NF-κB signaling in pro-remodeling-induction of cathepsin K activity, but there were no significant changes in active cathepsin V by NF-κB inhibition (Figure 6D). Treatment with the inhibitor...
did not significantly change total NF-κB protein levels seen in the immunoblots for the p65 subunit of NF-κB (Figure 6B and 6E).

**Discussion**

The findings from this study provide a mechanistic link for how endothelial cells integrate biomechanical shear stress and inflammation to differentially regulate cathepsin K in the proposed model (Figure 7). Specific kinase signaling pathways by which physiological shear stress mitigated or enhanced TNFα-stimulated cathepsin K in endothelial cells were investigated to identify how large artery endothelial cells integrate biochemical and biomechanical signals. TNFα-mediated cathepsin K activation via JNK signaling was significantly inhibited by vasoprotective shear stress (Figure 1), but not pro-remodeling shear stress, which was sufficient to increase active cathepsin K in endothelial cells (Keegan et al., 2012a). Vasoprotective shear stress reduction of active cathepsin K was not through JNK/c-Jun axis as both JNK and c-jun phosphorylation still occurred, but reduction of induced cathepsin K occurred by blocking bioavailable NF-κB with either reduced protein levels of NF-κB by vasoprotective shear stress (Figure 5) or with the inhibitor Bay 11–7082 inhibitor suppressed TNFα induced cathepsin K levels (Figure 6). Taken together, this indicates that cathepsin K is biochemically regulated distinctly from its biomechanical regulation and that endothelial cells must integrate this information to make decisions that impact cell and tissue physiology. A schematic of this conceptual model is shown in Figure 7.

Pathological effects of TNFα stimulation are commonly divided into pathways defined by either JNK or NF-κB activation. However, there is growing evidence that several genes require complementary activation of both AP-1 and NF-κB (Fujioka et al., 2004); studies have shown that activation of JNK via cytokine stimulation is accompanied by increased NF-κB nuclear translocation, and the AP-1 response is enhanced by the presence of NF-κB subunits (Stein et al., 1993; Verma et al., 1995), even in endothelial cells. Other groups have found NF-κB signaling to be increased in regions of pro-remodeling shear stress in mice, and *in vivo* inhibition of NF-κB protects against atherosclerotic lesion formation (Cuhlmann et al., 2011; Gareus et al., 2008; Hajra et al., 2000). In this study, we conclude that while TNFα does induce cathepsin K activity through biochemical pathways, it is predicated on the presence of available NF-κB such that the loss of NF-κB expression or signaling, as caused by the biomechanical stimuli of vasoprotective shear stress on endothelial cells, can block the induction of cathepsin K expression and activity.

Vascular biomechanical studies have long established that regions of the vasculature exposed to vasoprotective shear stress are protected from vascular remodeling, while regions exposed to pro-remodeling shear stress are regions where pathological arterial remodeling occurs. Work done by our group has previously implicated upregulation of cathepsin K and other cathepsins by endothelial cells at sites of disturbed flow in human coronary arteries and by contributing to increased elastase and gelatinase activity (Platt et al., 2006, 2007). TNFα and disturbed flow also can separately increase endothelial cell surface expression of VCAM-1 and ICAM-1 that promote monocyte adhesion to endothelial cells (Sorescu et al., 2004; Suo et al., 2007) and monocyte adhesion to endothelial cells signals production of cathepsins.
K and V (Keegan et al., 2012a,b), which are powerful elastases and collagenases that can remodel the collagen and elastin structures in the artery wall. NFκB signaling in response to shear stress has not yet been shown to affect other cathepsins. Our multiplex cathepsin zymography assay that was used here, detects active cathepsins K, L, S, and V, but there were no changes in cathepsins L or S with NF-κB inhibition. Others have reported that shear stress can regulate other pro-inflammatory responses through NF-κB including reactive oxygen species generation (Wang et al., 2013), VCAM-1 expression (Wang et al., 2011), and there are even reports that high shear stress can induce matrix metalloproteinases (MMPs) through NF-κB signaling in human umbilical vein endothelial cells (HUVECs) (Castier et al., 2009). While we did not find that interferon γ (IFNγ) did not upregulate cathepsins in HAECs (data not shown), it has been shown to induce production and secretion of cathepsins K and S in smooth muscle cells (Sukhova et al., 1998), indicating that there are cell-specific differences in responses to pro-inflammatory cytokines.

Beyond atherosclerosis, these results may have particular relevance for arterial remodeling due to sickle cell disease where there is chronic inflammation characterized by elevated serum levels of TNFα by as much as 20-fold and highly elevated levels of circulating monocytes (Keegan et al., 2012a). Children with sickle cell disease have a significantly elevated risk of stroke before the age of 16, with the highest risk between 2 and 5 years old (Hillery and Panepinto, 2004; Switzer et al., 2006). Mechanisms behind this rapid, pathological arterial remodeling and elastic lamina degradation are not clear as these are not lipid-laden plaques characteristic of atherosclerosis (Hassler, 1962; Merkel et al., 1978; Platt, 2005). Based on these findings, vasoprotective shear stress is sufficient to prevent cathepsin K activation or at a minimum to significant reduce it. However, if regions of pro-remodeling shear stress spontaneously form due to transient aggregation of red and white blood cells along the arterial wall due to increased monocyte numbers, endothelial activation, and monocyte adhesion seen in sickle cell disease (Barabino et al., 2010; Belcher et al., 2005; Chaar et al., 2010; Frenette, 2004), then a second, independent, pro-cathepsin K signal may be stimulated in the endothelium, allowing regions of the vasculature to become vulnerable to cathepsin-mediated elastin degradation and arterial remodeling. By identifying other mechanistic pathways by which cathepsin activity is regulated by the hemodynamic and inflammatory environment in the vasculature, new therapeutic targets may be identified for reduction in the risk of stroke for children with sickle cell disease or others for cardiovascular disease.

Materials and methods

Cell culture and actuation of physiological shear stress

Human aortic endothelial cells (HAECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth supplement (ECGS) isolated from bovine cerebral tissue. Vasoprotective and pro-remodeling shear stress profiles were generated by digitizing physiological the physiological shear stress waveforms (Dai et al., 2004). HAECs were grown to confluence in a 10cm dish, and stimulated with or without 10 ng/ml recombinant human TNFα (Invitrogen), then placed into a cone-and-plate bioreactor, and...
exposed to vasoprotective or pro-remodeling shear stress for 20 hours. For NF-κB inhibition studies, 5 μM of the inhibitor Bay 11–7082 (Sigma) which selectively and irreversibly inhibits NF-κB activation by blocking phosphorylation of IκBα, was added to cultures for 1 hour prior to stimulation with TNFα.

Multplex cathepsin zymography and Western blotting

Cells were lysed using zymography lysis buffer (20 mM Tris–HCl [pH 7.5], 5 mM ethyleneglycoltetraacetic acid [EGTA], 150 mM NaCl, 20 mM b-glycerol phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, and 0.1% Tween 20) with 0.1% leupeptin. To assess cathepsin activity, cell lysates were analyzed using multiplex cathepsin zymography, as described previously (Li et al., 2010; Wilder et al., 2011). Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Images were inverted in Adobe Photoshop and densitometry was performed using NIH ImageJ. Western blotting was used to determine phosphorylated and total c-Jun N-terminal kinase (JNK; Cell Signaling) and c-jun primary antibodies (Cell Signaling) and secondary antibodies to be visualized with a LI-COR Odyssey scanner. Densitometry of labeled nitrocellulose membranes was performed using NIH ImageJ (NIH).

Chromatin immunoprecipitation (ChIP) assay for Activator Protein 1 (AP-1)

After shear stress or TNFα stimulation, isolation of HAEC genomic DNA for ChIP assay was completed using the EZ-Chip system (Millipore). Briefly, after isolation of genomic DNA, fragmentation was completed via sonication for 5 seconds at 15% amplitude followed by a 10 second rest which was repeated 22 times for a total sonication time of 110 seconds. PCR primers were synthesized based on previously identified binding regions for activator protein 1 (AP-1) on the cathepsins K gene (Gelb et al., 1997). The forward sequence, 5’–TCC TAA CAG GAA AGG GGT AGG A-3’, and the reverse sequence, 5’-AGA CTG TCT TTG GTG GCA AAT-3’, were analyzed using the Basic Local Alignment Search Tool (BLAST; NIH) to ensure minimal cross reactivity with other sequences prior to synthesis (Integrated DNA Technologies).

Quantification of cathepsin K mRNA

Total RNA was isolated from shear stress conditioned HAEC cultures using RNeasy kits (Qiagen) and reverse transcribed to single stranded cDNA with random hexamers according to manufacturer’s instructions (Qiagen). Quantitative real time PCR was performed using the forward primer sequence 5’-ATA TGT GGG ACA GGA AGA GAG TTG T-3’ and the reverse primer sequence 5’-GGA TCT CTC TGT ACC CTC TGC ATT T-3’ with GAPDH cDNA used as a loading control. Relative cathepsin K mRNA levels were quantified using ΔΔCt methods for comparison.

Immunostaining

As described previously, confluent HAEC cultures were conditioned with 10 ng/ml TNFα, and maintained under static, vasoprotective, or pro-remodeling shear stress for 20 hours. Cells were rinsed with PBS three times, and fixed with 4% paraformaldehyde (PFA) for 10 minutes, rinsed with PBS, permeabilized using 0.2% Triton X, and blocked with 3%
BSA in PBS for 1 hour at room temperature. HAECs were incubated overnight at 4°C with rabbit anti-cathepsin K (Santa Cruz; 1:100) or rabbit anti-phosphorylated c-Jun, or NF-κB p65 (Cell Signaling; 1:100). Cells were rinsed three times with PBS, and then incubated with Alexa Fluor 488 conjugated secondary antibodies (Invitrogen; 1:150) for 1 hr at room temperature. Cells were counterstained with Alexa Fluor 568 phalloidin (10 μg/ml; Life Technologies), and Hoechst (10 mg/ml; Life Technologies), mounted, and covered. Images were acquired using a Zeiss LSM 700–405 confocal microscope.

**Statistical analysis**

Each experimental condition was repeated with a minimum of three biological replicates and each data point is presented as the mean value and standard error of the mean (SEM). Representative images are shown. Unpaired Student t-tests were used to determine statistical significance (p<0.05) between most experimental groups.

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**References**


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Figure 1.
Vasoprotective shear stress reduced TNFα-mediated increases in cathepsin K protein and activity.
(A) Shear stress profiles that were programmed into Servomotors are shown. Confluent HAECs were stimulated with 10 ng/ml TNFα, and cultured under static conditions, vasoprotective shear stress, or pro-remodeling shear stress for 20 hours using the shown shear stress profiles programmed into Servomotors. (B) TNFα stimulation induced cathepsin K activity under static and pro-remodeling shear stress, but vasoprotective shear stress significantly reduced the amount of active cathepsin K by TNFα by 50% as detected with multiplex cathepsin zymography quantified by densitometry (*p<0.05, n=6). (C) Densitometry for active cathepsin K signal and (D) densitometry for active cathepsin V signal.

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Figure 2.
Vasoprotective shear stress reduced TNFα-mediated increases in cathepsin K immunostaining and mRNA.

(A) After shear stress culture, HAECs were immunolabeled for cathepsin K protein (green), counterstained with phalloidin (red) for filamentous actin, and Hoechst (blue) for nuclei. Fluorescence for cathepsin K protein in HAECs was increased in response to pro-remodeling shear stress, but vasoprotective shear stress inhibited this (n=3, representative images shown), and endothelial cells aligned in the direction of flow. TNFα also stimulated an increase in cathepsin K activity under static and pro-remodeling shear stress, but this was substantially reduced under vasoprotective shear stress even when stimulated with TNFα.

(B) Quantitative real-time PCR for cathepsin K was performed on samples from vasoprotective shear stress or pro-remodeling shear stress. There was a significant reduction in cathepsin K mRNA after TNFα stimulation under vasoprotective shear stress (n=3, p<0.05).
Figure 3.
Vasoprotective shear stress does not block TNFα-mediated activation of JNK/c-Jun. Confluent HAEC cultures stimulated with or without 10 ng/ml TNFα, and maintained under static, pro-remodeling, or vasoprotective shear stress were lysed and probed for phosphorylated and total levels of JNK and c-Jun by (A) Western blotting, quantified by densitometry. TNFα induced a 3-fold increase in phosphorylation of JNK (B) and c-Jun (C), relative to total protein levels (n=3, p<0.05), and vasoprotective shear stress did not block JNK or c-Jun phosphorylation (n=3, p>0.8).
Figure 4.
Vasoprotective shear stress does not block c-jun binding to cathepsin K promoter. Confluent HAECs were stimulated with 10 ng/ml TNFα, and subjected to static conditions, vasoprotective, or pro-remodeling shear stress for 20 hours. (A) Immunostaining for phospho-c-Jun nuclear localization under both pro-remodeling and vasoprotective shear stress conditions (green-phospho-c-Jun, blue-nuclei, red-F-actin; representative images shown from 3 independent experiments). (B) In parallel, HAECs were also prepared for chromatin immunoprecipitation (ChIP) for AP-1 using antibodies against phosphorylated c-jun. TNFα increased binding of phosphorylated c-Jun to the cathepsin K promoter region by 2.7-fold under static conditions and by 28-fold after vasoprotective shear stress (*p<0.05, $p=0.05$ compared to vehicle control, n=5).
Figure 5.
Vasoprotective shear stress decreased NFkB protein levels even when stimulated with TNFα.

(A) Western blots for NF-κB p65 subunit were performed after pro-remodeling or vasoprotective shear stress on HAECs. There was a significant decrease in whole-cell NFκB protein levels in HAECs cultured under vasoprotective shear stress compared to that of pro-remodeling shear stress (*p<0.05, n=4). (B) Immunohistochemical labeling of NF-κB p65 subunit was performed on HAECs after shear stress and TNFα stimulation with green fluorescence indicating NF-κB, red being F-actin staining done with phalloidin, and blue for DAPI-stained nuclei.
Figure 6.
Shear stress-mediated activation of cathepsin K is dependent on NFκB activation. Confluent HAECs were pre-conditioned with 5 μM of Bay 11–7082, an inhibitor of NFκB activation, for 1 hour prior to being stimulated with or without 10 ng/ml TNFα, and exposed to static conditions or pro-remodeling shear stress for 20 hours. (A) HAECs were lysed and active cathepsins were determined by multiplex cathepsin zymography. Cathepsin K bands do not appear when Bay 11–7082 is incubated with the cells. (B) NF-κB protein levels were determined by Western blot, and (C) Pro-remodeling shear stress was sufficient to induce detectable levels of active cathepsin K, but inhibiting NF-κB activation with Bay 11–7082 significantly reduced the amount of active cathepsin K (*p<0.05, n=4). (D) There was no significant difference in the amount of cathepsin V, or (E) NF-κB in the presence or absence of Bay 11–7082.
Figure 7.
Schematic representation of working model of biomechanical and biochemical regulation of cathepsin K activity in endothelial cells. Proposed mechanism by which vasoprotective shear stress inhibits cathepsin K protein expression and activation downstream of TNFα stimulation via reduced NF-κB activation and expression. TNFα stimulation activates JNK/c-jun phosphorylation, but reduction of NF-κB protein prevents induction of cathepsin K transcription under vasoprotective shear stress conditions.