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RESEARCH PAPER

Oxidized low-density lipoprotein stimulates epithelial sodium channels in endothelial cells of mouse thoracic aorta

Correspondence Professor Zhi-Ren Zhang, Departments of Cardiology and Clinical Pharmacy, Harbin Medical University Cancer Hospital, 150 Haping Road, Harbin, 150081, P. R. China. E-mail: zhirenz@yahoo.com

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Chen Liang[1,*], Qiu-Shi Wang[1,*], Xu Yang[1], Na Niu[1], Qing-Qing Hu[1], Bao-Long Zhang[1], Ming-Ming Wu[1], Chang-Jiang Yu[1], Xiao Chen[1], Bin-Lin Song[1], Zhi-Ren Zhang[1] and He-Ping Ma[2]

[1]*Departments of Cardiology and Clinical Pharmacy, Harbin Medical University Cancer Hospital, Institute of Metabolic Disease, Heilongjiang Academy of Medical Science, Key Laboratories of Education Ministry for Myocardial Ischemia Mechanism and Treatment, Harbin, P. R. China, and* [2]*Department of Physiology, Emory University School of Medicine, Atlanta, Georgia, USA*

*These authors contributed equally to this work.

BACKGROUND AND PURPOSE

The epithelial sodium channel (ENaC) is expressed in endothelial cells and acts as a negative modulator of vasodilatation. Oxidized LDL (ox-LDL) is a key pathological factor in endothelial dysfunction. In the present study we examined the role of ENaC in ox-LDL-induced endothelial dysfunction and its associated signal transduction pathway.

EXPERIMENTAL APPROACH

Patch clamp techniques combined with pharmacological approaches were used to examine ENaC activity in the endothelial cells of a split-open mouse thoracic aorta. Western blot analysis was used to determine ENaC expression in the aorta. The aorta relaxation was measured using a wire myograph assay.

KEY RESULTS

Ox-LDL, but not LDL, significantly increased ENaC activity in the endothelial cells attached to split-open thoracic aortas, and the increase was inhibited by a lectin-like ox-LDL receptor-1 (LOX-1) antagonist (κ-carrageenan), an NADPH oxidase inhibitor (apocynin), and a scavenger of ROS (TEMPOL). Sodium nitroprusside, an NO donor, diminished the ox-LDL-mediated activation of ENaC, and this effect was abolished by inhibiting soluble guanylate cyclase (sGC) and PKG. Ox-LDL reduced the endothelium-dependent vasodilatation of the aorta pectoralis induced by ACh, and this reduction was partially restored by blocking ENaC.

CONCLUSION AND IMPLICATIONS

Ox-LDL stimulates ENaC in endothelial cells through LOX-1 receptor-mediated activation of NADPH oxidase and accumulation of intracellular ROS. Since the stimulation of ENaC can be reversed by elevating NO, we suggest that both inhibition of ENaC and an elevation of NO may protect the endothelium from ox-LDL-induced dysfunction.

LINKED ARTICLES

This article is part of a themed section on Spotlight on Small Molecules in Cardiovascular Diseases. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v175.8/issuetoc

Abbreviations

ECs, endothelial cells; EDR, endothelium-dependent relaxation; ENaC, epithelial sodium channel; eNOS, endothelial NOS; LOX-1, lectin-like oxidized-LDL receptor-1; ox-LDL, oxidized LDL; P_O, open probability; PTEN, phosphatase and tensin homolog; sGC, soluble guanylate cyclise

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Introduction

An elevation of plasma oxidized LDL (ox-LDL) is a hallmark of atherosclerosis (Zeibig *et al.*, 2011) and contributes to endothelial dysfunction (Pandey *et al.*, 2014). This dysfunction is one of the earliest indicators of atherosclerosis and involves numerous changes in endothelial cells, including an up-regulation of adhesion molecules (Feng *et al.*, 2014), an increase in their proliferation (Sun *et al.*, 2013), a down-regulation of endothelium-dependent vasodilatation (Yamamoto *et al.*, 2015) and NO production (Chan *et al.*, 2016), and modifications of their electrophysiological properties (Kuhlmann *et al.*, 2003). Several biological effects of ox-LDL are mediated via the lectin-like ox-LDL receptor-1 (LOX-1), the main receptor for ox-LDL on the endothelial lining of blood vessels (Pirillo *et al.*, 2013). ox-LDL stimulates LOX-1 transcription to positively feedback its effects, particularly in endothelial cells directly exposed to the blood and, therefore, greatly elevates ROS in these cells to cause vasoconstriction (Nishimura *et al.*, 2004; Zhou *et al.*, 2016). However, the signalling transduction pathway involved has not been elucidated.

It has been known for a long time that endothelial cells express the **epithelial sodium channel (ENaC)** (Golestaneh *et al.*, 2001; Jernigan *et al.*, 2008). Interestingly, a slight elevation of extracellular sodium induces the swelling and plasma membrane stiffening of endothelial cells, and these effects are dependent on a functional ENaC (Oberleithner *et al.*, 2004; Oberleithner *et al.*, 2007). Knockdown of the endothelial **ENaCα**-subunit leads to a significant softening of the endothelial cortex, suggesting that the ENaC may play a vital role in determining endothelial nanomechanics (Jeggle *et al.*, 2013; Warnock, 2013). In a recent *ex vivo* study it was shown, using atomic force microscopy, that elevated levels of extracellular sodium can mechanically stiffen endothelial cells of mouse aorta (Korte *et al.*, 2014). For the first time, our patch clamp single-channel recordings from split-open artery show that ENaC is functional in endothelial cells. Since ox-LDL elevates intracellular ROS (Cominacini *et al.*, 2000; Chen *et al.*, 2007; Thum and Borlak, 2008) and ROS can strongly stimulate ENaC in distal nephron principal cells (Ma, 2011; Zhang *et al.*, 2013), ox-LDL may stimulate ENaC in endothelial cells by elevating intracellular ROS.

More importantly, we have also shown that ENaC controls vascular relaxation by modulating **NO** levels in endothelial cells (Liu *et al.*, 2015; Zheng *et al.*, 2016). NO is an important signalling molecule and is produced endogenously from L-arginine in a reaction catalysed by **NOSs**. Subtle changes in its rate of production may critically affect cellular homeostasis and initiate a variety of cellular signalling processes. ox-LDL inhibits the expression of endothelial NOS (**eNOS**) and decreases NO production in endothelial cells (Xu *et al.*, 2015). LOX-1-overexpressing mice fed a high-fat diet show reduced endothelium-dependent relaxation as a result of decreased NO availability (Eichhorn *et al.*, 2009). We have shown that enhanced ENaC activity can also reduce NO production (Liu *et al.*, 2015; Guo *et al.*, 2016; Zheng *et al.*, 2016), suggesting that ENaC can mediate ox-LDL-induced endothelial dysfunction. Our data presented here suggest that ox-LDL stimulates ENaC in endothelial cells by elevating intracellular ROS and this accounts for the decreased aortic relaxation induced by ox-LDL.

Methods

Animals

All animal care and experimental procedures were approved by the Harbin Medical University Animal Supervision Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

C57BL/6 mice were purchased from the animal centre of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). A total of 215 male C57BL/6 mice aged 8–10 weeks (24–27 g) were used in the present study. Four to five C57BL/6 mice were housed in a cage and maintained under a 12/12 h light/dark cycle (lights on 0700 h) with the ambient humidity at 50–80% and the temperature at 21 ± 2°C. Food and water were provided *ad libitum*. Twenty mice were used to assess western blots of α-ENaC; the aorta pectoralis was isolated from 24 mice and preparations were used to investigate the vasodilatation. 171 C57BL/6 mice were used to record single-channel activity of ENaC.

Aorta pectoralis preparation

Briefly, mice (aged 8–10 weeks, 24–27 g body weight) were anaesthetized by an i.p. injection of 0.3% pentobarbital sodium (90 $mg \cdot kg^{-1}$, P3761; Sigma-Aldrich, St. Louis, MO, USA) and ventilated with room air using a small animal ventilator. Adequate anaesthesia was characterized by slow but regular breathing and no pedal withdrawal reflex. The aorta pectoralis was removed from each mouse immediately and rinsed with PBS to remove blood and improve the visibility of the tissue. Each aorta pectoralis was dissected from the surrounding fat using watchmaker's forceps in PBS solution and transferred to a 5 × 5 mm cover glass coated with polylysine to immobilize the aorta. The cover glass was then placed in a chamber mounted on an inverted microscope (Nikon, Tokyo, Japan) and incubated in a bathing solution.

In situ patch clamp recording

As described by Climent *et al.* (2011) and by Liu *et al.* (2015), *in situ* patch clamp recordings of ENaC single-channel currents were performed using intact vascular endothelia. Each dissected aorta pectoralis was placed in a Petri dish containing PSS. They were then placed on a 5 × 5 mm cover glass, coated with L-polylysine and transferred to a chamber mounted on an inverted Nikon microscope, allowing direct access to the endothelial cell layer. Single-channel ENaC currents were recorded in a cell-attached configuration with an Axon Multiclamp 200B amplifier (Axon Instruments, Foster City, CA, USA) at room temperature (22–24°C). Patch pipettes were pulled from borosilicate glass with a Sutter P-97 horizontal puller, and the resistance of the pipettes ranged from 6 to 10 MΩ when filled with the pipette solution (composition in mM: 135 NaCl, 4.5 KCl, 0.1 EGTA, 5 HEPES and 5 Na-HEPES; pH 7.2, adjusted with NaOH); for I–V curve measurements, NaCl was substituted with equimolar LiCl to potentiate the single-channel amplitude of ENaC. The bath

solution (composition in mM: 135 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES and 5 Na-HEPES; pH 7.2, adjusted with NaOH) was stationary, and the chamber volume was ~0.8 mL. Single-channel currents were recorded for at least 15 min immediately after gigaseal formation. For most experiments, data were acquired by applying 0 mV to the patch pipettes and sampling at 5 kHz with a low-pass filter at 1 kHz using Clampex 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Prior to analysis, single-channel traces were further filtered at 30 Hz. The current–voltage (I–V) relationship was constructed using the single-channel amplitude measured at the indicated pipette voltages as a function of voltages. $V_{pipette}$ represents the voltage applied to the electrodes during recordings; therefore, $-V_{pipette}$ indicates that the intracellular potential deviated from the resting potential of the apical membranes of the endothelial cells. Slope conductance was fit via linear regression using SigmaPlot software (Jandel Scientific, San Diego, CA, USA).

Western blotting

For western blot analysis, samples were homogenized with CelLyticM lysis reagent and a protease inhibitor cocktail (Sigma, Poole, Dorset, UK). Protein concentrations were determined by use of the Bradford assay. Equal amounts of total protein were loaded into SDS-PAGE wells. GAPDH was used as the internal standard to control for protein quantity. Samples prepared with 5× loading buffer were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes, which were rinsed with Tris-buffered saline Tween-20 (TBS-T) and then blocked in TBS-T containing 5% skimmed milk for 1 h at room temperature (22–24°C). Membranes were incubated with primary antibodies against α-ENaC (NB100–74357; Novus Biologicals, Littleton, CO, USA) and GAPDH (sc-20357; Santa Cruz Biotechnology, Santa Cruz, California, USA) overnight at 4°C, followed by washing in TBS-T and incubation with secondary antibodies (1:5000) for another 1 h at room temperature (22–24°C). Membranes were washed with TBS-T, and blots were detected using an ECL kit (Invitrogen, Carlsbad, CA, USA) and scanned for densitometric analysis (Bio-Rad, Richmond, CA, USA).

Myograph functional study

Aorta pectoralis muscles were surgically isolated from male C57BL/6 mice and placed in ice-cold Krebs solution (composition in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11 D-glucose). Aortas were isolated from connective tissues under a dissecting microscope and cut into 2 mm lengths. Each aorta pectoralis preparation was suspended in a wire myograph (Danish Myo Technology, Aarhus, Denmark) and bathed in oxygenated Krebs solution at 37°C. Each ring was stretched to 3 mN and then allowed to equilibrate for 60 min before beginning the experiment. After the 60 min stabilization period, KPSS (containing 60 mM K^+) was added to the chambers and washed out with PSS until a reproducible maximal contraction was achieved. EDR induced by **ACh** (1 nM to 100 μM) was recorded in **phenylephrine**-contracted rings in the presence or absence of 10 $\mu g \cdot mL^{-1}$ ox-LDL or 0.5 μM **amiloride**.

Data analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All data are presented as the mean ± SEM. Statistical analyses were performed with SigmaPlot and SigmaStat software (Jandel Scientific). One-way ANOVAs (followed by Student–Newman–Keuls *post hoc* tests) or Student's *t*-tests were used where appropriate for statistical analysis. Differences were considered statistically significant at $P < 0.05$.

Chemicals and reagents

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). ox-LDL was purchased from Alfa Aesar (Ward Hill, MA, USA). KT5823, **ODQ** and apocynin were dissolved separately in a minimum quantity of DMSO and further diluted with the bath solution to achieve the required concentration (the final dilution ratio did exceed 1:1000). The other reagents including ox-LDL, LDL, amiloride, κ-carrageenan, TEMPOL and **SNP** were dissolved in double-distilled H_2O. ox-LDL, SNP and TEMPOL were protected from light during their preparation and the experiments. All solutions were premade and stored in a −20°C freezer.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

ENaC is expressed and functional in endothelial cells of mouse thoracic aorta

The thoracic aorta was isolated and manually split open to access the apical membrane of endothelial cells. *In situ* cell-attached patch clamp experiments were performed to record single-channel currents of ENaC in the apical membrane of the intact endothelial cells. As shown in Figure 1A, single-channel currents with slow kinetics and very low amplitude were detected in these cells. The open probability (P_O) of this current was significantly reduced by amiloride, a potent blocker of ENaC ($P < 0.05$; $n = 7$). To further characterize the currents, current–voltage (I–V) relationships were constructed and fitted via linear regression (Figure 1B, C). The data show that the single-channel conductance of the currents was ~6.8 pS, which is similar to previously reported single-channel conductance of ENaC (Ma, 2011). The biophysical features and pharmacological profile of these currents are consistent with those previously reported for ENaC in human dermal endothelial cells (Wang *et al.*, 2009). Western blot was performed using isolated thoracic aorta with or without the endothelial layer. The data show that ENaCα, the major subunit of ENaC, is expressed both in endothelial cells and in smooth muscle cells (Figure 1D).

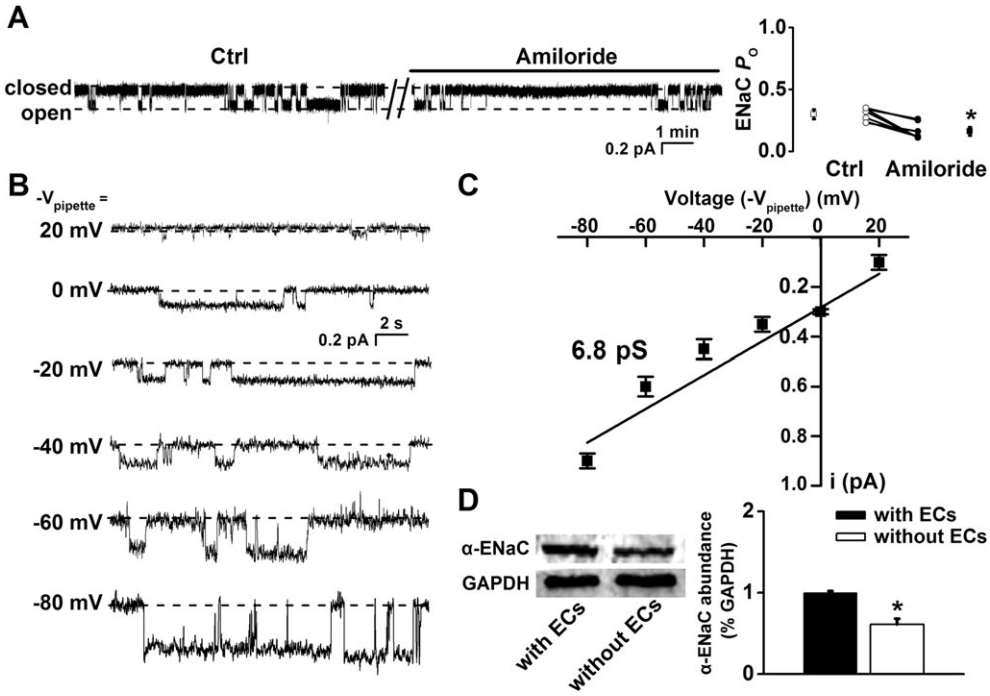

Figure 1

Mouse thoracic aorta expresses functional ENaC. (A) Representative ENaC single-channel currents recorded in endothelial cells attached to split-open aorta, before and after application of 0.5 μM amiloride (left). Summarized P_O values obtained from the recordings are shown on the right (*$P < 0.05$; $n = 7$). (B) Single-channel currents recorded at the indicated voltages. (C) I–V relationship constructed using single-channel current amplitude measured as a function of voltages applied to the patch pipette ($V_{pipette}$). Single-channel conductance was determined via linear regression as indicated by the black dashed line ($n = 8$ for the data point at $-V_{pipette} = 0$ mV; $n = 7$ for the data points at $-V_{pipette} = -20$ and -40 mV; $n = 6$ for the data points at $-V_{pipette} = 20$, -60 and -80 mV). (D) Western blot of α-ENaC from aortic tissue either with or without endothelial cells (ECs) attached ($n = 5$ for each group; *$P < 0.05$). Ctrl, control.

ox-LDL stimulates ENaC in aortic endothelia

Recent studies have shown that ox-LDL elevates intracellular ROS via its LOX-1 receptor (Cominacini *et al.*, 2000; Chen *et al.*, 2007; Thum and Borlak, 2008) and ROS can strongly stimulate ENaC (Ma, 2011; Zhang *et al.*, 2013). To determine whether ox-LDL can alter ENaC activity via this pathway, we performed patch clamp recordings from intact endothelial cells attached to isolated thoracic aorta. Exogenous ox-LDL was applied to the cells, while LDL was used as a control. ENaC activity was not altered by 10 μg·mL^{-1} LDL applied to the cells. ENaC P_O remained at control levels even when the cells were exposed to LDL for 30 min (0.36 ± 0.03 *vs*. 0.34 ± 0.03; $P > 0.05$; $n = 7$), suggesting that LDL has no acute effect on ENaC activity (Figure 2A, C). However, the application of 10 μg·mL^{-1} ox-LDL to the cells significantly elevated ENaC activity; ENaC P_O was increased from 0.31 ± 0.03 to 0.74 ± 0.04 ($P < 0.05$; $n = 7$) (Figure 2B, D). These results indicate that ox-LDL, but not LDL, stimulates ENaC activity.

To investigate whether ox-LDL stimulates ENaC in a concentration-dependent manner, endothelial cells attached to the split-open aorta were either investigated under control conditions or respectively treated with 1, 5, 10, 20, 40 and 50 μg·mL^{-1} of ox-LDL for 30 min, prior to the cell-attached patch clamp examination. The increasing the doses of ox-LDL gradually elevated ENaC activity, which almost reached a plateau at 50 μg·mL^{-1} of LDL. ENaC P_O was significantly increased by ox-LDL, from 0.29 ± 0.02 to 0.95 ± 0.02 (50 μg·mL^{-1}) (Figure 3A, B). The calculated mean P_O of ENaC was plotted as a function of different doses of ox-LDL and fitted with Pharmacology Standard Curves Analysis (the solid black line), having an EC_{50} of 8.60 ± 0.70 μg·mL^{-1} for ox-LDL to activate ENaC (Figure 3B; $n = 6$ for each data point). Therefore, 10 μg·mL^{-1} ox-LDL (a dose close to the EC_{50}) was used for the rest of the experiments. We also examined whether prolonged exposure to ox-LDL affects ENaC activity. Prior to the patch clamp experiments, the split-open aorta was either kept under control conditions or treated with 10 μg·mL^{-1} ox-LDL for 10, 30 or 60 min. As shown in Figure 3C, D, ENaC activity was significantly elevated at 30 min after the treatment and prolonged exposure of the aorta to 10 μg·mL^{-1} ox-LDL further increased ENaC activity ($P < 0.05$, $n = 6$). These data suggest that ox-LDL stimulates ENaC in endothelial cells of mouse aorta in a dose- and time-dependent manner.

ox-LDL elevates ENaC activity via LOX-1/NADPH oxidase/ROS

To determine whether the effect of ox-LDL on ENaC is due to the binding of ox-LDL to its receptor LOX-1, endothelial cells attached to the split-open aorta were either kept under control conditions or pretreated with 125 μg·mL^{-1}

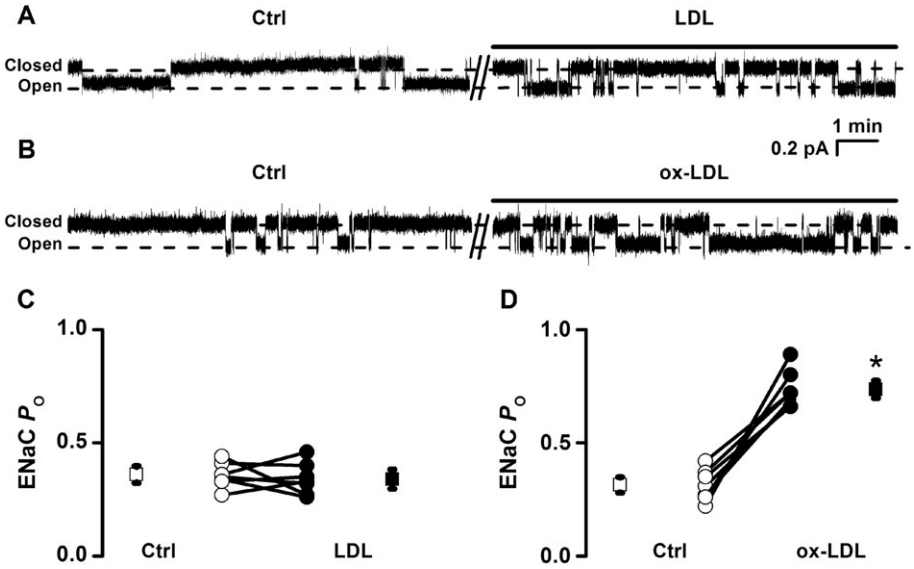

Figure 2

ox-LDL, but not LDL, stimulates ENaC in endothelial cells attached to a split-open aorta. (A, B) Representative ENaC single-channel currents recorded in endothelial cells before and after treatment with either 10 μg·mL^{-1} LDL (A) or 10 μg·mL^{-1} ox-LDL (B). (C, D) Summarized P_O values for ENaC before and after the application of LDL or ox-LDL to the cells are shown in the bottom of the figure (*$P < 0.05$ vs. respective controls (Ctrl); $n = 7$).

κ-carrageenan (a LOX-1 receptor inhibitor) for 10 min before the cell-attached recordings were performed. In the presence of κ-carrageenan, ox-LDL failed to increase ENaC activity (Figure 4A); ENaC P_O remained unchanged, 0.32 ± 0.02 (control) versus 0.35 ± 0.02 (30 min after 10 μg·mL^{-1} ox-LDL) ($P > 0.05$; $n = 7$). In contrast, κ-carrageenan itself did not alter ENaC activity. Since the binding of ox-LDL to LOX-1 can stimulate NADPH oxidase and rapidly increase intracellular ROS generation in articular chondrocytes (Nishimura *et al.*, 2004), we next examined whether ox-LDL stimulates ENaC in endothelial cells via a similar pathway. Therefore, the endothelial cells attached to split-open aorta were either kept under control conditions or treated with either 250 μM TEMPOL (an ROS scavenger) or 100 μM apocynin (an NADPH oxidase inhibitor). After these treatments, ox-LDL was no longer able to elevate ENaC activity. In the endothelial cells treated with TEMPOL or apocynin, ENaC P_O remained at control levels (Figure 4B and C respectively). Although apocynin itself did not alter ENaC activity, TEMPOL tended to decrease ENaC activity. These results suggest that ox-LDL stimulates ENaC in endothelial cells, at least in part, through its receptor LOX-1-mediated generation of intracellular ROS.

ox-LDL reduces endothelium-dependent aortic relaxation, which is partially dependent on ENaC activity

We previously showed that an elevation in ENaC activity reduces endothelium-dependent artery relaxation (Liu *et al.*, 2015). Therefore, we tested whether ENaC mediates ox-LDL-induced endothelial dysfunction. The experiments were performed using isolated aorta pectoralis with intact endothelia. As shown in Figure 5A, B, a contraction of the aorta pectoralis was induced by 1 μM phenylephrine; the EDR was induced by ACh at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M respectively. Experiments were then carried out to determine how amiloride (a potent ENaC blocker) and ox-LDL affect the ACh-induced relaxation. The data show that the ACh-induced relaxation of the aorta pectoralis was smaller after pretreatment with 10 μg·mL^{-1} ox-LDL. Amiloride did not alter the relaxation, indicating that basal ENaC activity does not affect aortic tone. However, the reduced relaxation induced by ox-LDL was partially but significantly reversed by blocking ENaC with amiloride. These data suggest that an elevated ENaC activity participates in the decreased endothelium-dependent aortic relaxation induced by ox-LDL.

NO reverses ox-LDL-induced ENaC activity via soluble guanylate cyclase and PKG

As it is well-known that NO can cause vascular relaxation, NO may antagonize the effect of ox-LDL on ENaC activity. Hence, we tested whether NO could reverse ox-LDL-induced ENaC activity. Consistently, ENaC P_O in endothelial cells attached to the split-open aorta was markedly increased after application of 10 μg·mL^{-1} ox-LDL to the cells, which was from 0.30 ± 0.02 (before) to 0.79 ± 0.03 (30 min after ox-LDL) ($P < 0.05$; $n = 6$); this increase was reversed by 1 mM SNP, an NO donor; ENaC P_O was reduced to 0.28 ± 0.03 ($P < 0.05$; $n = 6$). Furthermore, ox-LDL failed to stimulate ENaC when the cells were pretreated with SNP (Figure 6A). Therefore, we concluded that NO can prevent or attenuate activation of ENaC by ox-LDL. Since NO is known to stimulate **soluble guanylate cyclase** (sGC) and **PKG** (Shahidullah and Delamere, 2006), the endothelial cells attached to the split-open aorta were treated with either 10 μM ODQ (an sGC inhibitor) or 10 μM KT5823 (a PKG

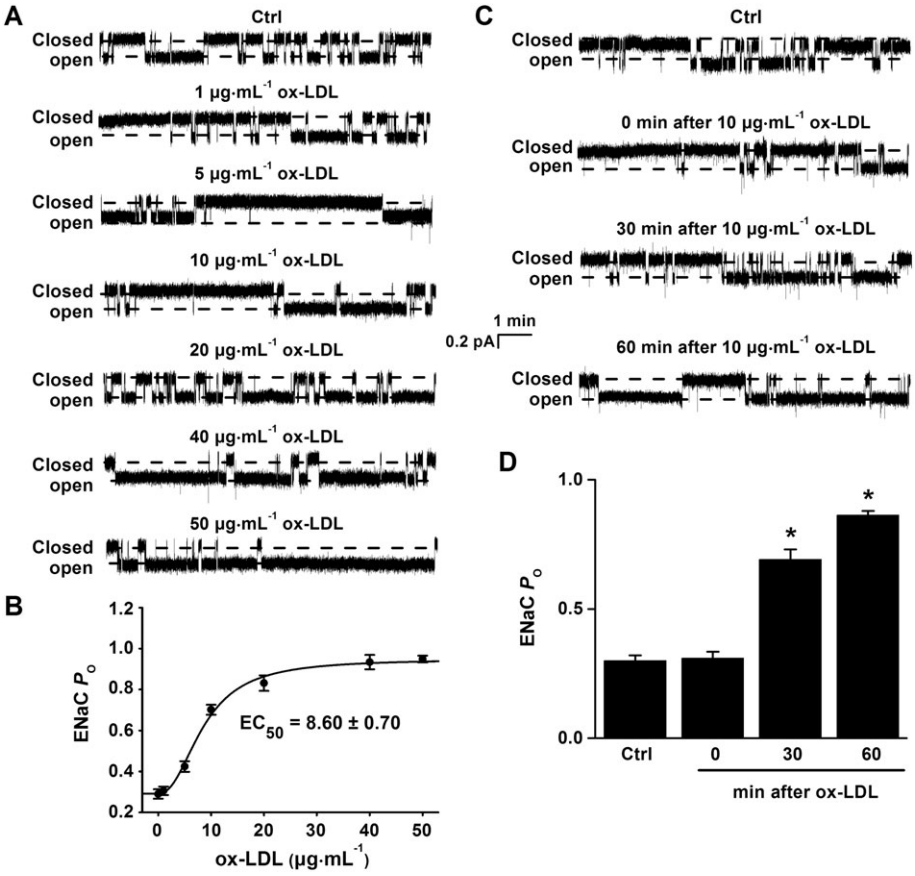

Figure 3

ox-LDL stimulates ENaC activity in a dose- and time-dependent manner. (A) Representative ENaC single-channel currents recorded in endothelial cells from a split-open aorta either under control (Ctrl) conditions or treated with ox-LDL at concentrations of 1, 5, 10, 20, 40 and 50 μg·mL^{-1} for 30 min. (B) ENaC P_O were plotted as a function of each corresponding concentration of ox-LDL and fitted with Pharmacology Standard Curves Analysis using SigmaPlot (n = 6 for each data point). (C) Representative ENaC single-channel currents recorded in endothelial cells from a split-open aorta either under control conditions or treated with 10 μg·mL^{-1} ox-LDL for 0, 30 or 60 min respectively. (D) Summarized ENaC P_O under the conditions shown in (C) respectively (*$P < 0.05$ vs. respective Ctrl; n = 6).

inhibitor) for 10 min before the patch clamp experiments. The data show that after inhibition of sGC with ODQ, SNP was no longer able to reverse ox-LDL-induced ENaC activity (Figure 6B). Similarly, after inhibition of PKG with KT5823, SNP failed to attenuate ox-LDL-induced ENaC activity (Figure 6C). These results suggest that NO reverses the activation of ENaC by ox-LDL via a pathway associated with sGC/PKG.

Discussion

The major findings of the present study are: (i) ENaC is expressed in endothelial cells of mouse thoracic aorta and its activity is significantly elevated by ox-LDL, but not by LDL; (ii) ox-LDL stimulates ENaC via LOX-1-mediated generation of intracellular ROS; (iii) blockade of ENaC significantly attenuates ox-LDL-induced decrease in endothelium-dependent aortic relaxation; and (iv) NO reverses ox-LDL-mediated increase in ENaC activity via the sGC/PKG pathway and, probably, also through NO inhibiting oxidative stress generated from NADPH oxidase. Our findings may provide a new therapeutic strategy for treating vascular dysfunction caused by ox-LDL.

It is well accepted that ox-LDL is a critical risk factor/biomarker for several concurrent pathophysiological processes, including chronic inflammation, metabolic disorder and oxidative stress, during atherogenesis, and a cause of vasculature dysfunction (Zhang *et al.*, 2016a). Our data show, for the first time, that ENaC is expressed and functional in the endothelial cells of thoracic aorta and mediates the decreased EDR induced by ox-LDL. The ox-LDL concentration in the circulation of healthy subjects is normally maintained at a very low level (~0.14 μg·mL^{-1}) (Wang *et al.*, 2007). However, ox-LDL levels are significantly elevated in patients with hyperlipidaemia (Owens *et al.*, 2012), type 2 diabetes mellitus (Nour Eldin *et al.*, 2014), unstable or stable angina (Neri Serneri *et al.*, 2013) or even coronary heart disease (Holm *et al.*, 2011). Not surprisingly, ox-LDL stimulates ENaC in aortic endothelial cells in a dose- and time-dependent manner, suggesting that the elevated ENaC activity evoked by the chronically enhanced ox-LDL

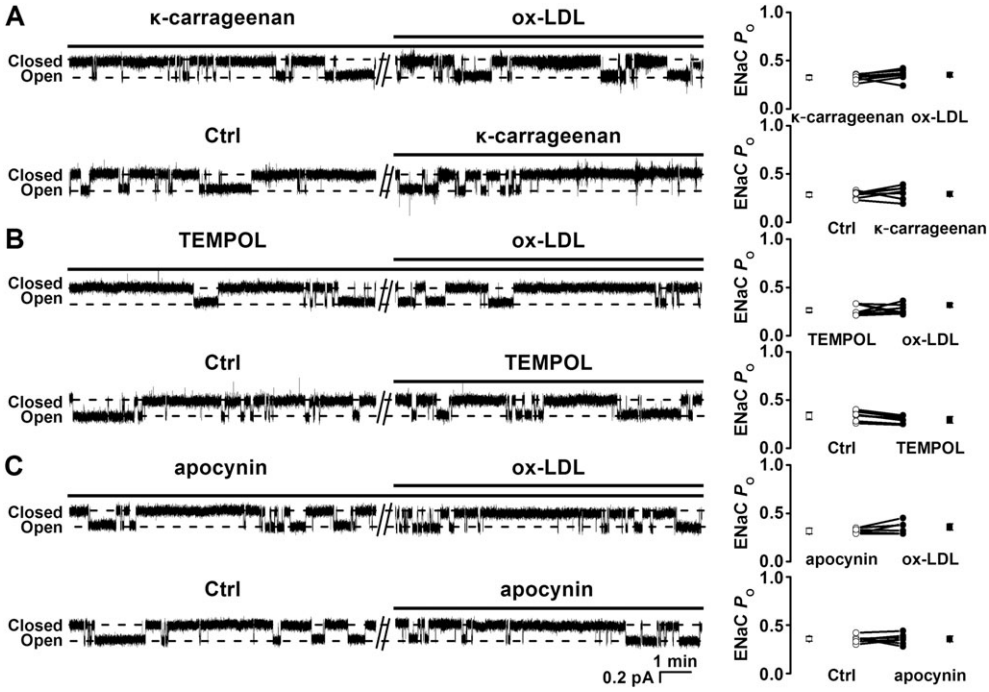

Figure 4

ox-LDL stimulates ENaC via LOX-1-mediated activation of NADPH oxidase. (A) Representative ENaC single-channel currents recorded in two separate endothelial cells; one was pretreated with 125 μg·mL^{-1} κ-carrageenan before being treated with10 μg·mL^{-1} ox-LDL (upper trace); the other served as a control (Ctrl) and was treated with 125 μg·mL^{-1} κ-carrageenan (lower trace). (B) Representative ENaC single-channel currents recorded in two separate endothelial cells; one was pretreated with 250 μM TEMPOL before the application of 10 μg·mL^{-1} ox-LDL (upper trace); the other served as a Ctrl and was pretreated with 250 μM TEMPOL (lower trace); (C) Representative ENaC single-channel currents recorded in two separate endothelial cells; one was pretreated with 100 μM apocynin before the application of 10 μg·mL^{-1} ox-LDL (upper trace); the other served as a Ctrl and was treated with 100 μM apocynin (lower trace). Summarized ENaC P_Os were plotted on the right (n = 7 in each experiment).

levels may contribute to vasculature dysfunction induced by oxidative stress.

Our recent studies demonstrated that elevated ENaC activity accounts for the reduced EDR by decreasing NO levels in endothelial cells from salt-sensitive rats fed a high-salt (HS) diet, and that blockade of ENaC by amiloride increased both phosphorylated eNOS and NO and, therefore, prevented the HS diet-induced loss of vasorelaxation and endothelial dysfunction (Wang *et al.* 2018). Others have also shown that inhibition of ENaC increases NOS phosphorylation and NO production in small-diameter rat mesenteric arteries (Perez *et al.*, 2009). Furthermore, in cultured alveolar type II monolayers, NO may inhibit ENaC-mediated Na^+ reabsorption (Guo *et al.*, 1998). In the present study, it was demonstrated that exogenous NO reverses the ox-LDL-induced increase in ENaC activity not only via the sGC/PKG pathway but also through inhibition of ROS, since NO can inhibit oxidative stress generated from NADPH oxidase (Shen *et al.*, 2010). Together, these findings suggest that there might be a feedback mechanism between ENaC activity and NO production. This feedback implies that enhanced ENaC activity reduces NO production in endothelial cells and that, conversely, a reduction in NO will relieve its inhibitory effects on ENaC activity. ox-LDL is a product of chronic oxidative stress; it is also a potent inducer of oxidative stress, as ox-LDL injections have been shown to increase both bone marrow and blood ROS levels in mice (Zhang *et al.*, 2016b). Importantly, incubation of cultured human aortic endothelial cells with ox-LDL enhances ROS via the phosphorylation of p66Shc, an important mediator of oxidative stress (Shi *et al.*, 2014). In a previous study, it was revealed that ox-LDL activates NADPH oxidase (both the gp91 phox and p47 phox subunits) expression and enhances ROS generation via LOX-1 in human coronary artery endothelial cells (Dandapat *et al.*, 2007). Therefore, these findings suggest that there might be another positive feedback mechanism between ox-LDL and ROS to elevate both ox-LDL and ROS.

In the present study, it was found that ox-LDL also stimulates ENaC. Therefore, a strong elevation of both ox-LDL and ROS due to the positive feedback mechanism should significantly enhance ENaC activity. The results described above provide a rationale for the hypothesis that ox-LDL probably stimulates endothelial ENaC through an ox-LDL/LOX-1-mediated accumulation of intracellular ROS. Our data show that the stimulating effects of ox-LDL on endothelial ENaC activity in the aorta were respectively abolished by TEMPOL (an ROS scavenger) and apocynin (an NADPH oxidase inhibitor), as well as by a LOX-1 antagonist. Together, these data suggest that ox-LDL regulates endothelial ENaC activity through its receptor, LOX-1,-mediated activation of NADPH oxidase.

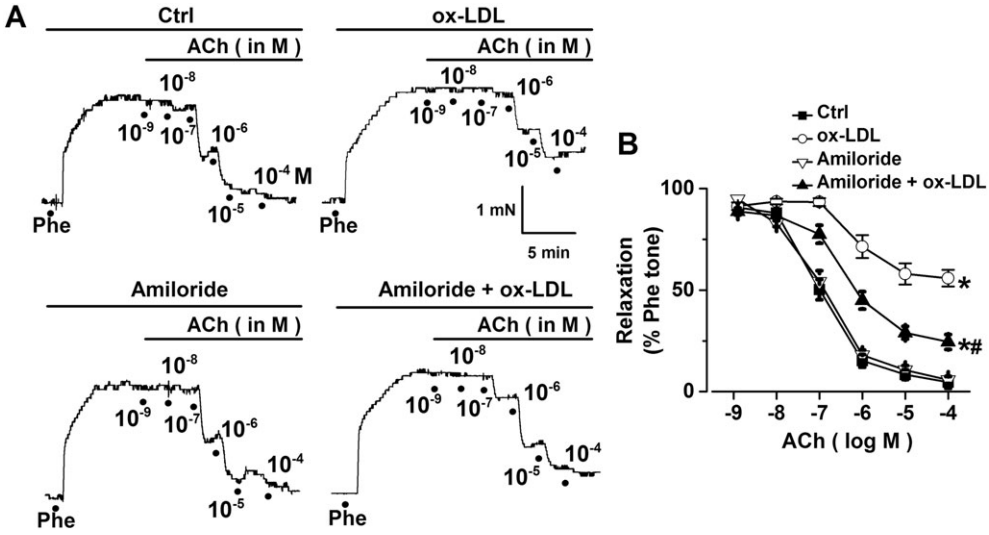

Figure 5

ox-LDL reduces ACh-induced aortic relaxation via an ENaC-dependent pathway. (A) Representative traces of ACh-induced relaxation of mouse aorta pectoralis either under control (Ctrl) conditions or after treatment with 10 μg·mL^{-1} ox-LDL, 0.5 μM amiloride or 0.5 μM amiloride +10 μg·mL^{-1} ox-LDL. Aorta pectoralis preparations were treated with 1 μM phenylephrine (Phe) to induce a contraction before examining the ACh-induced relaxation. (B) Summaries of relaxation induced by ACh at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M under the conditions described in (A) (n = 6 for each data point). The data for 10^{-4} M ACh were analysed, and showed *$P < 0.05$ versus Ctrl; #$P < 0.05$ versus ox-LDL.

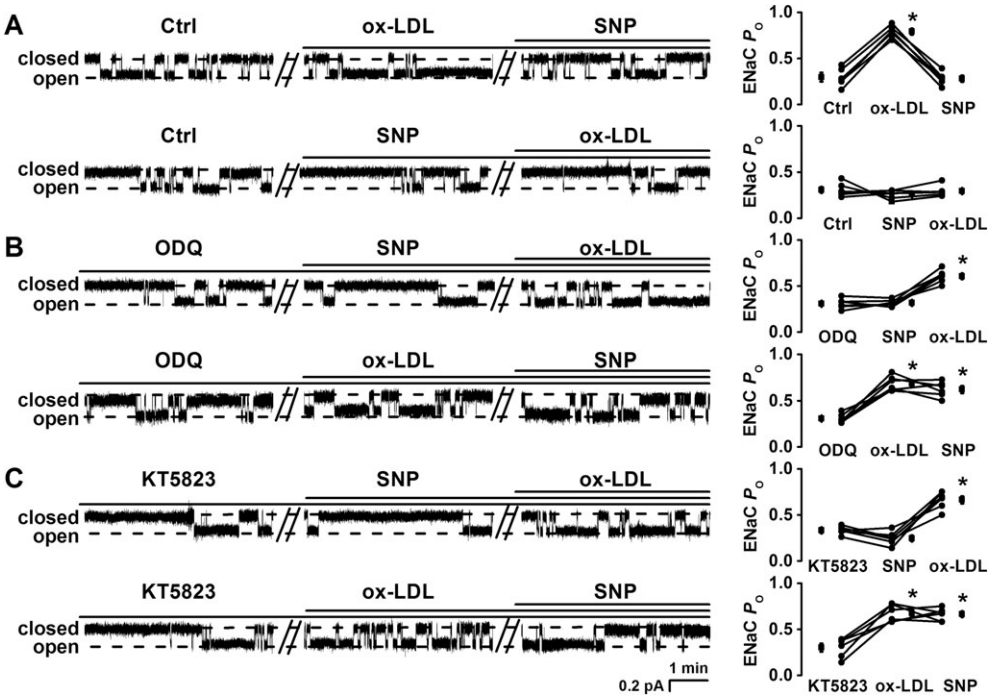

Figure 6

Exogenous NO reverses the ox-LDL-induced increase in ENaC activity via sGC/PKG. (A–C) Representative ENaC single-channel currents recorded in endothelial cells (left) and summarized ENaC P_O (right); basal ENaC activity in the same patch served as its own control (Ctrl). (A) SNP (an NO donor; 1 mM) reversed the increased ENaC activity induced by 10 μg·mL^{-1} ox-LDL under Ctrl conditions, no matter whether ox-LDL was applied to the cell first (upper trace) or SNP was applied to the cell first (lower trace); (B, C) SNP (1 mM) no longer reversed the increased ENaC activity induced by 10 μg·mL^{-1} ox-LDL in two separate cells; one was pretreated with 10 μM ODQ, an sGC inhibitor (B); the other was pretreated with 10 μM KT5823 (C), no matter whether SNP was applied to the cell first (upper traces in B and C) or ox-LDL was applied to the cell first (lower traces in B and C). *$P < 0.05$ vs. either Ctrl or pre-treament; n = 6 paired experiments.

In the present study, we did not explore the mechanisms by which ROS stimulates ENaC. However, in our previous studies we demonstrated that ROS stimulates ENaC via PI3K/PTEN-mediated increase in phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3) at the apical membrane in distal nephron cells (Ma, 2011; Zhang *et al.*, 2013). Therefore, it is possible that the ox-LDL-induced elevation of ROS might also increase PI(3,4,5)P_3 to stimulate ENaC in endothelial cells. Finally, there is accumulating evidence demonstrating that ox-LDL impairs ACh-mediated vasorelaxation (Valente *et al.*, 2014). Furthermore, we also demonstrated that blockade of ENaC attenuates the impaired EDR in aorta treated with ox-LDL, suggesting a direct involvement of ENaC in the regulation of endothelial and vascular function. Amiloride is known to inhibit the sodium–hydrogen exchanger (NHE). However, the binding affinity of amiloride to ENaC is ~100-fold higher than that to NHE (Weinbrenner *et al.*, 2003). Hence, it is unlikely that the concentration of amiloride (0.5 μM) used in the present study also affected the NHE.

Overall, the majority of data presented here were obtained from *ex vivo* experiments, which have limitations as regards their physiological relevance *in vivo*. Nevertheless, these findings provide the first direct evidence that the mouse thoracic aorta expresses a functional ENaC and that ox-LDL, but not LDL, stimulates ENaC in endothelial cells. The stimulating effects of ox-LDL on endothelial ENaC were significantly inhibited by the NO donor and amiloride. Thus, we suggest that blockade of ENaC or the elevation of NO may be useful approaches to prevent ox-LDL-induced vascular dysfunction.

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Author contributions

Z.-R.Z. conceived and designed the experiments; Z.-R.Z. and H.-P.M. revised the manuscript; C.L. and Q.-S.W. collected and analysed the data and drafted the manuscript; X.Y., N. N., Q.-Q.H., B.-L.Z., X.C., C.-J.Y., B.-L.S. and M.-M.W. collected the data; all authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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