Laminar Shear Stress Regulates Endothelial Kinin B1 Receptor Expression and Function

Potential Implication in Atherogenesis

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Objective—The proinflammatory phenotype induced by low laminar shear stress (LSS) is implicated in atherogenesis. The kinin B1 receptor (B1R), known to be induced by inflammatory stimuli, exerts many proinflammatory effects including vasodilatation and leukocyte recruitment. We investigated whether low LSS is a stimulus for endothelial B1R expression and function.

Methods and Results—Human and mouse atherosclerotic plaques expressed high level of B1R mRNA and protein. In addition, B1R expression was upregulated in the aortic arch (low LSS region) of $ApoE^{-/-}$ mice fed a high-fat diet compared to vascular regions of high LSS and animals fed normal chow. Of interest, a greater expression of B1R was noticed in endothelial cells from regions of low LSS in aortic arch of $ApoE^{-/-}$ mice. B1R was also upregulated in human umbilical vein endothelial cells (HUVECs) exposed to low LSS (0 to 2 dyn/cm^2) compared to physiological LSS (6 to 10 dyn/cm^2): an effect similarly evident in murine vascular tissue perfused ex vivo. Functionally, B1R activation increased prostaglandin and CXCL5 expression in cells exposed to low, but not physiological, LSS. IL-1β and ox-LDL induced B1R expression and function in HUVECs, a response substantially enhanced under low LSS conditions and inhibited by blockade of NFκB activation.

Conclusion—Herein, we show that LSS is a major determinant of functional B1R expression in endothelium. Furthermore, whereas physiological high LSS is a powerful repressor of this inflammatory receptor, low LSS at sites of atheroma is associated with substantial upregulation, identifying this receptor as a potential therapeutic target. **(*Arterioscler Thromb Vasc Biol*. 2009;29:1757-1763.)**

Key Words: atherosclerosis ■ laminar shear stress ■ inflammation ■ kinin B1 receptor

Cardiovascular disease (CVD) is the leading cause of death in developed countries with a major component of these deaths directly related to the consequences of atherogenesis (according to WHO statistics, 17 million people die of CVD each year, http://www.who.int). The past two decades has seen a growing appreciation that inflammatory mechanisms underlie the initiation and progressive development of an atheroma, and it is clear that the inner lining of the blood vessel wall, the endothelium, is a pivotal site at which these inflammatory events occur.[1] In particular, there is recognition that alteration of the phenotype of the endothelium, from protective (and maintaining homeostasis) to damaging, is likely to precipitate the atherogenic process.[2]

One of the major determinants of endothelial phenotype is laminar shear stress (LSS), defined as the frictional force engendered by blood flow on the endothelium. Indeed, variation in LSS has been identified as determining susceptibility of particular vascular sites to atheroma formation[2–4] and has been proposed to predominate above sex and dietary fat as a risk factor for atherosclerosis.[5,6] The levels of LSS vary throughout the circulation, however in large arteries (such as the aorta) the net unidirectional physiological levels of LSS are high (6 to 20 dyn/cm^2 in conduit vessels) and endow the endothelium with an antiinflammatory phenotype, whereas low LSS ($<$4 dyn/cm^2) levels found at sites of atheroma formation (ie, at

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bifurcations and curvatures such as the aortic arch) is thought to be proinflammatory and pathogenic in atherosclerosis.[2] However, the exact mechanisms stimulated by LSS that predispose a site to atheroma formation remain unclear.

Of particular relevance to the present study has been the finding that an inducible and proinflammatory G protein–coupled receptor (GPCR), the kinin B1 receptor (B1R), is localized to sites of human aortic atheroma,[7] although the functional significance of this expression has not been explored. The kinins are a family of inflammatory peptides, including bradykinin (BK) or Lys-BK and their metabolites, des-Arg^{9}BK (DBK) and Lys-DBK (LDBK), that interact with 2 specific GPCRs: B1 receptor (B1R) and B2 receptor.[8] Whereas the B2R, activated by BK and Lys-BK, is constitutively expressed, the B1R, activated by DBK or Lys-DBK, is weakly expressed normally but is induced under inflammatory conditions.[8] Functionally, B1R activation induces a number of proinflammatory effects, therefore we investigated whether low (atherogenic) LSS might be a stimulus for B1R expression and inflammatory function in the blood vessel wall; these data were complemented by an analysis of the mechanisms involved in endothelial B1R expression and function in atherosclerosis.

Materials and Methods

Full details of all methods can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Cell Culture and Application of Shear Stress

Steady unidirectional LSS of 10, 6, 2, or 0 dyn/cm^2 was applied on human umbilical vein (HUVECs) or aortic (HAECs) endothelial cells using a cone and plate viscometer.[9,10] Cells were left untreated or treated with B1R agonist (Lys-des-Arg^9-BK; 10 μMol/L), IL-1β (10 ng/mL) or with oxidized LDL (oxLDL, 20 μg/mL[12–14]) in the absence or presence of the B1R antagonist SSR240612[11] (1 μMol/L: 15 minutes before IL-1β application) or with the NFκB inhibitor BAY 11-7082[15] (20 μMol/L: 15 minutes before IL-1β application).

Perfused Mouse Mesentery Preparations

The mesentery was mounted in a 37°C water-jacketed organ bath and perfused with warmed physiological salt solution with varying amounts of dextran to achieve high (6 dyn/cm^2) or low (2 dyn/cm^2) levels of LSS.

Atherosclerosis in ApoE$^{-/-}$ Mice

Male atherosclerosis-prone ApoE$^{-/-}$ mice were fed a high-fat or chow diet. The whole aorta was removed, and in some instances the aortic arch separated from the thoracic aorta for separate analysis of regions subjected to low LSS and high physiological LSS, respectively. Blood was collected for lipid analysis.

Immunohistochemical Analysis

The aortic arches of ApoE$^{-/-}$ mice were embedded in paraffin and immunohistochemistry analysis performed.

Prostaglandin and Nitric Oxide Measurement in Endothelial Cell Culture Supernatant

Concentrations of prostaglandin (PG)I$_2$ and PGE$_2$ were measured using enzyme immunoassay kits. Nitrite production, as a measure of endothelial NO generation, was measured as previously described.[16]

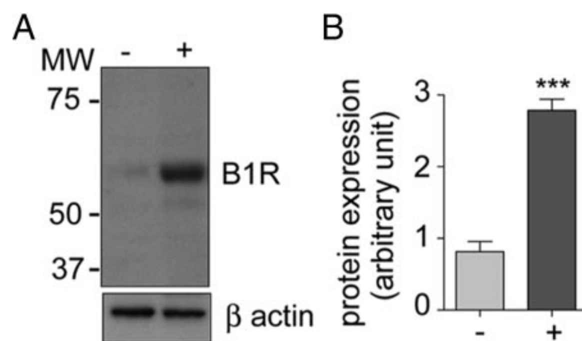

Figure 1. B1 receptor is induced in regions of atheroma in humans. A, Representative Western blot of B1R protein expression in human carotid endarterectomy tissue: sections: with (+; n=4) or without (−; n=4) plaque. B, Quantification of B1R expression normalized to β-actin. Data are shown as mean±SEM. ***$P<0.001$.

Quantitative Real-Time PCR

Total RNA was extracted from cells or tissue, cDNA synthesized, and quantitative real-time PCR conducted.

Western Blotting

Western blotting for B1R was determined in human carotid endarterectomy tissue and HUVEC samples using a selective antibody for B1R.[17] Samples were divided into sections containing lesion (+) and regions devoid of plaque (−) (supplemental Figure I).

Radioligand Binding Assay

In HUVECs, total B1R binding was determined by adding B1R agonist [^{3}H]-LDBK at 0.75 nmol/L, with nonspecific binding performed by cotreatment with LDBK in excess (10 μmol/L, 1 hour) on ice. Cells were dissolved and the radioactivity determined by liquid β-scintillation count.

Results

B1R Is Induced In Vivo in Vascular Regions Predisposed to Atheroma Formation

In tissue from human carotid endarterectomy B1R expression was more pronounced in regions of atheromatous plaque compared to regions devoid of plaque (Figure 1). B1R mRNA expression was also upregulated in a time-dependent fashion in aorta of ApoE$^{-/-}$ mice fed a high-fat diet ($P<0.05$; supplemental Figure IIA), an effect that was temporally associated with a rise in serum triglyceride level (supplemental Figure IIB). Mice fed a normal chow diet for 12 weeks had normal levels of both serum triglyceride and LDL cholesterol levels (supplemental Figure IIC through IID) and no change in B1R mRNA expression (Figure 2A). Further analysis of the different regions of the aorta (ie, regions of low LSS versus regions of high LSS), demonstrated that in ApoE$^{-/-}$ mice fed a high-fat diet a >3-fold increase in expression was evident in the arch region compared to the thoracic aorta (Figure 2B). Immunohistochemical assessment localised B1R to both smooth muscle and endothelial cells in sections of the inner curvature (ie, regions of low LSS, Figure 2C), with less intense expression evident in sections of the outer curvature.

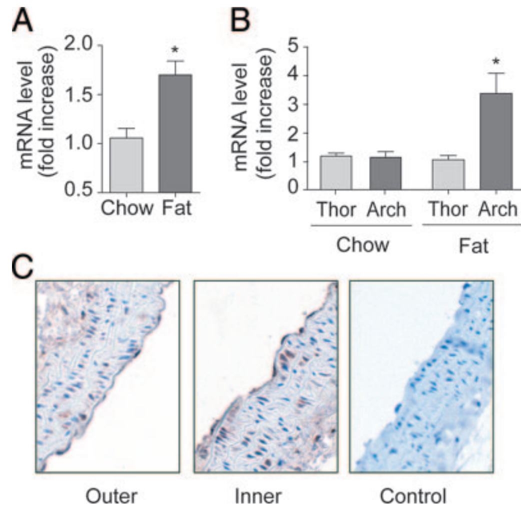

Figure 2. B1R is induced in regions of vessel curvature predisposed to atheroma formation in ApoE$^{-/-}$ mice. A through C, ApoE$^{-/-}$ mice fed a high-fat or normal chow diet for 12 weeks. B1R mRNA expression in whole aorta (A) or in aortic arch and thoracic aorta sections (B). C, B1R imunohistochemical expression in outer and inner curvatures of aortic arch of ApoE$^{-/-}$ mice fed a chow diet. Data are mean±SEM for n=9. **P*<0.05. Arch indicates aortic arch; Thor, thoracic aorta.

Low LSS Induces Kinin B1R Expression in Endothelial Cells and in Mesenteric Tissue Ex Vivo

Exposure of HUVECs to physiological (high) LSS but not low LSS resulted in a time-dependent alignment of cells (supplemental Figure IIIA). Low LSS was also associated with decreased nitrite production reflecting endothelial dysfunction (supplemental Figure IIIB). Under high LSS a suppression of B1R mRNA expression relative to static HUVECs occurred that was maximal at 8 hours (by ≈50%) and sustained for up to 16 hours (supplemental Figure IVA). Comparison between cells exposed to high and low LSS for 8 hours exposed a ≈2-fold increase in B1R mRNA expression in cells under low LSS conditions (Figure 3A). In contrast in HAECs, B1R mRNA was undetectable in both conditions (data not shown, n=4). All further cell experiments were conducted after 8 hours of LSS exposure, and using physiological (high) LSS as a reference control.

Western blotting of cell lysates demonstrated upregulated expression after low but not high LSS (Figure 3B and supplemental Figure IVC). Confirmation of antibody selectivity was achieved in preadsorption experiments in HEK-293 (supplemental Figure IVB). In addition [^{3}H]-LDBK binding was increased 20-fold (*P*<0.01) in cells exposed to low LSS (Figure 3C). HUVECs treated with IL-1β displayed binding with [^{3}H]-LDBK, which was displaced by increasing concentrations of cold LDBK confirming the validity of [^{3}H]-LDBK as tracer for these assays (supplemental Figure IVD).

LSS-induced regulation of B1R expression was also demonstrated in intact blood vessels; B1R mRNA was expressed at a very low level in mouse mesenteries exposed to physiological levels of LSS ex vivo, however exposure to low LSS caused a >5-fold elevation (*P*<0.05; Figure 3D).

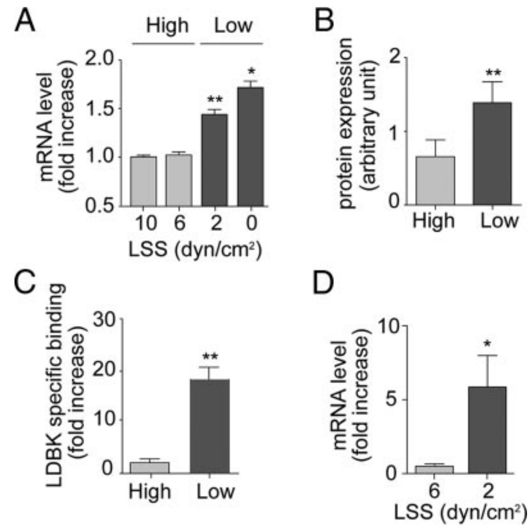

Figure 3. Low LSS induces B1R expression. B1R (A) mRNA expression, (B) protein expression, and (C) agonist binding in HUVECs subjected to low LSS or high LSS. Data are mean±SEM for n=6. ***P*<0.01, **P*<0.05 high vs low values. D, B1R mRNA expression in mesenteric tissue from WT mice perfused at high (6 dyn/cm^2) or low (2 dyn/cm^2) LSS for 4 hours. Data are mean±SEM for n=5. **P*<0.05.

Low LSS Increases B1R Functionality

Both PGI_2 and PGE_2 release and endothelial CXCL5 mRNA expression were significantly enhanced in response to LDBK in HUVECs exposed to low LSS but not high LSS (Figure 4). Neither COX-1 nor COX-2 expression were altered by LSS (supplemental Figure VA and VB). CXCL5 mRNA was also upregulated in aortic arch of ApoE$^{-/-}$ mice compared to thoracic aorta (>8-fold increase; data not shown, n=6).

Additive Effects of Low LSS and Inflammation on B1R Expression and Function

IL-1β caused a pronounced elevation of B1R mRNA expression in HUVECs under low LSS; an effect that peaked at 1

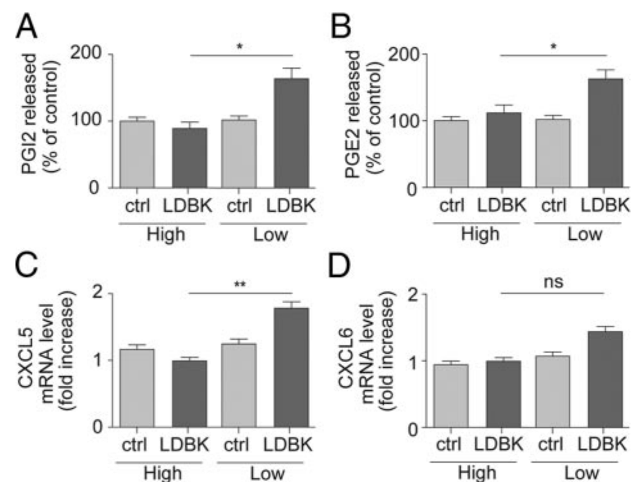

Figure 4. B1R activation stimulates PGE2, PGI2, and CXCL5 production only under low LSS. HUVECs were subjected to high or low LSS and stimulated or not with B1R agonist Lys-des-Arg^9-Bradykinin (LDBK, 10 μmol/L) and (A) PGI2 or (B) PGE2 release and endothelial (C) CXCL5 and (D) CXCL6 mRNA expression measured. Data are mean±SEM for n=6. ns indicates nonsignificant. **P*<0.05, ***P*<0.01.

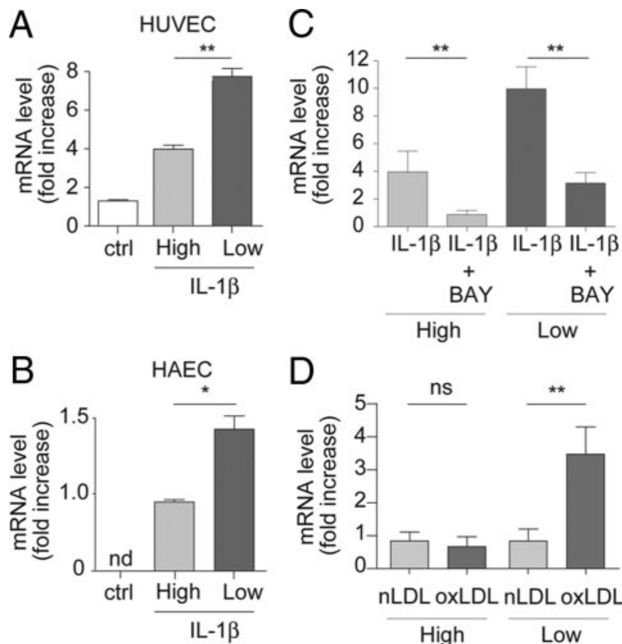

Figure 5. IL-1β–induced B1R expression is enhanced under low LSS conditions. B1R mRNA expression in (A) HUVECs (n=6) or (B) HAECs (n=6) subjected to varying LSS (0 to 10 dyn/cm^2, 12 hours), then stimulated or not with IL-1β (10 ng/mL, 4 hours) before or not treatment with (C) the NFκB inhibitor (BAY 11-7082, 20 μmol/L, n=6). D, After LSS HUVECs were treated with native LDL (nLDL, 20 μg/mL) or oxidized LDL (oxLDL, 20 μg/mL, 3 hour, n=3). *P<0.01, **P<0.05. nd indicates nondetermined; ns, nonsignificant.

hour and returned to basal by 8 hours (supplemental Figure VIA). In addition, although IL-1β (4 hour) caused ≈5-fold increase in B1R mRNA expression under low LSS conditions, this effect was substantially reduced (≈50%) under high LSS (Figure 5A). This enhanced expression in HUVECs exposed to low LSS was similarly evident in HAECs (Figure 5B), associated with enhanced LDBK-specific binding (supplemental Figure VIB and VIC) and inhibited by the NFκB inhibitor BAY 11-7082 (Figure 5C).

Confirmation that IL-1β-induced B1R expression was associated with enhanced B1R function was demonstrated by the finding that the elevated CXCL5 and CXCL6 mRNA expression evident in response to IL-1β under low LSS was suppressed by ≈50% by the B1R antagonist, SR240612 (Figure 6A-B). Finally, oxLDL caused a >3-fold increase in B1R mRNA expression compared to treatment with native LDL under low but not high LSS conditions (Figure 5D).

Discussion

LSS, the unidirectional frictional hemodynamic force, imposed on the endothelial cell surface as a result of blood flow plays a major role in maintaining homeostasis. Herein, we show that low LSS induces expression and functionality of the proinflammatory kinin B1R. Moreover, we demonstrate in vitro that inflammatory stimuli and low LSS, combined to mimic the atherogenic environment, synergize to enhance both expression and function of this receptor; a phenomenon also evident at sites of atheroma in both humans and a mouse model of atherosclerosis. Because activation of B1R is a

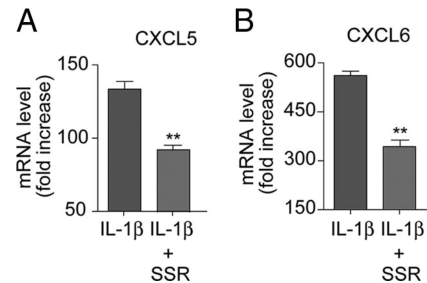

Figure 6. IL-1β–induced CXCL5 and CXCL6 expression is mediated by B1R activation and enhanced under low LSS conditions. HUVECs were subjected to low LSS for 12 hours and treated with IL-1β (10 ng/mL) for 4 hours in the absence or presence of the B1R antagonist SSR240612 (1 μmol/L, 15 minutes before IL-1β application). CXCL5 (A) or CXCL6 (B) mRNA expression was measured levels normalized to GAPDH. Data are mean±SEM for n=6. **P<0.01.

pivotal step in promoting leukocyte recruitment and endothelial permeability in inflammatory responses[9,18] and elevated B1R expression is evident at sites of human atheroma,[7] we propose that the targeting of this receptor represents an exciting prospect for atherosclerotic disease.

Previous evidence has demonstrated that kinin B1R expression is evident in human blood vessels and may be associated with atherosclerosis.[7] In this study, Western blotting of segments of human carotid artery, collected from individuals undergoing endarterectomy, demonstrated a ≈3-fold elevation of kinin B1R in those areas associated with substantial atheromatous plaque only. Analysis of the aorta of ApoE$^{-/-}$ mice fed a high-fat diet exposed a similar selectivity in localization of B1R expression. Although B1R expression was evident basally expression increased over time, with a near doubling of expression by 12 weeks; an effect not evident in mice fed a chow diet. Comparison of the levels of expression in the aortic arch (a region of substantial atheroma formation) with the longitudinal section of the thoracic aorta (a region of no significant atheroma formation) demonstrated a clear localization of B1R to regions of atheromatous plaque formation and, interestingly, at a similar level of intensity evident in human blood vessels (ie, ≈3-fold increase). Immunohistochemical analysis suggested that expression was particularly evident in endothelial cells, the site of LSS sensing in the blood vessel wall (expression was also evident in smooth muscle and diffuse within the intima likely reflecting inflammatory cell recruitment,[7] as previously reported).

Indeed, subjecting endothelial cells in culture to low (atherogenic) LSS raised kinin B1R expression above that measured in cells exposed to physiological levels of LSS. That the levels of LSS used accurately reflect atherogenic and physiological levels of LSS was demonstrated by the presence of "endothelial dysfunction" under low LSS, as evidenced by decreased endothelial NO synthesis; a key indicator of this phenomenon in CVD.[18] Similarly, in the arterial circulation of the mesentery, subjected ex vivo to varying LSS, minimal B1R expression was observed under physiological LSS but a >5-fold increase in expression occurred after exposure to atherogenic LSS. Together,

these findings imply a selective upregulation of B1R expression by low atherogenic LSS. An alternative interpretation of these findings, however, is that high LSS represses B1R expression. Indeed, physiological LSS, through specific transcription factor dependent pathways, represses expression of a number of proinflammatory proteins in endothelial cells in culture.[19] However, in the present study B1R expression was suppressed after inhibition of the proinflammatory transcription factor NF-κB, implying an induction by low LSS rather than an inhibition by high LSS.

To investigate whether enhanced expression was associated with enhanced function we measured the expression of downstream inflammatory molecules. Prostaglandins released during inflammation produce local vasodilatation, increasing regional blood flow and microvascular permeability, together facilitating leukocyte infiltration.[20] Prostaglandins have also been implicated in mediating, at least in part, B1R-induced increases in vascular permeability and blood flow in several different vascular beds.[8,21] PGE_2 and PGI_2, in particular, are prominent prostaglandins involved in mediating these effects, but are also molecules that have been implicated in atherogenesis.[22–24] We demonstrated that although B1R agonist treatment did not alter PGE_2 and PGI_2 production by endothelial cells exposed to physiological LSS, both were elevated under low LSS. This effect likely relates to an increase in enzymatic activity because no changes in expression of the principal vascular COX enzymes, COX-1 and COX-2, were evident. More recently, we have also reported that B1R-induced inflammatory leukocyte recruitment is, at least in part, attributable to endothelial chemoattractant cytokine ELR-CXCL chemokine, CXCL5/6, synthesis.[17] In the current study B1R agonist also induced CXCL5 production in cells subjected to low LSS while having no effect under high LSS. Indeed, in support of this finding we measured elevated levels of CXCL5 at sites of atheroma formation in $ApoE^{-/-}$ mice. Collectively, these findings intimate that the enhanced expression of B1R under low LSS conditions directly correlates with the enhanced inflammatory phenotype of endothelial cells exposed to B1R agonists.

The kinin B1R promoter possesses several potential shear stress response elements (SSRE) with consensus sequence GAGACC,[25] Barbie box (CTTT motif), and GAGA (GAGAG motif)[26] sites for binding of specific transcription factors, particularly noteworthy being the transcription factor NFκB that binds to GAGACC.[27] NF-κB has been implicated in mediating the enhanced expression of a number of proteins dually regulated by both inflammation and low LSS, including adhesion molecules (E-selectin, VCAM-1)[5,6] and chemokines (MCP1, IL8).[28–30] In the present study we demonstrate that inhibition of NF-κB activation using BAY 11-7082, a selective inhibitor of cytokine-inducible I-κBα phosphorylation,[15,31] inhibited B1R expression in response to IL-1β under both high and low LSS conditions implicating NF-κB in both low LSS and IL-1β–induced B1R expression and low LSS alike.

As mentioned previously, inflammation plays a pivotal role in all stages of the atherosclerotic disease process: initiation, progression, and plaque rupture.[1,32] An increasing body of evidence suggests that the prevailing hemodynamic conditions not only alters the expression of inflammatory genes within the endothelium but also determines the magnitude of the inflammatory response to pathogenic stimuli. Evidence suggests that low LSS is associated with an inflamed phenotype and enhanced responsiveness to diverse inflammatory stimuli including the cytokines IL-1β and TNFα, leading to enhanced expression of adhesion molecules and chemokines (eg, IL-8 and MCP-1)[5,29,33–36] as well as augmented inflammatory cell recruitment.[33,36] In line with these findings, in the current study, IL-1β produced a greater elevation of B1R expression, reflected by enhanced mRNA expression and agonist binding, and enhanced function in terms of prostaglandin and chemokine CXCL5 synthesis in cells subjected to low LSS compared to high LSS.

This enhanced activity was not limited solely to inflammatory cytokines but also to the molecule currently perceived to be the primary inflammatory stimulus in atherosclerosis: oxLDL.[37,38] At a concentration in line with those found in patients with CVD, and shown to be proinflammatory in endothelial cells,[12–14] oxLDL substantially elevated kinin B1R expression only in endothelial cells exposed to low LSS. In addition, our studies exposed the existence of a positive loop centered on B1R, whereby the effects produced by the cytokine in combination with low LSS were significantly attenuated by B1R blockade using SR240612. Equally interesting, SR240612 significantly inhibited IL-1β–induced CXCL6 as well as CXCL5 expression in cells subjected to low LSS, although the B1R agonist did not induce CXCL6 expression in HUVECs. This demonstrates that B1R expression and function were optimal when endothelial cells under low LSS were in a context of inflammation. SR240612 is a selective nonpeptidic antagonist with a Ki of 0.48 nmol/L at B1R and has an estimated pA_2 of 9.4 using standard organ bath assays for measurement of antagonist potency.[11] This antagonist has been tested against $>$100 other receptors and shows no or negligible activity at concentrations up to 1 μmol/L (the concentration we used for our experiments) and is at least 1000 times less potent at the kinin B2R. Thus, these findings clearly demonstrate the proinflammatory nature of B1R activation in endothelial cells and are in agreement with our previously published findings in B1R knockout mice,[17] demonstrating the essential role of the B1/CXCL5 pathway in inflammatory cell recruitment. Together, our data suggest that the contribution of the kinin B1R at sites of inflammation, in terms of both prostaglandins, CXCL5 and CXCL6 expression, is substantially enhanced at sites of low LSS and intimates a potential role for this pathway in the inflammatory events associated with atherogenesis.

A limitation of this work is that most of these data were produced with venular endothelium. However, to mitigate against this criticism, we investigated whether varying LSS could influence B1R expression in HAECs (cells relevant to clinical disease as demonstrated by the high prevalence of aortic lesions in patients, ie, $\approx$60%[39,40]). Indeed, although no expression was evident under basal

conditions, high LSS was a powerful suppressor of the raised expression under inflammatory conditions (ie, after IL-1β treatment). These findings intimate that the effect of LSS on endothelial B1R is likely a generalized feature of this cell type irrespective of vessel type.

Recent studies have implicated neutrophil recruitment in atherosclerosis.[41,42] Depletion of neutrophils, using an anti-PMN antibody, in ApoE$^{-/-}$ mice fed a high-fat diet significantly reduced plaque development.[42] Separate studies have also implicated neutrophil infiltration in promoting erosion and rupture of unstable plaque.[43,44] Studies investigating the pathways involved in the recruitment of this cell type clearly demonstrate that the interaction between neutrophils and endothelial cells in vivo occurs predominantly at sites of low shear.[34] Our previous studies have demonstrated an essential role for the kinin B1R in neutrophil recruitment to sites of inflammation, in particular we have demonstrated that Il-1β–induced neutrophil recruitment to the inflamed microvasculature was greatly diminished in B1R knockout mice.[17,45] Collectively, these data prompt us to speculate that a pathway centered on B1R expression and activation may underlie the neutrophil recruitment recently implicated in the process of atherogenesis in mouse models of disease,[41,42] although further studies are required to investigate this possibility more fully.

In conclusion, our data suggest that endothelial kinin B1R expression and function are tightly regulated by LSS, with expression being induced by low atherogenic levels of LSS, an effect substantially exacerbated under inflammatory conditions. Furthermore, we have identified a possible role for the kinin B1R in the pathogenesis of inflammatory CVD, particularly atherosclerosis; such findings imply that targeting the B1R pathway may prove beneficial in the therapeutic management of atherosclerotic disease.

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Disclosures

None.

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