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Low density lipoprotein induces upregulation of vasoconstrictive endothelin type B receptor expression

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Running title: Upregulation of vasoconstrictive ET_B receptors by LDL

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Abstract

Vasoconstrictive endothelin type B (ET_B) receptors promote vasospasm and ischemic cerebro- and cardiovascular diseases. The present study was designed to examine if low density lipoprotein (LDL) induces upregulation of vasoconstrictive ET_B receptor expression and if extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) signal pathways are involved in this process. Rat mesenteric artery segments were organ cultured in presence and absence of LDL with or without inhibitors for MAPK kinase 1 and 2 (MEK1/2), p38 and transcription. The upregulation of vasoconstrictive ET_B receptor expression was studied using a sensitive myograph, real-time PCR and Western blot. LDL (11, 22 and 44 mg protein/L) concentration-dependently induced upregulation of vasoconstrictive ET_B receptor expression with increase in the receptor-mediated vasoconstriction, elevated levels of the ET_B receptor mRNA and protein expressions, and activation of ERK1/2 and p38 MAPK. Blockage of ERK1/2 and p38 MAPK signal pathways using MEK1/2 inhibitors (PD98059 and U0126) or p38 inhibitors (SB203580 and SB239063) significantly abolished the LDL-induced upregulation of vasoconstrictive ET_B receptor expression. Actinomycin D (general transcriptional inhibitor) almost completely inhibited the LDL effects. In conclusion, LDL induces upregulation of vasoconstrictive ET_B receptor expression through activation of ERK1/2 and p38 MAPK signal pathways-dependent transcriptional mechanisms.

Key Words: LDL; MAPK; endothelin receptor; vasoconstriction; ischemia

Chemical compounds studied in this article

Sarafotoxin 6c (PubChem CID: 16132429); PD98059 (PubChem CID: 4713); U0126 (PubChem CID: 3006531); SB203580 (PubChem CID: 176155); SB239063 (PubChem CID: 5166); actinomycin D (PubChem CID: 2019).

1. Introduction

Vasoconstrictive endothelin type B (ET_B) receptors are pathogenic, and therefore upregulation of vasoconstrictive ET_B receptor expression induced by risk factors for cardiovascular disease may lead to vasospasm and ischemic cerebro- and cardiovascular diseases (Xu et al., 2010). Endothelin-1 (ET-1), a potent vasoconstrictor, induces strong and long-lasting vasoconstriction. It acts on two types of G protein-coupled receptors named the endothelin type A (ET_A) and type B (ET_B) receptors. In physiological condition, the ET_A receptors are expressed on vascular smooth muscle cells and mediate vasoconstriction, while the ET_B receptors mainly are located on the endothelial cells and induce vasodilatation via release of nitric oxide (NO) and prostacyclin (PGI₂). However, under pathogenic conditions, the ET_B receptors are induced to express on vascular smooth muscle cells and mediate vasoconstriction instead. Thus, there are two types of functionally distinct ET_B receptors i.e. vasorelaxant ET_B receptors located on vascular endothelial cells and vasoconstrictive ET_B receptors on vascular smooth muscle cells.

The upregulation of vasoconstrictive ET_B receptor expression with increased vasoconstriction are seen in coronary artery infarction (Wackenfors et al., 2004) and experimental ischemic stroke (Stenman et al., 2002). This increased vasoconstrictive ET_B receptor-mediated vasoconstriction are also found in the arterial segments treated with the risk factors such as cigarette smoke particles (Xu et al., 2008) or minimally modified low density lipoprotein (mmLDL) (Jie et al., 2012; Li et al., 2012). Blockage of extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK) signal pathways using Raf inhibitors has demonstrated that in cigarette smoke-exposed rats (Cao et al., 2012) and in rat

experimental subarachnoid hemorrhage model (Ansar et al., 2011), the upregulation of vasoconstrictive ET_B receptor expression is associated with activation of Raf/ERK/MAPK signal pathways. Previously, we have reported that mmLDL upregulates vasoconstrictive ET_B (Jie et al., 2012) and ET_A (Li et al., 2013a) receptors in rat coronary arterial smooth muscle cells mainly via activation of protein kinase C (PKC), ERK1/2 and the downstream nuclear factor-kappaB (NF-κB) signal pathways. However, at the same concentration (2.2 mg protein/L), low density lipoprotein (LDL) without modification has no such effects (Jie et al., 2012; Li et al., 2013a). Taken all together, this suggests although mmLDL is still recognized by LDL receptor (Berliner et al., 1990), it has different effects from LDL on regulation of vasoconstrictive ET_B and ET_A receptor expressions in the arterial smooth muscle cells. One explanation for the lack effects of LDL at concentration of 2.2 mg protein/L (Jie et al., 2012; Li et al., 2013a; Li et al., 2012) is due to far low concentration of LDL used in the experiments.

Oxidized LDL (oxLDL) levels in circulating plasma correlate with the severity of acute coronary syndromes (Ehara et al., 2001). It is believed that “fully oxLDL” does not exist in the circulation, and the presence of oxidation-specific epitopes on plasma LDL presumably represents mmLDL (Tsimikas and Witztum, 2001). There is a significantly positive association between the levels of circulating LDL and oxLDL (Calmarza et al., 2013). However, the circulating oxLDL is only about 0.25% of total LDL in patients with acute myocardial infarction (Ehara et al., 2001). For the first time, the present study was designed to investigate if LDL at concentrations of 11, 22 and 44 mg protein/L has similar effects as mmLDL (2.2 mg protein/L) on the regulation of vasoconstrictive ET_B receptor expression (Jie et al., 2012; Li et al., 2012) and if ERK1/2 and p38 MAPK signal pathways are involved in this process.

LDL is the most common and strongest cardiovascular risk factor associated with incident myocardial infarction and ischemic coronary heart disease (Isaacs et al., 2013). Many studies have demonstrated that LDL induces dysfunction of endothelium (Yu et al., 2013), causes vascular wall inflammation (Fenyo and Gafencu, 2013; Wang et al., 2013), promotes vasospasm (Miwa, 2003) and ischemic heart disease (Keenan and Rader, 2013). However, the underlying molecular mechanisms how LDL leads to vasospasm and ischemic cerebro- and cardiovascular diseases are not fully understood yet. Here, we report that LDL, through activation of ERK1/2 and p38 MAPK signal pathways-dependent transcriptional mechanisms, induces upregulation of vasoconstrictive ET_B receptor expression with increased vasoconstriction. This might contribute to LDL-associated vasospasm and ischemic cerebro- and cardiovascular diseases.

2. Materials and Methods

2.1 Chemicals and drugs

LDL from human plasma, selective ET_B receptor agonist Sarafotoxin 6c (S6c), inhibitors for MEK1/2 (PD98059 and U0126) and p38 (SB203580 and SB239063) as well as general transcriptional inhibitor actinomycin D (AcD) were obtained from Sigma (St. Louis, USA). The inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO (vehicle) used in experiments was 1 µl/mL that equals the volume of the inhibitor added to organ culture. The DMSO concentration was the same in all test conditions, and it presented in the organ culture without the inhibitors to serve as control. S6c was dissolved in

0.1% bovine serum albumin. LDL supplied as a lyophilized powder (5.0 mg protein/vial) was reconstituted with 1 ml of 150 mM NaCl, pH 7.4, and 0.01 % EDTA to prevent oxidation.

2.2 Tissue preparation and organ culture procedure

Sprague-Dawley rats (body weight 300-350 g) were anaesthetized with CO₂ and exsanguinated. The superior mesenteric artery was gently removed and freed from adhering tissue under a dissecting microscope. The endothelium was denuded by perfusion of the vessel for 10 seconds with 0.1% Triton X-100 followed by another 10 seconds with a physiologic buffer solution. The vessels were then cut into 1 mm long cylindrical segments and incubated at 37°C in humidified 5% CO₂ in air. Organ culture of the artery segments was performed in a 24-well plate, with two segments per well, each well containing 1 mL Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (584 mg/L), supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). The inhibitors (PD98059 10⁻⁵M, U0126 10⁻⁵M, SB203580 10⁻⁵M, or SB239063 10⁻⁵M) were added to the medium before the incubation. The present investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the experimental protocol was approved by the Ethic Committee of Lund University.

2.3 In-vitro pharmacology

Fresh or incubated segments were immersed in temperature-controlled (37°C) myograph individual baths (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 mL bicarbonate buffer solution (NaCl 119mM, KCl 4.6mM, NaHCO₃ 15mM, NaH₂PO₄ 1.2mM, MgCl₂ 1.2mM, CaCl₂ 1.5mM and glucose 5.5mM). The solution was continuously aerated

with 5% CO₂ in O₂, resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension with the Chart software (ADInstruments, Hastings, UK). A resting tone of 2.5 mN was applied to each segment, and the segments were allowed to stabilize at this tension for at least 1.5 hrs before exposure to a potassium-rich (60 mM K⁺) buffer solution with the same composition as the standard solution, except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for contractile capacity, and the segments were used only if potassium elicited reproducible responses over 1.0 mN. Concentration-response curves for S6c (a selective ET_B receptor agonist) were obtained by cumulative administration of the reagent.

2.4 Real-time PCR

The arteries for real-time PCR were frozen in liquid nitrogen and stored at -80 °C until the experiments were performed. Total RNA extracted from the arteries was reversely transcribed to cDNA. The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit in a GeneAmp 7300 sequence detection system (Perkin-Elmer, MA, USA). Specific primers for the rat ET_B receptor were designed as follows (GenBank accession no: NM_017333):

ET_B receptor: Forward: 5'-GATACGACAACTTCCGCTCCA-3'

Reverse: 5'-GTCCACGATGAGGACAATGAG-3'

Elongation factor-1 (EF-1) mRNA was used as a reference; the primers were designed as follows (GenBank accession no: NM_175838):

EF-1: Forward: 5'- GCAAGCCCATGTGTGTTGAA-3'

Reverse: 5'- TGATGACACCCACAGCAACTG-3'

2.5 Western blot

After organ culture for 6 hrs, the vessels were frozen in liquid nitrogen and homogenized in cell extract denaturing buffer (BioSource, USA) containing a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Sigma, USA). Protein concentration was measured with a Bio-Rad protein analysis kit and TECAN[®] infinite M200. Proteins (40 µg) were loaded and separated on 4-15% Ready Gel Precast Gels (Bio-Rad, USA), then probed with the One-Step[™] Complete Western Kit (GenScript Corporation, USA) according to the manual. Briefly, membranes were incubated with Pretreat Solution on a shaker for 5 min at room temperature and then with a primary antibody overnight at 4°C. After washing three times for 10 min, the membranes were developed with Working Solution (LumiSensor A + B) for 3 min, visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Stamford, CT, U.S.A.), and then analyzed with Image Gauge Ver. 4.0 (Fuji Photo Film Co., LTD., Japan). The antibodies for phospho-p44/42 MAP Kinase, phospho-p38 MAP Kinase, and β-actin were from Cell Signaling Technology (Beverly, MA, U.S.A.). The ET_B antibody was purchased from Abcam (Cambridge, UK). All antibodies were used at 1:1000, and the experiments were repeated 3 times.

2.6 Measurement of lipid Peroxidation

Reactive oxygen species are continuously formed in small quantities during the normal metabolism of cells and final products of lipid peroxidation are reactive aldehydes that are relatively stable (Gasparovic et al., 2013). LDL peroxidation in the arterial segments was

measured by thiobarbituric acid-reactive substances (TBARS) assay using Cayman TBARS Assay Kit (Cayman Chemical, MI, USA) following the manufacture's instruction.

2.7 Data analysis

All data are expressed as mean $\pm$ S.E.M. S6c-induced vasoconstriction are presented as a percentage of contraction induced by 60 mM K⁺. The level of ET_B receptor mRNA expression is calculated related to EF-1 in the same sample. When two sets of data were compared, the unpaired Student's t-test with Welch's correction or two-way ANOVA with Bonferroni post-test were used. One-way ANOVA with Dunnett's post-test was applied for comparisons of more than two data sets. A p-value of less than 0.05 was considered to be significant.

3. Results

3.1 LDL increased vasoconstrictive ET_B receptor-mediated contraction.

Fresh mesenteric artery ring segments isolated from rats showed none or only a negligible contractile response to S6c (<5% of K⁺-induced contraction, data not shown). Organ culture of the arterial segments with LDL for 24 hrs resulted in markedly increased maximal contractile responses to S6c in a dosage-dependent manner, compared to organ culture with vehicle control (Fig. 1). LDL at low concentration (11 mg protein/L) did not significantly increased vasoconstrictive ET_B receptor-mediated maximal contraction ($p>0.05$), which agreed well with our previous findings in rat coronary arteries that LDL 2.2 mg protein/L had no such effects (Jie et al., 2012) and ET_A (Li et al., 2013a). However, when LDL concentration was at 22 mg protein/L, it induced a significant increase in the vasoconstrictive ET_B receptor-mediated maximal contraction ($P<0.01$), and this increase was further enhanced by LDL at concentration

44 mg protein/L ($p < 0.001$). The K^+ -induced contraction did not differ between the groups, and incubation with vehicle (150 mM NaCl, pH 7.4, and 0.01 % EDTA) in the concentration used did not affect the contractile response to S6c.

3.2 LDL induced upregulation of protein expression for vasoconstrictive ET_B receptors via activation of ERK1/2 and p38 MAPK signal pathways.

Compared to vehicle, LDL (44 mg protein/L) increased phosphorylation (activation) of ERK1/2 protein ($p < 0.01$) and phosphorylated p38 protein (tendency) (Fig. 2A-C) measured with Western blot. The activation of ERK1/2 and p38 MAPK by LDL was parallel with a significant upregulation of protein expression for vasoconstrictive ET_B receptors (Fig. 2A and D, $p < 0.05$). To link activation of ERK1/2 and p38 MAPK signal pathways to the upregulation of vasoconstrictive ET_B receptors, specific inhibitors for MEK1/2 (PD98059 and U0126) and p38 (SB203580 and SB239063) at concentration 10^{-5} M were used for blocking ERK1/2 and p38 MAPK signal pathways. The used concentration of the inhibitors was chosen, according to our previous study that proved the inhibitors at the concentration were specific in our system (Xu et al., 2008). Blockage of ERK1/2 signal pathways using the MEK1/2 inhibitors (PD98059 and U0126) significantly decreased the LDL-induced upregulation of vasoconstrictive ET_B receptor protein expression (Fig. 2A and D, $p < 0.01$), and inhibition of p38 signal pathway using p38 inhibitors (SB203580 and SB239063) had similarly decreased effects (Fig. 2A and D, $p < 0.01$), suggesting that LDL induced upregulation of vasoconstrictive ET_B receptors via activation of ERK1/2 and p38 MAPK signal pathways. In addition, the transcriptional inhibitor (actinomycin D) significantly abolished the LDL induced upregulation of vasoconstrictive ET_B

receptor protein expression, indicating a transcriptional mechanism involved (Fig. 2A and D, $p < 0.01$).

3.3 LDL induced transcriptional upregulation of vasoconstrictive ET_B receptor mRNA expression.

To further study the transcriptional mechanism, mRNA expression for vasoconstrictive ET_B receptors was examined using real-time quantitative PCR. The results showed that LDL (44 mg protein/L) significantly increased mRNA expression for vasoconstrictive ET_B receptors (Fig. 3, $p < 0.001$). PD98059 inhibited the LDL-increased mRNA expression for vasoconstrictive ET_B receptors, but it did not reach statistical significance (Fig. 3, $P > 0.05$), while U0126, SB203580, SB239063 or actinomycin D significantly inhibited the LDL-increased mRNA expression for vasoconstrictive ET_B receptors (Fig. 3, $p < 0.001$). Thus, inhibition of transcription by general transcriptional inhibitor actinomycin D and blockage of ERK1/2 and p38 signal pathways using MEK1/2 inhibitor (U0126) and p38 inhibitors (SB203580, SB239063) significantly decreased the LDL-induced increase in mRNA expression for vasoconstrictive ET_B receptors, suggesting that LDL induced *de novo* transcription of vasoconstrictive ET_B receptor mRNA.

3.4 Inhibition of transcription and blockage of ERK1/2 and p38 signal pathways significantly abolished the LDL-increased vasoconstrictive ET_B receptor-mediated contraction.

Blockage of ERK1/2 signal pathways using the MEK1/2 inhibitors (PD98059 and U0126) resulted in a significant reduction of the LDL-increased contractile responses to S6c

particularly, U0126 almost completely abolished the LDL effects (Fig. 4A, $p<0.001$). The p38 inhibitors (SB203580 and SB239063) (Fig. 4B) and the general transcriptional inhibitor actinomycin D (Fig. 4C) had similar inhibitory effects as the MEK1/2 inhibitors ($p<0.001$). Taken all these results together, this demonstrated that inhibition of transcription and blockage of ERK1/2 and p38 signal pathways significantly abolished the LDL-increased vasoconstrictive ET_B receptor-mediated contraction, suggesting that LDL induced activation of ERK1/2 and p38 signal pathways-dependent transcriptional mechanisms, which subsequently resulted in *de novo* transcription and translation of vasoconstrictive ET_B receptors with increase in the receptor-mediated contraction.

3.5 LDL oxidation in the arterial wall.

Finally, LDL oxidation in the arterial segments was measured using TBARS assay to demonstrate that LDL was at least partly oxidized, during incubation with the arterial segments. The results showed there was a significant increase in malondialdehyde (MDA) levels in the arterial segments incubated with LDL 22 mg protein/L for 24 hrs, compared to the arterial segments incubated with vehicle (vehicle vs. LDL mean $\pm$ S.E.M.: 1.04 ± 0.19 vs. $1.86 \pm 0.15 \mu\text{M}$, $n=5-6$ segments, student *t*-test, $p<0.01$). However, MDA levels in cultured medium were no differences between vehicle and LDL groups at 24 hrs of incubation (Vehicle vs. LDL: mean $\pm$ S.E.M.: 0.94 ± 0.09 vs. 0.97 ± 0.10 , $n=5$ wells, student *t*-test, $p>0.05$). This suggests that the presence of EDTA in the vehicle for LDL prevents LDL oxidation in the culture medium outside the arterial segments.

4. Discussion

Ischemic heart disease is a leading cause of death worldwide and acute coronary ischemia is responsible for the largest number (Santos *et al.*, 2013). Elevated circulating LDL levels increase risk for ischemic cerebro- and cardiovascular diseases. Moreover, lipid-lowering therapy using statins has been proven to significantly reduce ischemic cardiovascular events and total mortality (Puri *et al.*, 2013). LDL is known to participate in the development of atherosclerotic lesions through the formation of macrophage-derived foam cells and/or through pro-inflammatory effect on vascular cells (Itabe and Ueda, 2007). However, how LDL promotes vasospasm and ischemic cerebro- and cardiovascular diseases is not fully understood yet. The present study, for the first time, has demonstrated that LDL induces upregulation of vasoconstrictive ET_B receptor expression with increased the receptor-mediated vasoconstriction. The LDL-increased vasoconstrictive ET_B receptor-mediated contraction occurs through activation of ERK1/2 and p38 MAPK signal pathways-dependent transcriptional mechanisms, which induce *de novo* transcription and translation of vasoconstrictive ET_B receptors with increase in the receptor-mediated contraction.

OxLDL acts via binding to a number of scavenger receptors, while mmLDL and LDL are recognized by LDL receptors. Interestingly, mmLDL may act on Toll-like receptor 4 and induce activation of inflammatory signaling pathways (Miller *et al.*, 2012). Besides, LDL can be modified by endothelial cells to oxidized form via hydroxyl radicals (Satoh and Tokunaga, 2002). Our previous studies have demonstrated that mmLDL at concentration 2.2 mg protein/L upregulates vasoconstrictive ET_B (Jie *et al.*, 2012) and ET_A receptors (Li *et al.*, 2013a) in rat coronary artery via activation of PKC, ERK1/2 and the downstream transcriptional factor NF-

κ B signal pathways. Here, we show that LDL at concentrations of 22 mg protein/L and 44 mg protein/L, but not 11 mg protein/L, induces a significant increase in the maximal vasoconstriction mediated by ET_B receptors. Interestingly, compared to the arterial segments incubated with vehicle, the levels of MDA in the arterial segments incubated with LDL are significantly elevated, suggesting that during the incubation, LDL has entered into the arterial wall where it is at least in part oxidized by the arterial cells. This is well in line with our previous findings that mmLDL induces upregulation of vasoconstrictive ET_B receptors via activation of MAPK signal pathways (Jie et al., 2012; Li et al., 2012).

Transcriptional factor NF- κ B is one of key regulators for gene expression. Excess or inappropriate activation of NF- κ B has been suggested to play an important role in human inflammatory diseases, including atherosclerosis (Madonna and De Caterina, 2012). In addition, NF- κ B serves as one of the down-stream transcriptional factors for MAPK signal pathways to induce *de novo* transcription of vasoconstrictive ET_B receptors in the arterial segments exposed to smoke particles (Xu et al., 2008). Similarly, organ culture of the arterial segments *per se* also induces activation of NF- κ B-dependent upregulation of vasoconstrictive ET_B receptor expression with increase in the receptor-mediated vasoconstriction (Zheng et al., 2010).

Recently, we have demonstrated that NF- κ B is involved in the mmLDL-induced upregulation of vasoconstrictive ET_B receptor expression (Jie et al., 2012; Li et al., 2012). In agreement with this, the present study shows that the general transcription inhibitor actinomycin D significantly inhibits the LDL-increased vasoconstrictive ET_B receptor expression, suggesting that a transcriptional mechanism is involved. However, in rat coronary artery, mmLDL induces upregulation of vasoconstrictive ET_B (Jie et al., 2012) and ET_A (Li et al., 2013a) receptor

expressions via activation ERK1/2 MAPK-dependent NF- κ B signal pathways, but not p38 MAPK. This difference is most likely due to different arteries used i.e. rat mesenteric arteries are used in the present study, while rat coronary arteries have been studied previously (Jie et al., 2012; Li et al., 2013a).

ET-1 overexpression exacerbates high-fat diet-induced atherosclerosis in apolipoprotein E knockout mice (Li et al., 2013b) and in patients with atherosclerosis (Fan et al., 2000; Iwasa et al., 1999). More interestingly, there is induced upregulation of vasoconstrictive ET_B receptor expression on porcine coronary artery smooth muscle cells in diet-induced hypercholesterolemia (Hasdai et al., 1997), in smoke exposed rats (Cao et al., 2012) and in hypertension (Li et al., 2007), ischemic cardiovascular disease (Wackenfors et al., 2004), ischemic stroke (Povlsen et al., 2012) as well as in suspected acute coronary syndrome in patients (Dimitrijevic et al., 2009). Both ET-1 overexpression and vasoconstrictive ET_B receptor upregulation contribute to vasospasms and decrease in local blood flow (Beg et al., 2006). Despite extensive knowledge of the physiology and pharmacology of vasoactive factors such as vasoconstrictors (ET-1 and thromboxane) and vasodilators (endothelium derived hyperpolarizing factor, NO and PGI₂), there is still plenty to find out about the ways in which synthesis or action of these mediators are selectively impaired in disease states; there is also still incomplete understanding of the nature of other endothelium-derived dilators and constrictors (Pearson, 2013).

In conclusion, LDL, the most common and strongest cardiovascular risk factor, induces ERK1/2 and p38 MAPK signal pathways-dependent *de novo* transcription and translation of

vasoconstrictive ET_B receptors, which might be relevant to vasospasm and ischemic cerebro- and cardiovascular diseases. Identifying the intracellular signal mechanisms that mediate the upregulation of vasoconstrictive ET_B receptor expression induced by risk factors like LDL may provide new therapeutic targets for the treatment of vasospasms and ischemic cerebro- and cardiovascular diseases.

Conflict of interest

The authors declare no conflict of interest.

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References

- Ansar, S., Maddahi, A., Edvinsson, L., 2011. Inhibition of cerebrovascular raf activation attenuates cerebral blood flow and prevents upregulation of contractile receptors after subarachnoid hemorrhage. *BMC Neurosci* 12, 107.
- Beg, S.A., Hansen-Schwartz, J.A., Vikman, P.J., Xu, C.B., Edvinsson, L.I., 2006. ERK1/2 inhibition attenuates cerebral blood flow reduction and abolishes ET(B) and 5-HT(1B) receptor upregulation after subarachnoid hemorrhage in rat. *J Cereb Blood Flow Metab* 26, 846-856.
- Berliner, J.A., Territo, M.C., Sevanian, A., Ramin, S., Kim, J.A., Bamshad, B., Esterson, M., Fogelman, A.M., 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 85, 1260-1266.
- Calmarza, P., Trejo, J.M., Lapresta, C., Lopez, P., 2013. LDL Oxidation and Its Association With Carotid Artery Intima-Media Thickness and Other Cardiovascular Risk Factors in a Sample of Spanish General Population. *Angiology*. May 9, DOI:0003319713488639
- Cao, L., Zhang, Y., Cao, Y.X., Edvinsson, L., Xu, C.B., 2012. Cigarette smoke upregulates rat coronary artery endothelin receptors in vivo. *PLoS One* 7, e33008.
- Dimitrijevic, I., Ekelund, U., Edvinsson, M.L., Edvinsson, L., 2009. Increased expression of endothelin ET(B) and angiotensin AT(1) receptors in peripheral resistance arteries of patients with suspected acute coronary syndrome. *Heart Vessels* 24, 393-398.
- Ehara, S., Ueda, M., Naruko, T., Haze, K., Itoh, A., Otsuka, M., Komatsu, R., Matsuo, T., Itabe, H., Takano, T., Tsukamoto, Y., Yoshiyama, M., Takeuchi, K., Yoshikawa, J., Becker, A.E., 2001. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation* 103, 1955-1960.
- Fan, J., Unoki, H., Iwasa, S., Watanabe, T., 2000. Role of endothelin-1 in atherosclerosis. *Ann N Y Acad Sci* 902, 84-93; discussion 93-84.
- Fenyo, I.M., Gafencu, A.V., 2013. The involvement of the monocytes/macrophages in chronic inflammation associated with atherosclerosis. *Immunobiology* 218, 1376-1384.
- Gasparovic, A.C., Jaganjac, M., Mihaljevic, B., Sunjic, S.B., Zarkovic, N., 2013. Assays for the measurement of lipid peroxidation. *Methods Mol Biol* 965, 283-296.
- Hasdai, D., Mathew, V., Schwartz, R.S., Smith, L.A., Holmes, D.R., Jr., Katusic, Z.S., Lerman, A., 1997. Enhanced endothelin-B-receptor-mediated vasoconstriction of small porcine coronary arteries in diet-induced hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 17, 2737-2743.
- Isaacs, A., Willems, S.M., Bos, D., Dehghan, A., Hofman, A., Ikram, M.A., Uitterlinden, A.G., Oostra, B.A., Franco, O.H., Witteman, J.C., van Duijn, C.M., 2013. Risk Scores of Common

Genetic Variants for Lipid Levels Influence Atherosclerosis and Incident Coronary Heart Disease. *Arterioscler Thromb Vasc Biol*.

Itabe, H., Ueda, M., 2007. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *J Atheroscler Thromb* 14, 1-11.

Iwasa, S., Fan, J., Shimokama, T., Nagata, M., Watanabe, T., 1999. Increased immunoreactivity of endothelin-1 and endothelin B receptor in human atherosclerotic lesions. A possible role in atherogenesis. *Atherosclerosis* 146, 93-100.

Jie, L., Yong-Xiao, C., Zu-Yi, Y., Cang-Bao, X., 2012. Minimally modified LDL upregulates endothelin type B receptors in rat coronary artery via ERK1/2 MAPK and NF-kappaB pathways. *Biochim Biophys Acta* 1821, 582-589.

Keenan, T.E., Rader, D.J., 2013. Genetics of lipid traits and relationship to coronary artery disease. *Curr Cardiol Rep* 15, 396.

Li, J., Cao, L., Xu, C.B., Wang, J.J., Cao, Y.X., 2013a. Minimally modified LDL upregulates endothelin type A receptors in rat coronary arterial smooth muscle cells. *Mediators Inflamm* 2013, 656570.

Li, J., Cao, Y.X., Liu, H., Xu, C.B., 2007. Enhanced G-protein coupled receptors-mediated contraction and reduced endothelium-dependent relaxation in hypertension. *Eur J Pharmacol* 557, 186-194.

Li, J., Cao, Y.X., Liu, Y., Xu, C.B., 2012. Minimally modified LDL upregulates endothelin type B receptors in rat basilar artery. *Microvasc Res* 83, 178-184.

Li, M.W., Mian, M.O., Barhoumi, T., Rehman, A., Mann, K., Paradis, P., Schiffrin, E.L., 2013b. Endothelin-1 Overexpression Exacerbates Atherosclerosis and Induces Aortic Aneurysms in Apolipoprotein E Knockout Mice. *Arterioscler Thromb Vasc Biol*.

Madonna, R., De Caterina, R., 2012. Relevance of new drug discovery to reduce NF-kappaB activation in cardiovascular disease. *Vascul Pharmacol* 57, 41-47.

Miller, Y.I., Choi, S.H., Wiesner, P., Bae, Y.S., 2012. The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL. *Br J Pharmacol* 167, 990-999.

Miwa, K., 2003. Low density lipoprotein particles are small in patients with coronary vasospasm. *Int J Cardiol* 87, 193-201.

Pearson, J.D., 2013. The endothelium in vascular pharmacology--an overview of 2011-2012. *Vascul Pharmacol* 58, 335-336.

Povlsen, G.K., Waldsee, R., Ahnstedt, H., Kristiansen, K.A., Johansen, F.F., Edvinsson, L., 2012. In vivo experimental stroke and in vitro organ culture induce similar changes in

vasoconstrictor receptors and intracellular calcium handling in rat cerebral arteries. *Exp Brain Res* 219, 507-520.

Puri, R., Nissen, S.E., Ballantyne, C.M., Barter, P.J., Chapman, M.J., Erbel, R., Libby, P., Raichlen, J.S., St John, J., Wolski, K., Uno, K., Kataoka, Y., Nicholls, S.J., 2013. Factors underlying regression of coronary atheroma with potent statin therapy. *Eur Heart J* 34, 1818-1825.

Santos, E.S., Aguiar Filho, L.D., Fonseca, D.M., Londero, H.J., Xavier, R.M., Pereira, M.P., Minuzzo, L., Souza, R.D., Timerman, A., 2013. Correlation of risk scores with coronary anatomy in non-ST-elevation acute coronary syndrome. *Arq Bras Cardiol* 100, 511-517.

Satoh, T., Tokunaga, O., 2002. Intracellular oxidative modification of low density lipoprotein by endothelial cells. *Virchows Arch* 440, 410-417.

Stenman, E., Malmsjo, M., Uddman, E., Gido, G., Wieloch, T., Edvinsson, L., 2002. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. *Stroke* 33, 2311-2316.

Tsimikas, S., Witztum, J.L., 2001. Measuring circulating oxidized low-density lipoprotein to evaluate coronary risk. *Circulation* 103, 1930-1932.

Wackenfors, A., Emilson, M., Ingemansson, R., Hortobagyi, T., Szok, D., Tajti, J., Vecsei, L., Edvinsson, L., Malmsjo, M., 2004. Ischemic heart disease induces upregulation of endothelin receptor mRNA in human coronary arteries. *Eur J Pharmacol* 484, 103-109.

Wang, X.H., Wang, F., You, S.J., Cao, Y.J., Cao, L.D., Han, Q., Liu, C.F., Hu, L.F., 2013. Dysregulation of cystathionine gamma-lyase (CSE)/hydrogen sulfide pathway contributes to ox-LDL-induced inflammation in macrophage. *Cell Signal* 25, 2255-2262.

Xu, C.B., Sun, Y., Edvinsson, L., 2010. Cardiovascular risk factors regulate the expression of vascular endothelin receptors. *Pharmacol Ther* 127, 148-155.

Xu, C.B., Zheng, J.P., Zhang, W., Zhang, Y., Edvinsson, L., 2008. Lipid-soluble smoke particles upregulate vascular smooth muscle ETB receptors via activation of mitogen-activating protein kinases and NF-kappaB pathways. *Toxicol Sci* 106, 546-555.

Yu, J., Han, J.L., He, L.Y., Feng, X.H., Li, W.H., Mao, J.M., Gao, W., Wang, G., 2013. Low density lipoprotein cholesterol level inversely correlated with coronary flow velocity reserve in patients with Type 2 diabetes. *J Geriatr Cardiol* 10, 159-164.

Zheng, J.P., Zhang, Y., Edvinsson, L., Hjalt, T., Xu, C.B., 2010. NF-kappaB signaling mediates vascular smooth muscle endothelin type B receptor expression in resistance arteries. *Eur J Pharmacol* 637, 148-154.

Figure Legends

Figure 1

The maximal contractile response to S6c in rat mesenteric arteries without endothelium after organ culture for 24 hrs with vehicle (control) or LDL (11, 22, 44 mg protein/L). The maximal contractions induced by S6c are presented as the percentage of 60 mM K⁺-induced contraction. Each data point was derived from 8 segments (n=8) and represented as mean $\pm$ S.E.M.. Statistical analysis is performed using one-way ANOVA with Dunnet's post-test. n.s. = not significant, ** p < 0.01 and *** p < 0.001 vs. vehicle.

Figure 2

The expression of phosphorylated ERK1/2 (p-ERK1/2), p38 (p-p38) MAPK and ET_B receptor proteins in presence and absence of the specific MEK1/2 inhibitors (PD98059 10⁻⁵M and U0126 10⁻⁵M), p38 inhibitors (SB203580 10⁻⁵M and SB239063 10⁻⁵M) and transcriptional inhibitor actinomycin D (AcD 5 mg/L). Western blot experiments were performed in rat mesenteric arteries without endothelium after organ culture in presence LDL (44 mg protein/L) with DMSO (vehicle) or with the inhibitors for 6 hrs. (A) A typical western blot experiments traces are representative of three experiments with similar results. (B) Semi-quantitation of p-ERK1/2 protein in rat mesenteric arteries; (C) Semi-quantitation of p-p38 protein in rat mesenteric arteries; (D) Semi-quantitation of ET_B receptor protein expression in rat mesenteric arteries. The protein expression was normalized to beta-actin and percentage of fresh (uncultured) arterial segments (% of fresh). The arteries were collected from total of 16 rats for one experiment. Each data point is derived from 3 identical experiments (n=3) and presented as

mean $\pm$ S.E.M.. Unpaired Student's *t*-test with Welch's correction or one-way ANOVA with Dunnet's post-test to compare all inhibitor groups vs. control were used for statistical analysis, [#]*p* < 0.05, ^{##}*p* < 0.01 vs vehicle; * *p* < 0.05, and *** *p* < 0.001 vs. LDL.

Figure 3

The mRNA expression of ET_B receptors in rat mesenteric arteries without endothelium after organ culture with LDL (44 mg protein/L) for 24 hrs in presence of MEK1/2 inhibitors (PD98059 10⁻⁵M and U0126 10⁻⁵M), p38 inhibitors (SB203580 10⁻⁵M and SB239063 10⁻⁵M), general transcriptional inhibitor Actinomycin D (AcD 5 mg/L) or vehicle DMSO respectively. Data were obtained from real-time PCR studies and expressed as mean $\pm$ S.E.M. relative to EF-1. Each data point were derived from 6-7 segments (n=6-7). Statistical analysis was performed using unpaired Student's *t*-test with Welch's correction or one-way ANOVA with Dunnet's post-test to compare all inhibitor groups vs. control, ^{###}*p* < 0.001 vs vehicle; *** *p* < 0.001 vs. LDL+DMSO, n.s. = not significant.

Figure 4

S6c-induced contractile response curves of rat mesenteric arteries without endothelium after organ culture with LDL (44 mg protein/L) for 24 hrs in presence of (A) MEK1/2 inhibitors (PD98059 10⁻⁵M and U0126 10⁻⁵M), (B) p38 inhibitors (SB203580 10⁻⁵M and SB239063 10⁻⁵M) and (C) general transcriptional inhibitor Actinomycin D (AcD 5mg/L), or vehicle DMSO respectively. The contractile responses are presented as the percentage of 60 mM K⁺-induced contractile response. Each data point was derived from 8 segments (n=8) and represented as

mean $\pm$ S.E.M.. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. *** $p < 0.001$ vs. LDL+DMSO.

Figure 1

Figure 2

Figure 3

Figure 4