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**Apolipoprotein B of low-density lipoprotein impairs nitric oxide-mediated
endothelium-dependent relaxation in rat mesenteric arteries**

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Abstract

Apolipoprotein B (ApoB) of low-density lipoprotein (LDL) causes endothelial dysfunction in the initial stage of atherogenesis. The present study was designed to explore the underlying molecular mechanisms involved. Rat mesenteric arteries were organ cultured in presence of different concentrations of ApoB or LDL. Vasodilation induced by acetylcholine was monitored by a sensitive myograph. Nitric oxide (NO), endothelium-dependent hyperpolarizing factor (EDHF) and prostacyclin (PGI₂) pathways were characterized by using specific pathway inhibitors. Real-time PCR and immunohistochemistry with confocal microscopy were used to examine alteration of mRNA and protein expressions for NO synthases (eNOS and iNOS) and cyclooxygenase (COX), respectively. Lipid peroxidation was measured by thiobarbituric acid reactive substances. In presence of either LDL or ApoB for 24 h concentration-dependently attenuated the endothelium-dependent vasodilation. Immunohistochemistry staining of endothelial cell marker CD31 were weaker in presence of LDL, indicating that LDL induced damage to the endothelium. Using the pathway specific inhibitors demonstrated that LDL-induced impairing vasodilation was mainly due to attenuation of NO pathway. This was supported by decreasing mRNA (real-time PCR) and protein expression (immunohistochemistry) for eNOS and iNOS, but not COX, in presence of LDL. In addition, the levels of lipid peroxidation significantly increased in presence of LDL for 24 h. In conclusion, ApoB of LDL impairs vasodilation with damaging the endothelium and attenuating the NO-mediated endothelium-dependent relaxation, which might associate with lipid peroxidation and contribute to the development of cardiovascular disease.

Key words: Apolipoprotein B; low-density lipoprotein; endothelial dysfunction; vasodilation; lipid peroxidation; cardiovascular disease

Chemical compounds studied in the present study:

Acetylcholine (PubChem CID: 187); 5-hydroxytryptamine (PubChem CID: 5202);
indomethacin (PubChem CID: 3715); charybdotoxin (PubChem CID: 56842037); apamin
(PubChem CID: 16129677); N_ω-nitro-L-arginine methyl ester (PubChem CID: 39836).

1. Introduction

Endothelium-derived nitric oxide (NO) is a paracrine factor that controls vascular tone, inhibits platelet function, prevents adhesion of leukocytes and reduces proliferation of the intima (Forstermann, 2010). Impaired endothelium-dependent vasodilation represents an early manifestation of atherosclerosis and ischemic vascular disease (Egashira, 2002). Normal vasodilation is mediated by a number of endothelial systems including NO, prostaglandins (PGI₂ and PGE₂), and a family of endothelial-derived hyperpolarizing factors (EDHF) (Giles et al., 2012). NO is produced by nitric oxide synthase (NOS) in endothelial cells and relaxes smooth muscle cells by activating guanylate cyclase in the vascular smooth muscle cells. This enzyme can be inhibited by the NOS inhibitors such as N^ω-nitro-L-arginine methyl ester (L-NAME) (Moncada et al., 1991). Prostacyclin (PGI₂) is formed by cyclooxygenase (COX) from arachidonic acid in endothelial cells. It relaxes smooth muscle cells by activating adenylate cyclase. Formation of PGI₂ can be inhibited by indomethacin, a general COX inhibitor (Moncada, 1982). EDHF is an endothelium-derived vasodilation mediator, distinct from NO and PGI₂, which hyperpolarizes vascular smooth muscle cells. A combination of the potassium channel inhibitors, charybdotoxin and apamin, inhibits both EDHF-mediated vascular smooth muscle cell hyperpolarization and the subsequent vasodilation (Chataigneau et al., 1998; Doughty et al., 1999).

Endothelial cells are the first cells to experience the impact of hyperlipidemia in lesion-prone areas (Ivan and Antohe, 2010). Abundant data have confirmed the exacerbating effects of low-density lipoprotein (LDL) and its modified forms on endothelial dysfunction in cells (Ji et al., 2006), animals (Dai et al., 2004) and human (Cosentino et al., 2008). LDL is mainly oxidized in the subendothelial layer of the vascular wall (Zhang et al., 2010). LDL oxidation is the key event in hyperlipidemia-induced dysfunction of endothelium and vascular damage,

which includes attenuation of endothelium-dependent vasodilation (Hein et al., 2000), increased production of intracellular reactive oxygen species, and activation of transcription factor nuclear factor-kappaB (NF- κ B) in cultured human endothelial cells (Matsunaga et al., 2003). However, exposure to LDL for only few hours has not seen such effects on vascular endothelial function (Galle and Bassenge, 1991), suggesting that longer time exposure is needed for oxidation of LDL and dysfunction of endothelium.

We have developed an organ culture model for studying functions of intact arterial ring segments (Adner et al., 1996), which provides the possibility for evaluating the effects of cardiovascular risk factors on pathophysiological changes at both functional and molecular levels. This model has been frequently used to study increased vasocontractility by cardiovascular risk factors such as smoke particles (Sandhu et al., 2010) and minimally modified LDL (Li et al., 2013; Li et al., 2012). In addition to this, dysfunction of the endothelium and reduced endothelium-dependent relaxation also contribute to the increased vasocontractility, while this is relatively less studied.

LDL particles have two major compartments, an apolar lipid core and an outer amphipathic shell. The shell composed of a phospholipid monolayer and one single copy of apolipoprotein B (ApoB) that is atherogenic (Prassl and Laggner, 2009). In contrast, high-density lipoproteins contain exchangeable apolipoproteins of the A, C and E families that are antiatherogenic (Lund-Katz and Phillips, 2010). Clinical studies demonstrate that the atherogenic lipoprotein can cause endothelial dysfunction in the initial stage of atherogenesis with impairing endothelium-dependent vasodilation (Kraml et al., 2004). The ApoB/A1 levels, but not LDL-cholesterol, were inversely related to endothelium-dependent vasodilation (Lind, 2007), indicating that the ApoB might be an early marker of structural

vascular changes, whereas high-density lipoprotein specifically relates to endothelial vasodilatory function (Steer et al., 2002). However, the underlying molecular mechanisms involved in ApoB of LDL-impaired endothelium-dependent vasodilation are not fully understood. The present study was designed to explore how ApoB of LDL impairs vascular endothelium-dependent vasodilation. We have demonstrated that 24 h of exposure to ApoB and LDL induces damage to the endothelium and impairs endothelium-dependent vasodilation mainly by attenuating the NO-pathway. This might be relevant to the early stage of cardiovascular pathogenesis in clinical patients.

2. Material and Methods

2.1. Drugs and reagents

Acetylcholine, 5-hydroxytryptamine (5-HT), indomethacin, charybdotoxin, apamin, N_ω-nitro-L-arginine methyl ester (L-NAME), LDL (lyophilized powder reconstituted with solution of 150 mM NaCl and 0.01% EDTA, pH 7.4) and ApoB (lyophilized powder) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LDL and ApoB were diluted / dissolved in solution with 150 mM NaCl and 0.01% EDTA, pH 7.4, according to the manufacture's instruction. All other drugs were dissolved in double distilled water. The confection and storage of the reagents were according to the product information sheet and preparation guide.

2.2. Tissue preparation and organ culture procedure

Male Sprague-Dawley rats (weighting 300-350 g) were anaesthetized with CO₂ and exsanguinated. The vessels were cut into 1 mm long cylindrical segments and incubated at 37 °C in humified 5% CO₂ in air for different time periods. Culture was carried out in a 96-well plate, one segments in each well, containing 250 µL of Dulbecco's modified Eagle's

medium (DMEM) supplying with L-glutamine (584 mg/L) and supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). The organ culture experiments were performed with LDL or ApoB or vehicle (solution with 150 mM NaCl and 0.01% EDTA, pH 7.4). The experimental protocol was approved by Lund University Animal Ethics Committee.

2.3. In-vitro pharmacology

Following the incubation in DMEM, the vessel segments were transferred to buffer solution (without LDL or ApoB) for functional results. Arterial segments were immersed in temperature-controlled (37°C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution. 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 by the Chart software (ADInstruments, Hastings, UK). A resting tone of 2.5 mN was applied to each segment. The segments were allowed to stabilize at this tension for at least 1.5 h. After equilibration, the vessels were pre-constricted with 5-HT (10^{-5.5} or 10^{-5.7} M). Once the sustained tension was obtained, acetylcholine (from 10⁻¹⁰ to 10⁻⁴ M) was cumulatively added to the tissue baths and the isometric tension was recorded. Vasodilation was expressed as percentage of pre-constriction with 5-HT (Alm et al., 2002).

2.4. Characterization of endothelium-dependent vasodilation

Acetylcholine-induced vasodilation mainly consists of NO-, PGI₂- and EDHF-mediated pathways. NO-mediated dilatation induced by acetylcholine was studied in presence of indomethacin (10⁻⁵ M), charybdotoxin (10^{-7.3} M) and apamin (10⁻⁶ M). EDHF was studied in presence of L-NAME (10⁻⁴ M) and indomethacin. PGI₂ was studied in presence of L-NAME,

charybdotoxin and apamin. The inhibitors were added 20 minute before administration of 5-HT pre-constriction as described before (Alm et al., 2002; Zhang et al., 2006).

2.5. Real-time PCR

Arterial segments were homogenized in 1 ml of the RNapro solution (Q-BIOgene, CA, USA) by using a FastPrep instrument (Q-BIOgene, CA, USA). The total RNA was extracted following a protocol from the FastRNA Pro kit (Q-BIOgene, CA, USA) supplier. Reverse transcription of total RNA to cDNA was carried out using TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA, USA). The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit in a GeneAmp 7500 sequence detection system (PE Applied Biosystems, CA, USA) (Xu et al., 2008). Specific primers for the rat cyclooxygenase-1 (COX-1) mRNA, cyclooxygenase-2 (COX-2) mRNA, endothelial NO synthase (eNOS) mRNA and inducible NO synthase (iNOS) mRNA were designed as follows, respectively:

COX-1 Forward: 5'- GTGAATGCCACCTTCATCCG -3'

Reverse: 5'- CACCGTGAGTACCCAGCCC -3'

COX-2 Forward: 5'- CAGAGCAGAGAGATGAAATACCAGTC -3'

Reverse: 5'- GAAGCGTTTGCGGTACTCATT -3'

eNOS Forward: 5'- TCCTGGTGCGTCTGGACACT -3'

Reverse: 5'- CTCCTGCTGTTCGCTGGACT -3'

iNOS Forward: 5'- AACAGTGGCAACATCAGGTCG -3'

Reverse: 5'- CATGCTTCCCATCGCTCC -3'

Elongation factor-1 (EF-1) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) mRNA were used as references. The primers were designed as follows:

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

GAPDH Forward: 5'- GGCCTTCCGTGTTCTACC -3'
 Reverse: 5'- CGGCATGTCAGATCCACAAC -3'

2.6. Immunohistochemistry with confocal microscopy

The cultured arterial segments were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C. After fixation, the specimens were dehydrated in 20 % sucrose of phosphate buffer (0.1 M, pH 7.4) for 24 h at 4°C, then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and stored at -80°C. Sections were cut at 10-μm thickness in a cryostat and mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemistry was carried out using standard protocols (Xu et al., 2008; Zhang et al., 2008).

2.7. Lipid peroxidation assay

LDL peroxidation in arterial segments was measured by thiobarbituric acid-reactive substances (TBARS) assay, a commonly used approach (Wallin et al., 1993). The measurement was carried by using Cayman TBARS Assay Kit (Cayman Chemical, MI, USA) following the manufacture's instruction.

2.8. Statistics

All data are expressed as mean ± S.E.M. The amount of enzyme mRNA is expressed as percentage of the control group. Unpaired Student's *t*-test was used to compare two sets of

data or two-way analysis of variance (ANOVA) with Bonferroni post-test for comparisons of more than two data sets. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. Effects of LDL and ApoB on acetylcholine-induced vasodilation

Organ culture in presence of LDL (50, 100, 200 mg/L) for 24 h significantly reduced the vasodilatory responses to acetylcholine, compared with vehicle (150 mM NaCl, pH 7.4, 0.01% EDTA) (Fig. 1A, n=10). However, short-term incubation (6 h) with LDL (50 mg/L) (Fig. 1B, n=8) or acute administration of LDL into tissue bath did not alter the vasodilatory responses (data not shown). Thus, the effects observed were due to in presence of LDL.

In order to test the possible role of ApoB, the major component of LDL, equivalent amounts of ApoB (11 mg/L) as contained in LDL 50 mg/L, was administered. Co-incubation with ApoB for 24 h resulted in attenuation of acetylcholine-induced vasodilation with decreased maximal relaxation from 75% to 27% (Fig. 1C, n=8-12), suggesting that the reduced endothelium-dependent relaxation is caused by ApoB in LDL.

In addition, the ring segments of mesenteric arteries were stable in 60 mM K⁺ and 5-HT (10^{-5.5} M)-induced contractions, which were not significantly altered in presence of LDL or ApoB (data not shown).

3.2. Involvement of NO, EDHF and PGI₂ pathways

The acetylcholine-induced vasodilation in the arterial segments was mainly derived from the activation of pathways involved in the formation of NO, EDHF and PGI₂. To determine if LDL modified these pathways, specific inhibitors were added into the tissue bath 20 min

before 5-HT pre-constriction as described previously (Alm et al., 2002). The results showed that, organ culture with LDL (100 mg/L) for 24 h significantly reduced the NO-derived relaxant responses (Fig. 2A), while the EDHF-derived and PGI₂-derived responses (Fig. 2B and 2C) were not altered significantly. This indicates that the role of LDL during 24 h of culture is to suppress NO-mediated vasodilation, which could play an important role in attenuation of endothelium-dependent vasodilatory functions.

3.3. Effects of LDL on COX and NOS mRNA expression

To obtain further knowledge about the attenuated vasodilation induced by LDL at the molecular level, mRNA expression of COX-1, COX-2, eNOS and iNOS were studied by real-time PCR. The identity of EDHF is still unclear (Feletou and Vanhoutte, 2006).

Compared with vehicle, organ culture with LDL (100 mg/L) for 24 h significantly suppressed eNOS and iNOS mRNA expression, but did not affect COX-1 and COX-2, no matter if it is expressed as percentage of EF-1 or GAPDH, two house-keeping genes used for normalization (Fig. 3A and 3B).

3.4. Effects of LDL on eNOS and iNOS protein expression

The expression of eNOS, iNOS, CD31 (endothelial cell marker) and α -actin (smooth muscle cell marker) proteins were visualized by immunohistochemistry with confocal microscopy in smooth muscle cells and endothelial cells. Compared to control (vehicle, Fig. 4A), organ culture in presence of LDL resulted in diminished CD31 immunostaining, as a sign of endothelium damage (Fig. 4B). The iNOS protein expression was clearly observed in both smooth muscle cells and endothelium in the control (Fig. 4C) and LDL treatment decreased the expression of iNOS (Fig. 4D). The expression of eNOS was only seen in the endothelium (Fig. 4 E and 4F) and not observed in the smooth muscle cells. LDL treatment significantly

reduced the immunostaining of eNOS (Fig. 4F and 4H), compared with controls (Fig. 4E and 4G). Semi-quantification of the eNOS and iNOS protein expressions in the endothelium demonstrated that LDL significantly decreased eNOS ($P<0.001$) and iNOS ($P<0.05$) expression (Fig. 4I). The decreased expression of the NOS proteins in the endothelium paralleled with the reduced NOS mRNA levels after LDL treatment, which suggests that a transcriptional mechanism is involved.

3.5. LDL peroxidation

TBARS assay was used for evaluation of lipid peroxidation. The level of malondialdehyde (MDA) in the arterial tissue was significantly elevated after exposed to LDL (100 mg/L) for 24 h during organ culture, compared with the vehicle group. Interestingly, there was no difference between vehicle and LDL groups at time points up to 12 h (Fig. 5).

4. Discussion

The present study for the first time has demonstrated that 24 h exposure of arterial ring segments to ApoB of LDL resulted in impairing endothelium-dependent vasodilation via a transcriptional down-regulation of eNOS in the endothelial cells. Similar effects were obtained from LDL and ApoB respectively, suggesting that the effect of LDL was mediated by ApoB, the atherogenic protein contained in LDL. After LDL treatment, the immunoreactions of endothelial cell marker (CD31) were weaker, indicating the endothelium damage. Characterization of endothelium-dependent vasodilation of the artery revealed that a reduction of NO-mediated signal pathway was mainly incriminated in the LDL-impaired vasodilation, which correlated well with the decreased mRNA and protein expression of eNOS and iNOS, but not COX-1 and COX-2. In addition, LDL oxidation took place in presence of LDL. Our findings are supported by clinical studies showing that the apoB/A1

levels, but not LDL-cholesterol, are inversely related to endothelium-dependent vasodilation in a population-based study of 1016 subjects aged 70 years (Lind, 2007) and the atherogenic lipoprotein can impair endothelium-dependent vasodilation (Kraml et al., 2004).

The acetylcholine-induced endothelium-dependent vasodilation mainly consists of NO, EDHF and PGI₂ pathways (Giles et al., 2012). Previous investigations demonstrated that, when artery segments are denuded of the endothelium or pre-treated with a combination of NO, EDHF and PGI₂ inhibitors, the vasodilatory responses to acetylcholine are abolished completely (Alm et al., 2002). In the present study, both NO- and EDHF-mediated vasodilations dominated (with a negligible role for PGI₂) and were depressed by LDL treatment. However, only the decreased NO-mediated vasodilation reached the statistical significance. This is paralleled with the decreased mRNA and protein expression for eNOS and iNOS, suggesting that LDL treatment resulted in a transcriptional down-regulation of NOS mRNA and protein expression that led to the decreased NO-mediated endothelium-dependent vasodilation. As an important endothelium-derived signal mediator, NO plays key roles in vasodilation (Schulz et al., 2008) and cardiovascular disease pathogenesis (Cromheeke et al., 1999). A report showed that atherosclerotic lesions containing foam cells were induced in a model of atherosclerosis in rabbits with moderate hypercholesterolaemia by chronic inhibition of NOS (Kitahara et al., 2010), revealing the importance of NO system in the progression of atherosclerotic diseases. Different LDL species including native LDL from healthy subjects, oxidized LDL (formed by native LDL oxidation), and native LDL from type 2 diabetic patients, significantly inhibited NOS expression and activity in human umbilical vein endothelial cells (Ji et al., 2006). We did not measure the NOS activity in our model, but the results showed functional, mRNA and protein data to support the transcriptional down-regulation of NOS induced by LDL.

Oxidized LDL has been extensively investigated and demonstrated to elicit a direct cytotoxic effect on vascular endothelial cells (Xavier et al., 2004) and could selectively attenuate the NO-mediated relaxation in isolated porcine coronary arterioles (Hein et al., 2000). In hypercholesterolemic rabbits, vitamin E administration improves endothelial function in parallel with reduced MDA level in the aorta, suggesting that the antioxidant effect of vitamin E could prevent the endothelium from oxidized LDL-induced damage (Ribeiro Jorge et al., 1998). It has been concluded that, LDL is mainly oxidized in the subendothelial layer of the vascular wall (Zhang et al., 2010). The endothelial cells could modify LDL to the oxidized form via hydroxyl radicals (Sato and Tokunaga, 2002). The present study showed that, exposure of the arterial segments to LDL for up to 24 h significantly increased lipid peroxidation level in the tissue, indicating the involvement of LDL oxidation in the impaired endothelial function which may eventually lead to cardiovascular events. Previous studies regarding the effects of LDL on the arteries drew a conclusion that LDL treatment does not contribute to endothelial dysfunction (Galle and Bassenge, 1991; Galle et al., 1998; Xavier et al., 2004). However, those studies utilized short-term exposure (not more than 6 h). In the present study, exposure of the arterial segments to LDL for 6 h did not alter the endothelium-dependent vasodilation either, while enhanced lipid peroxidation did not occur until 24 h of organ culture. Moreover, an *in-vivo* study has showed that a single injection of LDL for 48 h markedly reduces endothelium-dependent vasodilation to acetylcholine in thoracic aortic rings in rat (Dai et al., 2004). Hence, longer time incubation of LDL seems to be obligatory for LDL-induced endothelial dysfunction as observed in our study. The organ culture model with intact vessels could provide the possibility for investigating the direct effect of LDL at both functional and molecular levels, and assist in the understanding of the underlying mechanisms linking hyperlipidemia and cardiovascular disease.

24 h incubation with ApoB alone (equivalent to the amount of ApoB in LDL) resulted in depressed endothelium-dependent vasodilation during organ culture, indicating that ApoB is responsible (to a major degree) for LDL-induced endothelial dysfunction. Previous studies focused on the effects of LDL *per se* or its modified forms, with little concern about ApoB, the major component of LDL. Interestingly, a clinical investigation concluded that ApoB could be an early marker of structural vascular change in healthy subjects as it was an independent predictor of both attenuated endothelium-dependent and -independent vasodilation with high correlation (Steer et al., 2002). Also known as “bad cholesterol”, LDL exerts multiple effects including endothelial damage which greatly contribute to pathogenesis of cardiovascular diseases, and ApoB seems to be the malefactor despite that extensive works are required to elucidate some mechanisms behind.

In conclusion, 24 h of exposure to ApoB of LDL impairs the endothelium-dependent NO-mediated vasodilation mainly via attenuation of NOS expression in rat mesenteric arterial segments. The endothelium damage and dysfunction by ApoB of LDL might associate with LDL oxidation and contribute to the development of cardiovascular disease.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1

(A) Acetylcholine (ACh)-induced vasodilation in 5-HT ($10^{-5.5}$ M) pre-contracted rat mesenteric arterial segments after organ culture with vehicle or LDL (50, 100, 200 mg/L) for 24 h. All concentrations of LDL significantly suppressed ACh-induced vasodilation ($n = 10$). (B) Co-incubation with LDL (50 mg/L) for 6 h did not alter the vasodilation responses in the arteries ($n = 8$). (C) ACh-induced vasodilation in 5-HT ($10^{-5.5}$ M) pre-contracted rat mesenteric artery segments after organ culture in presence of vehicle or ApoB (11 mg/L) for 24 h. ApoB concentration were equivalent as in LDL 50 mg/L ($n = 8-12$). Data are expressed as mean \pm S.E.M. Two-way ANOVA with Bonferroni post-test, *** $P < 0.001$ versus vehicle (control).

Fig. 2

Characterization of endothelium-derived dilatory mediators in 5-HT ($10^{-5.7}$ M) pre-contracted rat mesenteric artery segments after organ culture with vehicle or LDL (100 mg/L) for 24 h. The inhibitors were added 20 minute before administration of 5-HT pre-constriction, respectively. Administration of different inhibitors resulted in vasodilation responses mediated by (A) NO, (B) EDHF and (C) PGI₂. Data are expressed as mean \pm S.E.M ($n = 7$). Two-way ANOVA with Bonferroni post-test, * $P < 0.05$, *** $P < 0.001$ versus vehicle (control).

Fig. 3

Real-time PCR quantification of COX-1, COX-2, eNOS and iNOS mRNA expression relative to house-keeping genes: (A) EF-1 and (B) GAPDH in rat mesenteric artery after

organ culture with vehicle (control) or LDL (100 mg/L) for 24 h. Data are expressed as mean \pm S.E.M (n=6). Unpaired Student's *t*-test with Welch's correction, ***P* < 0.01, ****P* < 0.001 versus control.

Fig. 4

Immunohistochemistry with confocal microscopy representation of CD31, α -actin, iNOS and eNOS protein expressions in the mesenteric artery segments organ cultured with vehicle (A, C, E, G) or LDL (100 mg/L, B, D, F, H) for 24 h. Bar indicates 10 μ M.

(A, B) Double staining with specific antibodies against CD31 (endothelial cell marker) and α -actin (smooth muscle cell marker) visualizes locations of the endothelium (green; pink arrow), smooth muscle cells (orange; white arrow head), collagen bands (yellow; white arrow) and the lumen (L). After LDL treatment, the immunoreaction of CD31 staining (green) was much weaker, indicating that LDL induced endothelium damage.

(C, D) Double staining with specific antibodies against iNOS and α -actin (smooth muscle cell marker). The expression of iNOS (green) was seen in both the endothelium (pink arrow) and smooth muscle cells (white arrow) of control (C), while LDL (D) treatment decreased the immunostaining of iNOS.

(E, F) The expression of eNOS (green, white arrow) was only seen in the endothelium, and not in the smooth muscle cells. Compared to control (E), LDL (F) treatment significantly reduced the eNOS immunostaining.

(G, H) Double staining with specific antibodies against eNOS (green; white arrow) and α -actin (orange) confirmed the decreased eNOS protein expression induced by LDL (H) in the endothelium, compared with the control (G).

(I) Semi-quantification of eNOS and iNOS protein expression levels in the endothelium.

Each data point is derived from 6 experiments. Unpaired Student's *t*-test with Welch's correction, ****P* < 0.001 and **P* < 0.05 versus vehicle (control).

Fig. 5

LDL peroxidation determined by TBARS assay kit in rat mesenteric arterial segments incubated for different time points in presence of vehicle or LDL (100 mg/L). Co-incubation with LDL for 24 h resulted in significant elevation of LDL peroxidation in the arterial tissue, compared with vehicle. Data are expressed as mean ± S.E.M (n = 5-6). Two-way ANOVA with Bonferroni post-test, **P* < 0.05 versus vehicle (control).

Fig. 1

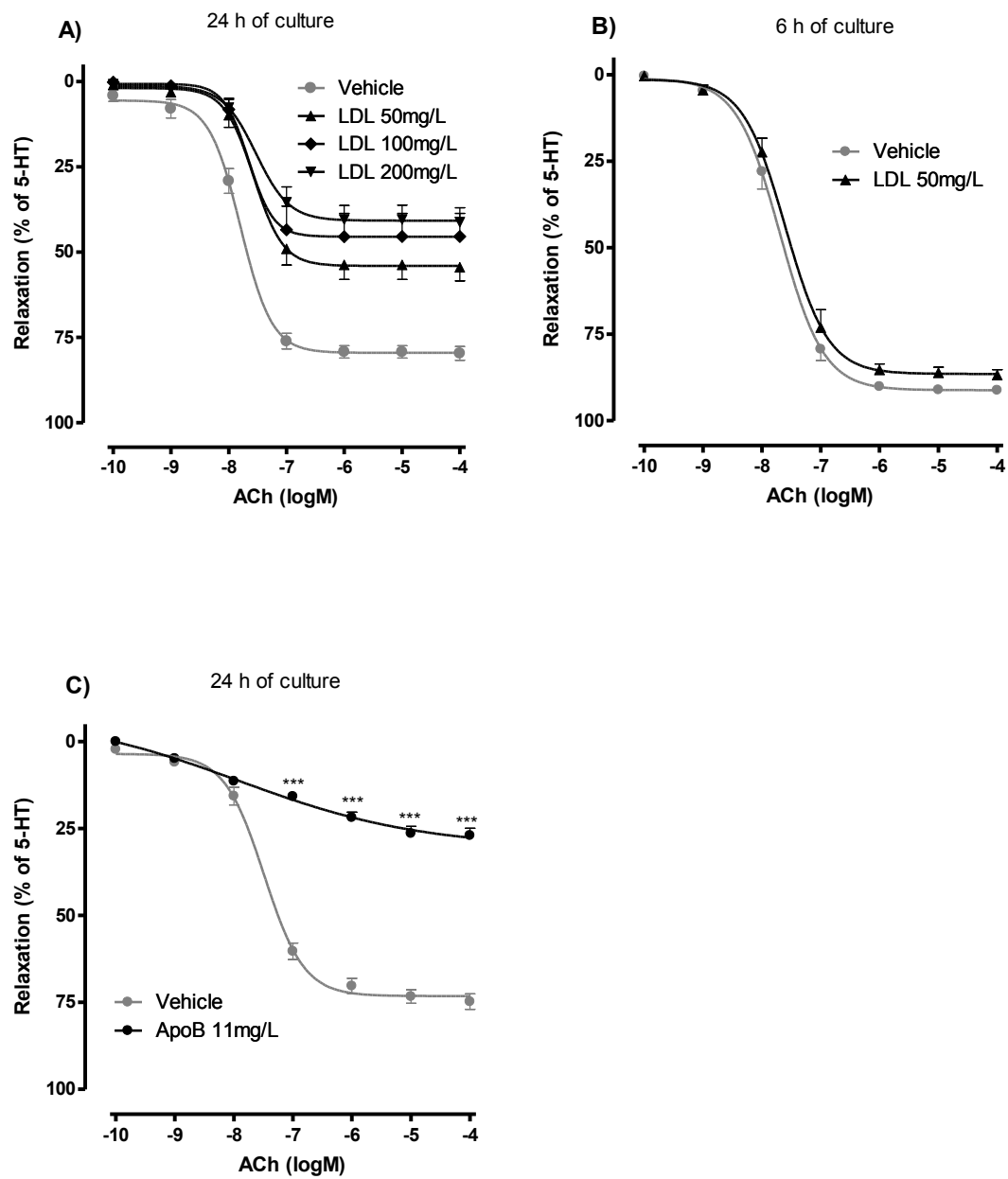


Fig. 2

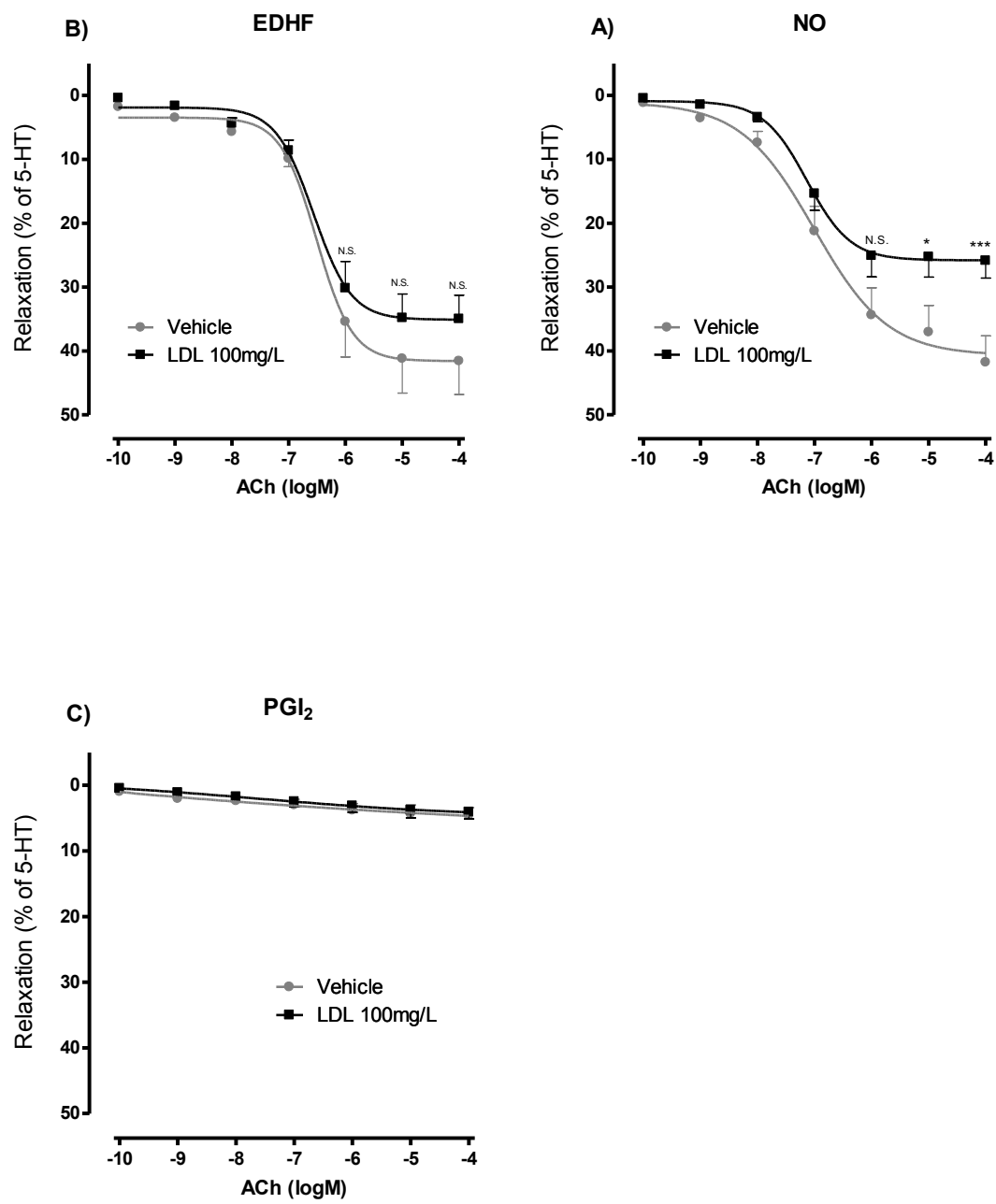


Fig. 3

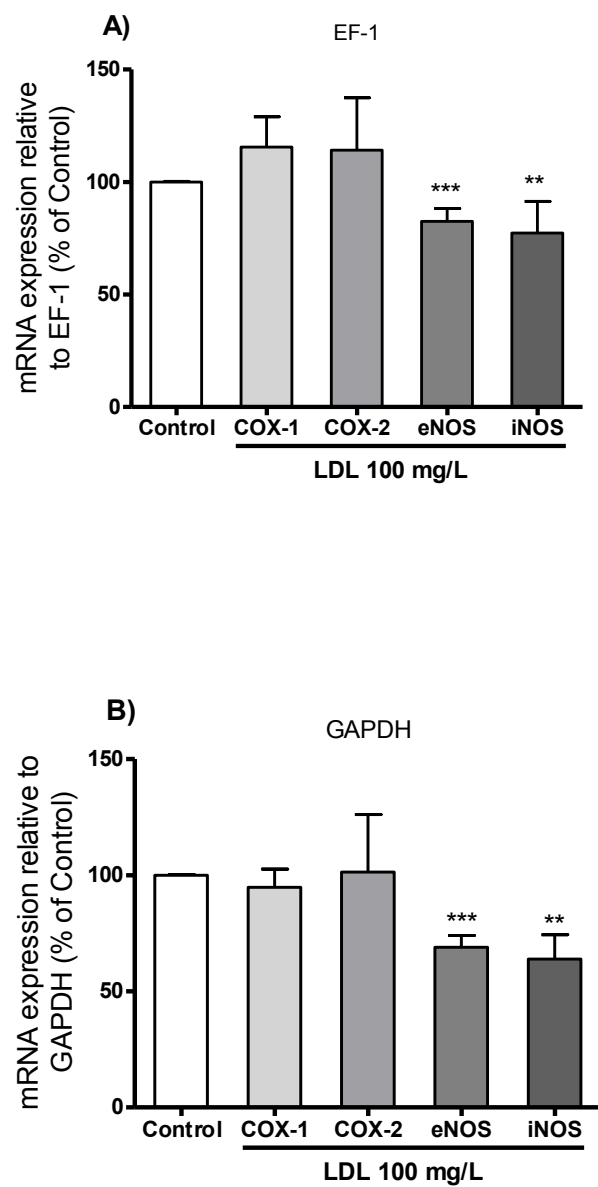


Fig. 4

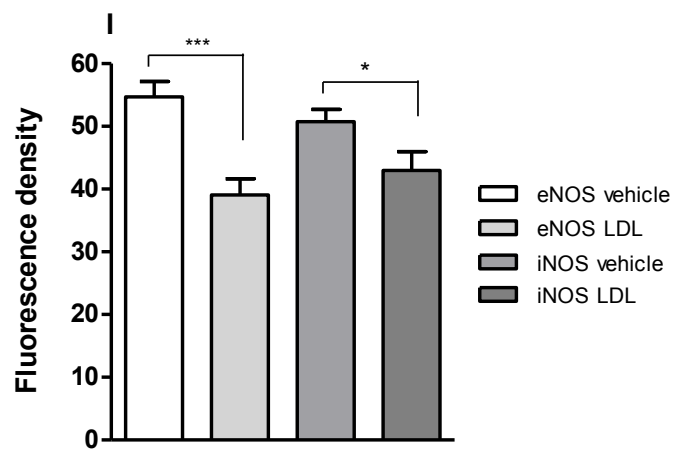
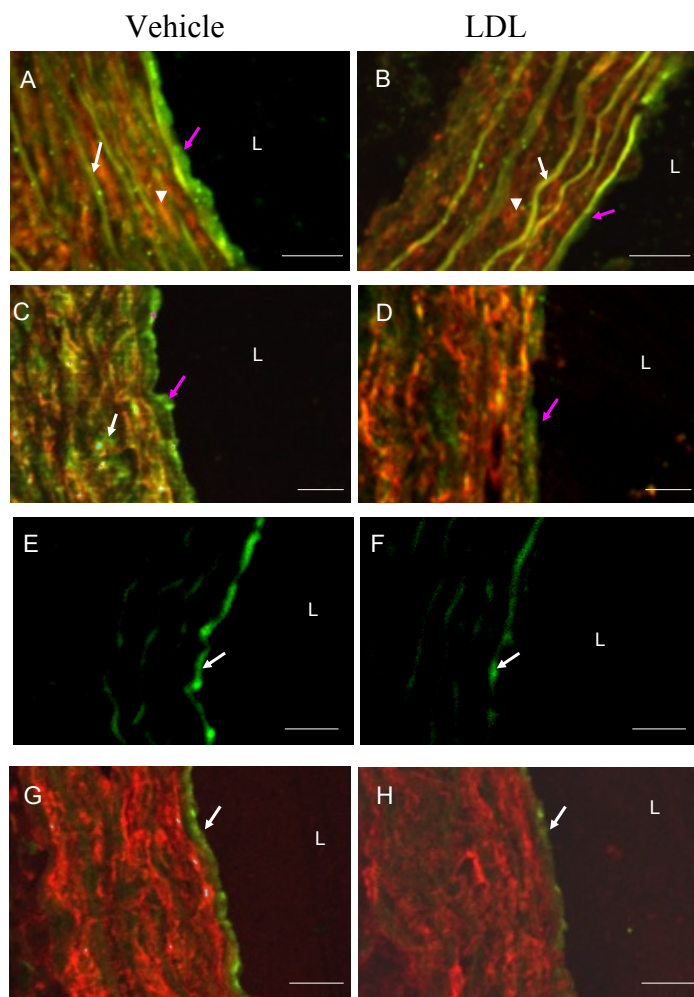


Fig. 5

