SUPPLEMENT MATERIAL

Laminar shear stress regulates endothelial kinin B1 receptor expression and function: potential implication in atherogenesis

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MATERIALS AND METHODS

Human carotid endarterectomy

We studied four, not previously examined, surgical in-patients enlisted to undergo carotid endarterectomy for extracranial high-grade internal carotid artery stenosis. Carotid endarterectomy was performed in patients and tissue was immediately snap-frozen at -80^{o}C until analysis of protein expression (see below). The study was approved by local Italian ethics review committees. Written informed consent was obtained from all patients before each examination.

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

Male atherosclerosis-prone ApoE$^{-/-}$ mice (bred in-house, breeding pairs Jackson Labs USA) were fed a high fat diet (21% fat, 0.5% cholesterol, Harlan,UK) for 0, 3, 6 and 12 weeks or with chow diet for 12 weeks. Mice were killed by cervical dislocation the aorta was removed, snap frozen in liquid nitrogen and stored at -80ºC until use. In some experiments the aortic arch was separated from the longitudinal section of the thoracic aorta for separate analysis of regions of the aorta subjected to low (atherogenic) levels of LSS and high physiological levels of LSS respectively.

Cell culture and application of shear stress

Human Umbilical Vein Endothelial Cells (HUVECs; pooled donors, Lonza, UK) and Human Artery Endothelial Cells (HAEC, Lonza, UK) were cultured in EGM-2 endothelial growth

medium (Lonza, UK) at 37^{o}C , 5% CO_2 in Falcon 6-well plates. Cells at passage 4 and 80-90% confluency were used in all experiments. Steady unidirectional LSS of 10, 6, 2 or 0 dyn/cm^2 was applied, using a cone and plate viscometer as previously described[1,2]. Calculated values of LSS in large blood vessels suggest that physiological levels range from 5-20dyn/cm^2, thus for our studies cells were exposed to both 6 and 10 dyn/cm^2 to simulate physiological conditions. In order to mimic the levels of low LSS thought to be present at sites of atheroma cells were subjected to 2dyn/cm^2 or 0dyn/cm^2 (static). Evidence clearly link levels of unidirectional shear stress with alterations in endothelial phenotype supporting the use of this technique in our measurements[3,4]. Since we demonstrated that steady expression levels of B1R were achieved only after 8h and sustained up until 16h following application all analyses were conducted on cells exposed to shear stress for 16h unless stated otherwise. Since interleukin-1β (IL-1β) is the optimal inflammatory stimulus for kinin B1 receptor expression, to simulate an inflammatory stress cells were treated with IL-1β (10ng/ml, Preprotech EC, UK) for 4h (i.e at 12h following initiation of LSS) in the absence or presence of the B1R antagonist SSR240612[5] (1μM, 15 min prior to IL-1β application, kindly provided and synthesised by Jerini AG). Cells were also treated with oxidized LDL (oxLDL, 20μg/ml, 8h)[6-8] being introduced 8h into the LSS stimulus. Native LDL (nLDL) was used as control. After shear exposure and/or IL-1β/oxLDL treatment cells were rinsed twice in PBS and frozen at -80^{o}C until analysis of mRNA or protein expression (see below).

Transfected HEK-293

cDNA for the human wild-type B1 receptor cloned into pcDNA3.1 (Invitrogen) was obtained from Missouri S&T cDNA Resource Center. HEK-293 cells were transiently transfected with

JetPEI as described by the manufacter (Polyplus-transfection). Briefly 3ng of the plasmid was incubated with 6µL of JetPEI for 30min at room temperature. The mix was then added to HEK-293 cultured in 6wells-plate (200,000-300,000 cells per wells) for 24h with RPMI medium (Lonza,Uk). The cells were rinsed in PBS and frozen at -80^{o}C until analysis protein expression (see below)

Serum triglyceride and cholesterol analysis

Triglyceride and LDL cholesterol levels were determined in serum of ApoE-/- mice as described by the manufacturers (TR0100 Serum Triglyceride Determination Kit; Sigma and LDL/VLDL Cholesterol ELISA Kit (Abcam, UK).

Perfused mouse mesentery preparations

All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Male C57BL/6 mice were killed by cervical dislocation, the superior mesenteric artery cannulated and the mesentery isolated and then mounted in a 37ºC water-jacketed organ bath and perfused with warmed (37ºC), oxygenated (5% CO_2 in O_2) physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, CaCl2.2H2O 2.5, MgSO4·7H2O 1.2, NaHCO3 25, KH2PO4 1.2, glucose 5.5, containing 1% Dextran (MW=64,000-76,000). Perfusion pressure was measured through an in-line transducer (P23XL, Becton Dickinson). The mesenteric bed was perfused at either physiological (1ml/min equivalent to an LSS of 6dyn/cm^2 within the superior mesenteric artery) or low (0.5ml/min equivalent to 2dyn/cm^2) flow rate. Decreases in flow rate below

0.5ml/min had profoundly depressed vascular constrictor responses to KCL and therefore was not used for experimentation. Neither flow rate significantly altered basal vasoactive responses. Constrictor responses to KCl (125mM) in PSS (equimolar substitution for NaCl) were unaffected by flow rate (37 ± 10.5mmHg, n=6 and 43 ± 11.1mmHg, n=5 for 1ml/min and 0.5ml/min, respectively) indicating no significant alteration in smooth muscle reactivity under these conditions of flow. Flow rate did not affect basal perfusion pressure (12±1.7, n=6; and 12±4.4 mmHg, n=5 for 1ml/min and 0.5ml/min, respectively). Each preparation was perfused for 4h, snap frozen in liquid N_2, and RNA extracted for real-time quantitative PCR.

Assessment of kinin B1-receptor-induced functionality: measurement of prostaglandin and chemokine synthesis

After exposure to shear stress or IL-1β cells were treated with the selective B1 agonist: Lys-des-Arg^9-BK (LDBK, 10μM, Bachem). For prostaglandin measurement medium was collected 30 min after LDBK application, centrifuged for 20 min at 16000 rpm, 4ºC and the supernatant stored at -80ºC. Concentrations of 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2) and PGE_2 were measured using enzyme immunoassay kits (Cayman Chemical Co) according to the manufacturer's protocol. For PGE_2 measurement, prostaglandins were extracted with Sep-pak cartridges by solid phase extraction (Sep-Pak ® Vac C18, Waters Corporation) prior to assay. 6-keto-$PGF_{1\alpha}$ and PGE_2 concentrations were expressed relative to the cell protein concentration measured by Bradford assay. The effects of B1R activation on prostaglandin levels were expressed as a percentage of the level without agonist treatment for each condition. For assessment of B1-induced chemokine synthesis cells were collected, by scraping, at 4h

following treatment with Lys-DABK, snap frozen and stored at -80ºC until mRNA extraction and assessment of CXCL5 and CXCL6 mRNA expression as described below**.**

Ozone chemiluminescence for determination of nitrite

After exposure to shear stress medium was collected centrifuged for 20 min at 16000 rpm, 4ºC and the supernatant stored at -80ºC. Samples were analysed for nitrite using chemiluminescence as described previously[9]. Briefly, samples and standards containing nitrite were first reduced to NO, which was then quantified using a NO analyser (NOA 280, Sievers). Nitrite concentrations were determined by addition of samples to 1.5 % potassium iodide in glacial acetic acid under nitrogen at room temperature.

Quantitative real time PCR

Total RNA was extracted, cDNA synthesised and subjected to quantitative RT-PCR using SYBR green reagents (ABgene, UK). To quantify B1 receptor and CXCL5/CXCL6 chemokine mRNA expression, the following primers were used:

human B1 sense: 5'-ACG CCT TCA TTT TCT GCC TG-3', antisense: 5'-GCT GGC TCT GGT TGG AGG AT-3',

murine B1 sense: 5’-TGG AGT TGA ACG TTT TGG GTT T-3’, antisense: 5’-GTG AGG ATC AGC CCC ATT GT-3’,

human CXCL5 sense: 5’-GAG AGC TGC GTT GCG TTT G-3’ and antisense: 5’- TTT CCT TGT TTC CAC CGT CCA-3’,

human CXCL6 sense: 5'-GGT CCT GTC TCT GCT GTG C-3' and antisense: 5'-GGG AGG CTA CCA CTT CCA-3',

human GAPDH sense primer: 5'-CAT GTT CGT CAT GGG TGT GAA-3'; antisense primer: 5'-ATG GAC TGT GGT CAT GAG TCC TT-3',

murine β-actin: sense 5'-GAA ATC GTG CGT GAC ATC AAA G-3' and antisense 5'-TGT AGT TTC ATG GAT GCC ACA G-3'.

In HUVEC/HAEC B1 mRNA expression was normalised for each sample with respect to the corresponding (GAPDH) mRNA expression which is unaffected by alterations in fluid shear stress and in murine tissue the comparison was made to actin. The comparative Ct method of Livak and Schmittgen[10] was applied to compare gene expression levels between samples. Using the AB SDS2.1™ system software, the amplification threshold cycle values (CT) were obtained. The data were analysed using the equation $2^{-\Delta\Delta Ct}$ where ΔΔCt=(CtB1 or CXCL5/6-CtGAPDH)Treated-(CtB1 or CXCL5/6- CtGAPDH)Control.

Western blotting

Lysates were prepared from human carotid endarterectomy tissue or cells (HUVEC subjected to LSS and HEK-293 transfected with pcDNA3.1-B1R) and protein concentration determined as previously described[11] Lysate samples were subjected to western blotting to detect B1 receptor expression. Blots were probed with with the following antibodies: the rabbit polyclonal anti-B1 receptor antibody (K21N[12] dilutions of 1/2000 or 1/5000 in 2% milk were used for tissue or cell samples respectively). Secondary peroxidase-coupled sheep anti-rabbit

antibody (dilution 1/10000; Prepotech) or goat anti-rabbit antibody (dilution 1/2000; DakoCytomation) were used for tissue or cell samples respecively. 1/5000 of purified rabbit antiserum directed against a segment of the C-terminal sequence of the B1 receptor (termed K21N)[12]. For quantification of protein expression all blots were reprobed for β-actin or α-tubulin expression using rabbit anti-β-actin (Sigma-Aldrich, UK, 1/500 dilution) or 1/5000 dilution of mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich, UK) for tissue or cell samples respectively. The specificity of the B1R band was determined in pcDNA3.1-B1R transfected HEK-293 cells and HUVEC by pre-incubating the K21N antibody with the immunizing peptide prior to reaction with the membrane. Densitometric analysis was performed on scanned images (Hewlett Packard) and analyzed using TotalLab™.

Immunohistochemistry

Immunohistochemistry for B1R was performed on 3-µm-thick paraffin-embedded sections of aortic arch from ApoE-/- mice. Following rehydration, antigen was unmasked for 45 minutes at 95°C using Dako Target retrieval solution (pH 6; Dako). Endogenous peroxidase was blocked for 10 minutes with Dako peroxidase blocking reagent, and nonspecific binding was blocked for 20 minutes with Dako protein block. The primary antibody anti-B1R (K21N, 1/250 dilution) was added and incubated for 1 hour at room temperature. Following 3 washes with Tris buffered saline, slides were incubated with biotinylated anti-rabbit IgG (1/10000 dilution; Dako) was used as a secondary antibody for 60 minutes at room temperature. Omission of primary antibody and staining with isotype-matched control immunoglobulins served as negative control. After 3 washes with PBS-Tween, we treated individual sections with horseradish

peroxidase-labeled streptavidin (Dako) for 30 minutes, washed the sections 3 times with PBS-Tween, and determined peroxidase activity with 3,3-diaminobenzidine tetrahydrochloride (Dako). The slides were lightly counterstained with hematoxylin before dehydration and mounting in DePex (VWR International,Uk).

Radioligand binding assay

Radioligand binding was performed on whole cells exposed to 0-2 dyn/cm^2 or 6-10dyn/cm^2 (for 8h) treated or not with IL-1β (10ng/mL, 4h). In each experiment, total binding was determined by adding B1 agonist [^{3}H]-LDBK at 0.75nM and non-specific binding was performed by co-treatment with LDBK in excess (10µM, 1h) on ice. Following two washes with ice-cold TRIS buffer, cells were dissolved with 0.3N NaOH and the radioactivity determined by liquid β-scintillation count (1900TR, Packard). All measurements were conducted in triplicate in each experiment i.e. 3 separate wells, each containing 350,000 cells for each n. Specific binding was calculated by subtracting the non-specific binding from the total binding and expressed as fold change compare to cells subjected to physiological LSS (6-10dyn/cm^2). The specificity of [^{3}H]-LDBK binding was confirmed by constructing a competitive binding curve with increasing concentration of cold ligand, LDBK.

Statistical analysis

Values are given as means ± SEM where n represents the number of animals or the number of experiments conducted for cells. Statistical comparisons were conducted using paired or unpaired Student's t test for 2 groups or one way ANOVA for more than 2 groups. Differences

were considered significant when $p<0.05$. All statistics were calculated using Graph Pad Prism™ 4.1.

SUPPLEMENTAL FIGURE

Figure SI. Representative human carotid endarterectomy tissue divided in two sections: with (+) or without (-) plaque.

Figure SII. ApoE$^{-/-}$ mice fed either a high fat or normal chow diet for 0, 3, 6 and 12 weeks. B1 receptor mRNA expression was measured by qPCR in whole aorta (A) and (B) serum triglyceride levels were measured in serum. Comparison of (C) triglyceride and (D) LDL cholesterol levels in serum after 12 weeks normal chow or high fat diet fed ApoE-/- mice. . Data are mean ± SEM for n=9, ** P<0.01, * P<0.05 chow diet (Chow) vs fat diet (Fat).

Figure SIII. HUVECs subjected to low LSS (0 or 2 dyn/cm^2) or physiological levels of LSS (6 or 10 dyn/cm^2) for 8h. (A) Representative image of cell alignment and (B) levels of NO synthesis measured by chemiluminesence for nitrite in endothelial culture medium following exposure to low or high LSS. Data are mean ± SEM for n=6, ***P<0.001.

Figure SIV. (A) Time dependency of B1 receptor expression in response to a physiological LSS stimulus. HUVECs were subjected to physiological levels of LSS (6 dyn/cm^2) for 2, 4, 8 and 16h, (B) B1 receptor protein expression was assessed by western-blotting in HEK-293 transfected with pcDNA3-B1R and HUVEC subjected to antibody preadsorbed with (+) or without (-) the B1R peptide against which the antibody was raised. (C) Typical B1 receptor protein expression as assessed by western-blotting and (D) B1 receptor binding was measured with [^{3}H]-LDBK in cells subjected stimulated with IL-1β (10ng/mL, 4h). Data are mean ± SEM for n=6.

Figure SV. HUVECs were subjected to physiological (6-10 dyn/cm^2) or low LSS (0-2 dyn/cm^2) for 12h and (A) COX-1 and (B) COX-2 expression determined by western blotting. Data are mean ± SEM for n=6, protein expression was normalized to α-tubulin.

Figure SVI. (A) HUVECs were subjected to low LSS (0-2 dyn/cm^2) for 12h and stimulated with IL-1β (10ng/mL) for different times (0, 1, 2, 4, 8 and 16h). HUVECs were subjected to varying LSS (0 to 10 dyn/cm^2) for 12h, then stimulated or not with IL-1β (10ng/mL) and (B) B1 receptor protein expression or (C) radioligand binding determined. Protein expression was normalized to α-tubulin. Data are mean ± SEM for n=6. * P<0.01, ** P<0.05, 6-10 dyn/cm^2 versus 0-2 dyn/cm^2 values or control versus treated value.

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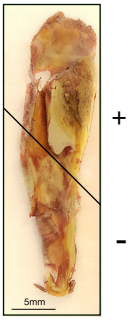

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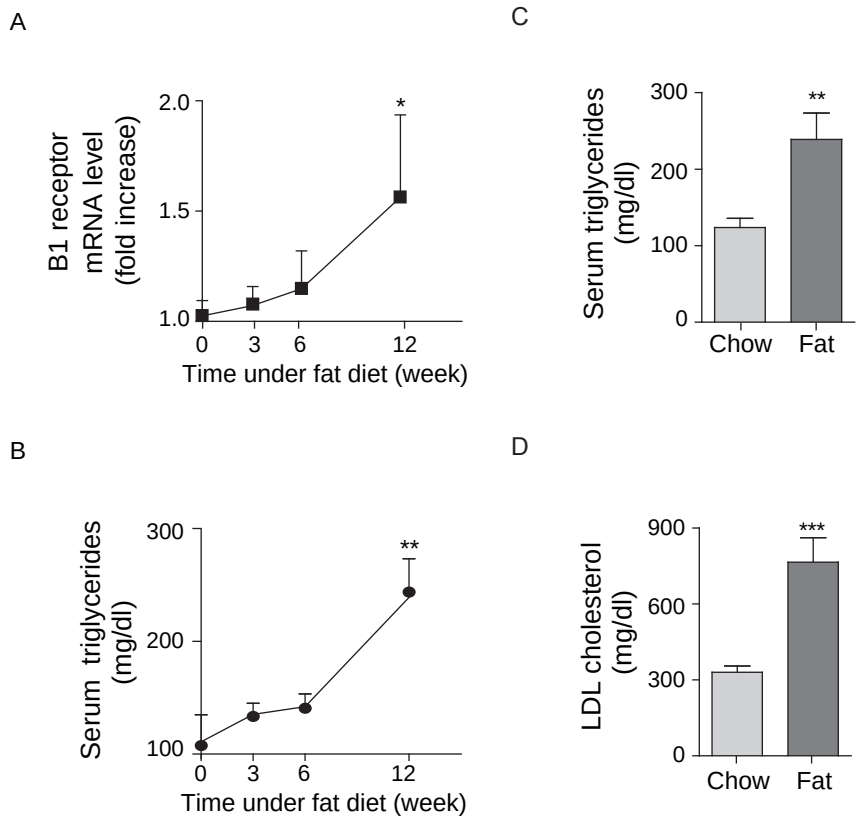

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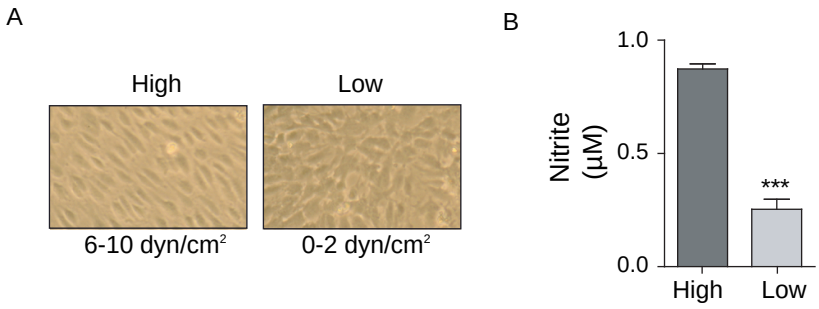
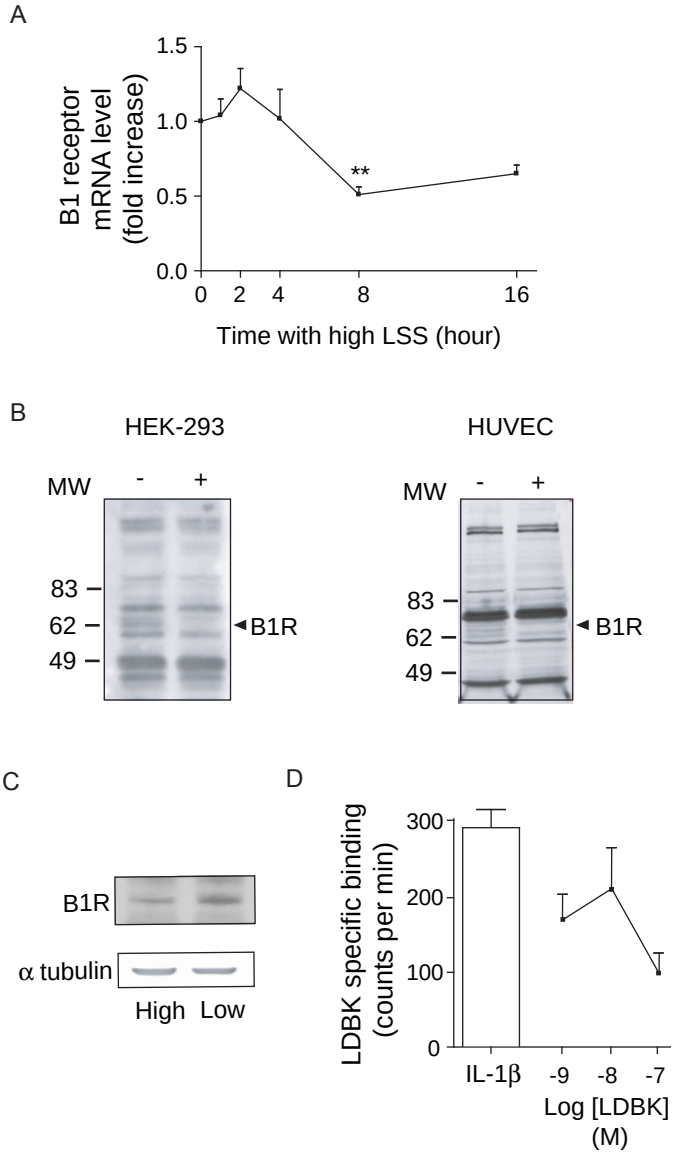
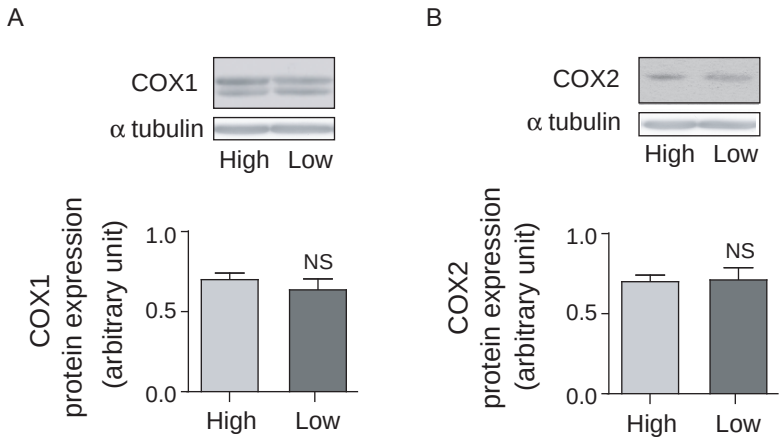


FigIV Duchene J *et al*



FigV Duchene J *et al*



FigVI Duchene J *et al*