Supplementary Material to Bajari, Winnicki, et al. “Known players, new interplay in atherogenesis: Chronic shear stress and carbamylated-LDL induce and modulate expression of atherogenic LR11 in human coronary artery endothelium” (Thromb Haemost 2014; 111.3)

Supplementary Methods

Antibodies

Monoclonal antibodies (mAb) against human LR11 (BD Transduction Laboratories™, Franklin Lakes, NJ, USA), and human β-actin was used from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal rabbit anti-p38MAPK, anti-IkB-α, and anti-phospho-p38MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and anti-NFkB-p65 and p-ERK from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). As second antibodies a horseradish peroxidase (HRP) coupled goat anti-rabbit-IgG, and HRP-coupled goat anti-mouse-IgG were used (Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry

Paraffin specimens from explanted human hearts were retrieved from our archives. After deparaffinization in xylene and rehydration, endogenous peroxidase activity was blocked by hydrogen peroxide followed by treatment with Tris/EDTA buffer (pH 9.0) at 1 bar and 121°C for 5 min. UltraV block (UltraVision Kit, Thermo Fisher Scientific, Fremont, CA, USA) was used for 5 min followed by incubation with anti-LR11 mAb (1:300) for 60 min. Visualization was performed by HRP polymer UltraVision Kit (Thermo Fisher Scientific, Fremont, CA, USA) and 3-Amino-9-Ethylcarbazole substrate. Human brain samples were used as positive controls. All histological samples were fully anonymized according to the Bioethics Committee guidelines of The Medical University of Vienna.

Preparation of LDL

Native LDL was isolated from human blood plasma as described previously (1). LDL
carbamylation was performed with cyanate according to Ok et al (2).

Native and cLDL samples were analyzed by agarose gel electrophoresis. Relative electrophoretic mobility (REM) of untreated LDL (REM = 1) increased to 4.35 +/- 0.1 by carbamylation.

Based on previously shown features, copper oxidation was the method of choice for LDL oxidation (3). Two hundred to 500 µg/mL LDL was incubated with copper ions (60 µM/mg protein) at 37°C in PBS pH 7.4 for 4h (4). The oxidation reaction was stopped by addition of EDTA (120 µM/mg protein) and the preparation was subjected to gel chromatography to eliminate copper/EDTA. The resulted product was a heavily oxidized LDL as was shown by relative electrophoretic mobility (REM) on 1.2% agarose gels. REM ox-LDL versus untreated LDL (REM 1) was 1.5 +/- 0.1 (n =3).

**Chronic shear stress experiments**

Cartridges containing 20 permeable polysulfone hollow fibers as artificial capillaries and the pulsatile pump unit “Quad” were from FiberCell Systems (Frederick, MD, USA) (Supplemental Figure 1). Shear stress was regulated by pressure and volume of each pulse. Six to 8 x 10⁶ primary HCAEC (passages 3-4, Promocell, Heidelberg, Germany) were seeded onto the fibronectin (2.5 µg/cm², Sigma-Aldrich) pre-coated inner surface of capillaries following the manufacturer’s instructions. After 8 hours with no or low linear flow (0.5 mL/h), cartridges were subjected to a shear stress of 2.5 dynes/cm² for 2 days. This level was kept for chronic low shear stress (LSS) cartridges during the entire experiment, while it was raised to 25 dynes/cm² in chronic high shear stress (HSS) cartridges starting at the 3rd day (Supplemental Figure 2). Starting day 8, cells were kept in serum free endothelial cells medium (PAA, Vienna, Austria). On day 9, nLDL or cLDL (25 µg/mL) was added to the medium for 24 hours. For p38MAPK phosphorylation inhibition 2.5 µmol/L of the specific p38MAPK phosphorylation inhibitor SB202190 (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium for 4 hours prior to cell elution. Medium was changed in 2 day intervals, and discarded medium was frozen at -80°C for further detection of soluble LR11 (see
Immunoblotting) after lactate measurement using a calibrated blood gas apparatus (Drott ABL 800 Flex, Wr. Neudorf, Austria). Toxicity of nLDL, cLDL or SB202190 was tested using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Mannheim, Germany). Successful culture of endothelial monolayers on the inner capillary surface was shown in H&E stained sections from fibers of an additional cartridge for each setting (Supplemental Figure 3).

**Quantitative Real-Time PCR**

An ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used. Reactions (10 µL) were performed using the TaqMan Fast-Universal Master Mix and FAM-labeled probe for LR11 obtained from Assays On Demand (Applied Biosystems, Foster City, CA, USA) after cDNA was generated from HCAEC using the FastLane Cell cDNA kit (QIAGEN, Hilden, Germany). Beta-actin was used as endogenous control gene (Applied Biosystems, Foster City, CA, USA). All qRT-PCR were performed for 40 cycles (3 sec. at 95°C, 30 sec. at 60°C). Expression of LR11 was calculated according to the 2^ΔΔCt method (5).

**Immunoblotting**

Western blotting was performed according to standard protocols (6). Twenty µg HCAEC lysate/lane were subjected to 5% (LR11), 10% SDS-PAGE (p38MAPK and phosphorylated p38MAPK) or 12% SDS-PAGE (I kappa B, nuclear factor kappa B (NF-kB) and phosphorylated extracellular signal-regulated kinase (p-ERK) under reducing conditions. For blotting of sLR11, 100 µL of cell culture medium from HCAEC (HSS, LSS or static conditions) was incubated with receptor associated protein-glutathione-S-transferase affinity beads overnight at 4°C before equal amounts of beads-bound material were subjected to SDS-PAGE. Bound secondary antibodies were visualized by an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech, Vienna, Austria) and band intensities were analyzed using ImageJ software (NIH, Bethesda, MD, USA). For quantification band intensities were normalized to β actin in Figure 2 B in the manuscript.
**Wound healing assay**

Wound healing assay was performed by using VSMC (passages 3-4, Promocell, Heidelberg, Germany) cultured in “Smooth Muscle Cells Medium” (Promocell, Heidelberg, Germany), and the CytoSelect 24-well Wound-Healing Assay Kit (Cell BioLabs Inc. San Diego, CA, USA) according to the manufacturer’s protocol. Influence on VSMC migration of concentrated conditioned medium (1:20) (7) from HCAEC cultured with nLDL and HSS versus cLDL and LSS was compared. Fetal bovine serum (FBS, 1%), basic fibroblast growth factor (bFGF, 2 ng/mL) and epidermal growth factor (EGF, 0.5 ng/mL) with their known pro-migratory or stabilizing effect on VSMC (8, 9) were used as controls. Wound areas on images at 11 and 21 hours were quantified by using Java's ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA). For blocking experiments, concentrated conditioned medium from each setting was pre-incubated with anti-LR11 mAb (1:10) for 1 hour at 37°C.

**Vascular smooth muscle cell migration assay**

A 24-well double chamber assay was employed (Corning Inc., NY, USA). Polystyrene membrane inserts with pores of 8 µm were chosen due to the better cell visibility. In short, vascular smooth muscle cells were starved for 24 hours prior the experiment. 2x10⁴ cells were then transferred into the migration inserts which were precoated with fibronectin (2.5 µg/cm²). Platelet-derived growth factor (PDGF) at a final concentration of 5 ng/mL was added into the receiver wells as a chemoattractant, while VSMC in the upper chamber were incubated with the appropriate conditioned medium (as in the wound healing assay) in the presence or absence of anti-LR11. After a migration period of 6 hours in the incubator (37°C; 5% CO2) the non-migrated cells were discarded and each insert was stained with crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes and left at room temperature for drying thereafter. The average number of cells from 5 randomly chosen high-power (×400) fields on the lower surface of the filter was counted and the total number of migrated cells was calculated. Results are shown in Supplemental Table 1.
Vascular smooth muscle cell proliferation assay

The non-radioactive EZ4U cell proliferation assay (Biomedica, Vienna, Austria) was performed according to the manufacturer’s instructions. In short, 1500 VSCMC were seeded in 96-well plates and incubated in smooth muscle cell medium (Promocell, Heidelberg, Germany) containing 1% FBS for 24 h. The cells were then treated with fresh 1% FBS versus with conditioned medium from HCAEC grown under LSS with cLDL or HSS with nLDL for 16 hours. The VSMC medium in the wells was then replaced with 200 µL of the substrate (1:10 dilution of tetrazolium). PDGF-AB at the concentration of 100 ng/mL served as positive control. After an incubation period of 3 hours at 37°C allowing the conversion of tetrazolium salts into formazan derivates by living cells, the absorbance at 450 nm and 620 nm (background) was measured. Cell numbers were determined by using a standard curve drawn using absorbance values from known cell numbers: 500 / 1000 / 1500 / 2000 / 2500 / 3000 / 4000 / 5000 /10000. Results are shown in Supplemental Table 1.

Statistical analysis

Each qRT-PCR reaction and wound-healing assay was performed in triplicates. Data were calculated from five independent experiments (n=5). Statistical analysis was performed using Microsoft Excel 12.2.7 and one-sample t test. Migration and proliferation experiments were performed three times in triplicates (n=3) and were analyzed by a two-sample t test using Excel Microsoft 12.2.7; p≤0.05 were considered statistically significant. All results are shown as mean ± SEM.
Suppl. Figure 1: Shear stress unit. a) Electric pump unit. b) Endothelial cell cartridge.

Arrow: Direction of flow.
Suppl. Figure 2: Profile of chronic shear stress. Human coronary endothelial cells were cultivated under very low linear flow (0.5 mL/hr) during the first 4 hours. Pulsatile shear stress at 2.5 dynes/cm² was applied thereafter. Cells were kept at this level for chronic low shear stress experiments for 10 days (continuous line). For chronic high shear stress, shear stress level was increased to 25 dynes/cm² at day 3 and was kept for 7 days (dashed line) until cell harvest at day 10.
Suppl. Figure 3: Cross-section of H&E stained fiber after 10 days of shear stress. a) Polysulfone fiber. b) Endothelial cell monolayer. c) Lumen of the fiber (photographed at a 50x magnification).
Table 1: Results from vascular smooth muscle cell migration and proliferation assays. bFGF, basic fibroblast growth factor; cLDL, carbamylated LDL; EGF, epidermal growth factor; FBS, fetal bovine serum; HSS, high shear stress; LSS, low shear stress; nLDL, native LDL. Statistically significant when p<0.05.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Migration Mean ± SEM [number of cells]</th>
<th>Proliferation Mean ± SEM [number of cells]</th>
<th>p-value</th>
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<tbody>
<tr>
<td>1% FBS</td>
<td>2137.67 ± 333.10</td>
<td>1894.00 ± 49.52</td>
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<tr>
<td>1% FBS + antiLR11</td>
<td>1970.00 ± 406.37</td>
<td>1980.00 ± 50.69</td>
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<td>nLDL / HSS</td>
<td>2517.67 ± 519.38</td>
<td>1852.33 ± 119.17</td>
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<td>nLDL / HSS + antiLR11</td>
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<td>1962.00 ± 62.17</td>
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<td>cLDL / LSS</td>
<td>7125.33 ± 195.85</td>
<td>2244.67 ± 223.80</td>
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<tr>
<td>cLDL / LSS + antiLR11</td>
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<td>EGF+bFGF</td>
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<td>EGF+bFGF+antiLR11</td>
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<td>1918.67 ± 92.67</td>
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Suppl. References


