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*Published in:*

Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids

*DOI:*

[10.1016/j.bbalip.2011.12.001](https://doi.org/10.1016/j.bbalip.2011.12.001)

2012

[Link to publication](#)

*Citation for published version (APA):*

Li, J., Cao, Y.-X., Yuan, Z.-Y., & Xu, C.-B. (2012). Minimally modified LDL upregulates endothelin type B receptors in rat coronary artery via ERK1/2 MAPK and NF-kappa B pathways. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1821(4), 582-589. <https://doi.org/10.1016/j.bbalip.2011.12.001>

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4

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**Minimally modified LDL upregulates endothelin type B receptors in rat coronary artery  
via ERK1/2 MAPK and NF- $\kappa$ B pathways**

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## **Abstract**

Minimally modified low density lipoprotein (mmLDL) is a well-known risk factor for coronary artery disease. Upregulation of vascular endothelin type B (ET<sub>B</sub>) receptors on the vascular smooth muscle cells is predicted to be the molecular mechanism that leads to cardiovascular pathogenesis. The objective of the present study was to examine the hypothesis that mmLDL upregulates ET<sub>B</sub> receptors in rat coronary artery. The contractile responses to sarafotoxin 6c (ET<sub>B</sub> receptor agonist) were studied using a sensitive myograph. ET<sub>B</sub> receptor mRNA and protein expression was determined using real-time PCR and Western blot analysis. The results showed that organ culture increased the contractile responses induced by sarafotoxin 6c and the levels of ET<sub>B</sub> receptor mRNA and protein. This increase was further enhanced by the addition of mmLDL (10 µg/mL). Specific ERK1/2 inhibitors (SB386023 and U0126) and an NF-κB inhibitor (wedelolactone) attenuated the mmLDL-increased ET<sub>B</sub> receptor-mediated contraction and ET<sub>B</sub> receptor mRNA and protein levels. Wedelolactone significantly attenuated the mmLDL-decreased IκBα protein expression. Consistent with this result, IκBα protein expression was significantly decreased by culture with mmLDL compared to the level of expression in the organ culture group. However, the JNK inhibitor, SP600125 or p38 pathway inhibitor, SB203580 did not inhibit mmLDL-enhanced effects. The PKC inhibitor, staurosporine attenuated only culture-alone-increased effects. In conclusion, mmLDL upregulates the ET<sub>B</sub> receptors in rat coronary arterial smooth muscle cells, mainly via activation of the ERK1/2 MAPK and the downstream transcriptional factor NF-κB.

**Keywords:** Minimally modified low density lipoprotein; Coronary artery; Endothelin type B receptor; Extracellular signal related kinases 1/2; Nuclear factor-κB

## 1. Introduction

Minimally modified low density lipoprotein (mmLDL) comes from low density lipoprotein (LDL). When only the lipid region of LDL is oxidized, mmLDL will be formed. The mmLDL induces monocyte adhesion to endothelial cells and endothelium dysfunction, promotes the formation of oxidized low density lipoprotein (oxLDL) and foam cells, enhances vascular cell migration and proliferation [1–3], and participates in atherosclerotic lesion formation; these effects increase the risk of cardiovascular disease, such as coronary heart disease, the leading cause of death worldwide. These biological effects occur through a mechanism involving the stimulation of the receptor-mediated signal transduction pathways [4]. The endothelin system plays important roles in cardiovascular disease pathogenesis. There are two types of endothelin receptors in the vessel, endothelin type A (ET<sub>A</sub>) and endothelin type B (ET<sub>B</sub>). The ET receptors on the vascular smooth muscle cells are involved in the atherosclerotic process by enhancing contraction and proliferation of the smooth muscle cells [5–7]. The ET<sub>B</sub> receptors exhibit plasticity to adapt to the environmental changes. The upregulation of ET-1 and its receptors plays an important role in the pathogenesis of coronary vascular diseases and in the formation of atherosclerotic lesions in animals [8,9] and in man [7,10]. The ET<sub>B</sub> receptor is the principal receptor involved in the progression of coronary vascular diseases [5,7]. We previously developed an organ culture model that can mimic the upregulation of ET<sub>B</sub> receptors in cardiovascular disease [11–15]. Using this model, we demonstrated that the upregulation of ET<sub>B</sub> receptors induced by organ culture of arteries is via activation of mitogen-activated protein kinase (MAPK) and the downstream nuclear factor-kappaB (NF-κB) intracellular signal transduction pathways [14,15].

MAPK consists of 3 main pathways, the extracellular signal-regulated protein 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 cascades [16]. The different MAPK pathways represent another group of serine/threonine kinases that act downstream from protein kinase C (PKC) in the smooth muscle cell regulatory cascade [17]. MAPKs play an important role in the intracellular signaling that occurs in response to extracellular stimuli [16]. Activated MAPK causes phosphorylation and activation of transcription factors in the cytoplasm or nucleus [18]. NF- $\kappa$ B is a pivotal transcription factor down-stream of the MAPK and PKC pathways [19–21]. PKC participates in signal transduction events in response to specific hormonal, neuronal and growth factor stimuli [22]. Activation of NF- $\kappa$ B is essential for controlling the inducible expression of several genes involved in inflammation and cell proliferation [23].

Although both mmLDL and ET<sub>B</sub> receptor upregulation are involved in the pathogenesis of coronary heart disease, their relationships are unknown. The effects of mmLDL on ET<sub>B</sub> receptor expression in rat coronary artery and the underlying molecular mechanisms remain unclear. The aim of the present study was to determine if mmLDL upregulates ET<sub>B</sub> receptors in rat coronary artery and if the MAPK-related intracellular signaling pathways are responsible for the upregulation.

## **2. Materials and methods**

### **2.1. Animal**

Sprague–Dawley rats weighing 300–350 g were purchased from the Animal Center of Xi'an Jiaotong University College of Medicine, China. All animals were handled according to the guidelines specified by the Animal Care and Use Committee at Shaanxi Province. The

experimental protocols for using the animals were reviewed and approved by the Animal Ethics Committee at Xi'an Jiaotong University.

## **2.2. Chemicals and reagents**

The mmLDL and LDL were purchased from the Xiehe Research Institute (Beijing, China).

The sarafotoxin 6c (S6c) (Auspep, Parkville, Australia) was dissolved in 0.9% saline with 0.1% bovine serum albumin. The staurosporine, SB386023, U0126, SP600125, SB203580 and

wedelolactone (Sigma, St Louis, MI, USA) were dissolved in DMSO. Analytical grade chemicals and double-distilled water were used throughout the experiments. All drugs were further diluted in buffer solution just before the experiments. The concentrations were expressed as the final molar concentration in the tissue baths.

## **2.3. Preparation of coronary arterial segments and organ culture**

The rats were anesthetized with CO<sub>2</sub>. The hearts were quickly removed and chilled in ice-cold bicarbonate buffer solution (for composition, see below). The left anterior descending coronary artery was excised from the myocardium gently [24], immersed in cold buffer solution, and freed from adhering tissue under a dissection microscope. The vessels were then cut into approximately 1–2 mm long cylindrical segments.

For organ culture, the coronary artery ring segments were placed in 24-well plates, two segments in each well, containing 1 mL of Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and supplemented with penicillin and streptomycin [25]. As organ culture per se enhances the function and expression of ET<sub>B</sub> receptors in the coronary artery [26, 27], we added an organ culture group as a control in the experiments. The culture of the

arterial segments was performed in the mmLDL (10  $\mu$ g/mL) or LDL (10  $\mu$ g/mL). To examine the mechanism of effects, the specific inhibitors of different intracellular signal transduction pathways were used. The inhibitors and mmLDL were added to DME simultaneously at the beginning of the organ culture process. In previous experiments, we found that the addition of 1  $\mu$ l DMSO to the 1 ml DMEM did not modify the upregulation of receptors. The PKC pathway inhibitor was staurosporine (0.1  $\mu$ M) [28], and the inhibitors selected to target different kinases leading to ERK1/2 activation were U0126 (10  $\mu$ M) [13] and SB386023 (10  $\mu$ M) [29]. U0126 is an inhibitor of the MAP kinase/ERK kinase (MEK) 1/2, the MAPKK of ERK1/2, while SB386023 inhibits raf, the MAPKKK of ERK1/2. The specific JNK and p38 MAPK inhibitors were SP600125 (10  $\mu$ M) [30] and SB203580 (10  $\mu$ M) [31], respectively. To inhibit downstream transcription, the NF- $\kappa$ B-inhibitor wedelolactone (10  $\mu$ M) [32] was used. Each inhibitor was added and incubated for 24 h. Thereafter, the artery segments were mounted in myograph baths. For examination by real-time PCR or Western blot, the vessels were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processed.

#### **2.4. Analysis of mmLDL modification**

Incubation of mmLDL in organ culture induces minimal lipoprotein oxidization. We estimated the oxidation rate of mmLDL with 24 h culture by measuring thiobarbituric acid-reactive substances (TBARS). Quantification of TBARS in conditioned media was performed by comparison to a standard curve of malondialdehyde (MDA) equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane in the same conditions as the experimental samples.

#### **2.5. Myograph study**

A myograph was used to record the isometric tension in the isolated coronary arterial segments. The vessel segments were threaded on two 40- $\mu$ m-diameter stainless steel wires and mounted in a Mulvany–Halpern myograph (Danish Myo Technology A/S, Denmark). One wire was connected to a force displacement transducer attached to an analog-digital converter unit (ADInstruments, Hastings, U.K.). The other wire was attached to a movable displacement device allowing fine adjustments of the vascular tension by varying the distance between the wires. The experiments were recorded using the software program Chart™ (ADInstruments, U.K.). The arterial segments were immersed in temperature-controlled (37 °C) tissue baths containing Krebs's solution. The solution was continuously bubbled with 5% CO<sub>2</sub> in O<sub>2</sub>, resulting in a pH of 7.4. The arterial segments were given an initial tension of 1.2 mN and were left alone to adjust to this level of tension for 1 h. The contractile capacity was determined by exposure to a potassium-rich (63.5 mM) Krebs's solution that had the same composition as the solution except that NaCl was exchanged for KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the segments were used only if potassium elicited reproducible responses over 0.8 mN. Concentration–response curves were obtained by cumulative application of the ET<sub>B</sub> receptor agonist S6c (10<sup>-10</sup> M–10<sup>-7</sup> M).

## **2.6. Real-time PCR**

Total RNA was extracted from fresh or cultured coronary artery segments using the RNeasy 200 kit (Qiagen, Shanghai, China) according to the supplier's instructions. The resulting pellet was finally washed with 75% ethanol, air-dried, and dissolved in 40  $\mu$ L diethylpyrocarbonate-treated water. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer Applied Biosystems) in a Perkin-Elmer DNA thermal cycler. First strand cDNA was synthesized from total RNA in a 40  $\mu$ L reaction



volume with random hexamers as primers. The reaction mixture was incubated at 25 °C for 10 min, heated to 42 °C for 15 min, heated further to 99 °C for 5 min, and chilled to 5 °C for 5 min. Real-time PCR was performed in a GeneAmp 5700 sequence detection system using the GeneAmp SYBR GREEN kit (Toyobo Co., LTD, Osaka, Japan) with the previously synthesized cDNA as the template in a 25 µL reaction volume. A no-template control was included in all experiments. All primers were designed using the Primer Express 2.0 software (Applied Biosystems) and synthesized by Beijing Sunbiotech Co. Ltd (Beijing, China). Specific primers for the ET<sub>B</sub> receptor (GenBank accession no. NM\_017333) were as follows: ET<sub>B</sub> receptor forward primer: 5'-TGACGCCACCCACTAAGACC-3'

reverse primer: 5'-GGCACGGAGGAGGGAAGG-3'

β-actin (GenBank accession no. NM\_031144) mRNA was used as a house-keeping gene. The primers used were as follows:

β-actin forward: 5'-ACTATCGGCAATGAGCGGTTCC-3'

reverse: 5'-CTGTGTTGGCATAGAGGTCTTTACG-3'

The real-time PCR was carried out with the following profile: 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s, at 60 °C for 15 s and at 72 °C for 45 s. Dissociation curves were run after the real-time PCR to identify the specific PCR products.

## **2.7. Western blotting**

Each sample was a pool of 4 coronary arteries in Western blots. Fresh or cultured coronary artery segment samples were frozen in liquid nitrogen and immersed in cold RIPA lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) (Upstate USA, Inc., Charlottesville, VA USA) containing complete protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN, USA). Total protein was quantified using the BCA Protein Assay Kit (Pierce Biotechnology) according to the manufacturer's

instructions, separated on SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk. The immunoblots were incubated with primary antibodies for ET<sub>B</sub> (1:100) (Millipore, CA, USA) and IκBα (1:1000) (Proteintech, USA). Detection of protein bands was performed using Super Signal Chemiluminescent Substrate (Pierce Biotechnology) after incubation with a horseradishperoxidase-conjugated secondary antibody (1:5000). β-actin was used as an internal loading control. Densitometric analysis was performed using Image Gauge Ver. 4.0 (Fuji Photo Film Co., Ltd, Japan).

## 2.8. Calculations and statistics

The E<sub>max</sub> values refer to maximum contraction calculated as a percentage of the contractile capacity of 63.5 mM K<sup>+</sup> and the pEC<sub>50</sub> values refer to the negative logarithm of the molar concentration that produced half-maximum contraction. Each agonist's concentration–effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism 4, San Diego, USA), to provide estimates of the E<sub>max</sub> and pEC<sub>50</sub> values. All real-time PCR experiments were performed in duplicate and the mean values were used. The amount of ET<sub>B</sub> receptor mRNA was calculated relative to the mRNA expression of the housekeeping gene β-actin in the same sample. The following formula was used for calculating the amount of ET<sub>B</sub> receptor mRNA:  $X_0/R_0 = 2^{CtR - CtX}$ , where X<sub>0</sub> = original amount of endothelin ET<sub>B</sub> receptor mRNA, R<sub>0</sub> = original amount of β-actin mRNA, CtR = C<sub>T</sub>-value for β-actin and CtX = C<sub>T</sub>-value for the endothelin ET<sub>B</sub> receptor. The amount of receptor protein relative to the internal control is expressed as a percentage of the control group. All data are expressed as the mean ± SEM. Student's t-test was used to compare two sets of data and one-way analysis of variance (ANOVA) or two-way ANOVA followed by Dunnett's test (GraphPad Prism) for comparisons of more than two data sets. A p-value less than 0.05 was considered to be significant. The decreased ratio is expressed as an equation:

$$\text{Decreased ratio} = \frac{\text{Value}_{(\text{mmLDL/DMSO})} - \text{Value}_{(\text{mmLDL/DMSO+inhibitor})}}{\text{Value}_{(\text{mmLDL/DMSO})}} \times 100\%.$$

### 3. Results

#### 3.1. Analysis of mmLDL modification

In the present study, the MDA values of mmLDL before and after 24 h of culture were  $10.84 \pm 0.79$  nM MDA/mg LDL protein and  $14.02 \pm 1.73$  nM MDA/mg LDL protein ( $p > 0.05$ ,  $n = 3$ ).

#### 3.2. Effect of mmLDL on upregulation of the ET<sub>B</sub> receptors

The viability and general contractility of the arteries during organ culture in the presence and absence of mmLDL and LDL and inhibitors were examined by their contractile responses to 63.5 mM K<sup>+</sup>. There were no significant differences in the E<sub>max</sub> of the contractile responses induced by K<sup>+</sup> among the groups (i.e., fresh  $1.88 \pm 0.12$  mN, organ culture  $2.04 \pm 0.15$  mN, organ culture in the presence of mmLDL  $1.98 \pm 0.16$  mN, organ culture in the presence of LDL  $1.84 \pm 0.17$  mN,  $n = 8$ ,  $p > 0.05$ ).

In the fresh coronary arteries, S6c induced only negligible contractions. After organ culture for 24 h, S6c induced strong contraction of coronary arteries in a concentration dependent manner, with an E<sub>max</sub> value of  $163\% \pm 15\%$  and a pEC<sub>50</sub> value of  $8.37 \pm 0.10$  (Fig. 1A).

Culture with LDL had a trend to shift the S6c-induced concentration–contraction curve of organ culture toward the left, but there was no significant difference of either E<sub>max</sub> of  $188\% \pm 14\%$  or pEC<sub>50</sub> of  $8.39 \pm 0.08$  between LDL group and organ culture control group ( $p > 0.05$ ,  $n = 8$ ) (Fig. 1A), suggesting that organ culture per se enhances the contraction of the coronary artery induced by S6c. Culture with mmLDL further shifted the S6c-induced concentration–contraction curve of organ culture artery toward the left and significantly

increased  $E_{\max}$  ( $264\% \pm 20\%$ ,  $p < 0.01$ ) and  $pEC_{50}$  ( $8.75 \pm 0.10$ ,  $p < 0.05$ ,  $n=8$ ) (Fig. 1A), suggesting that mmLDL enhances the contraction of the coronary artery induced by S6c.  $ET_B$  receptor mRNA and protein expression was assessed using real-time PCR and Western blot. There were low levels of the  $ET_B$  receptor mRNA and protein in fresh coronary artery segments. Organ culture alone elevated the  $ET_B$  receptor mRNA and protein levels compared to the fresh group ( $p < 0.01$ , Fig. 1B–D). Furthermore, mmLDL significantly enhanced the organ-culture-elevated mRNA and protein levels of  $ET_B$  receptors (Fig. 1B–D).

### **3.3. Inhibition of PKC on mmLDL-upregulated $ET_B$ receptors**

Culture with mmLDL and staurosporine, a specific inhibitor of PKC, markedly inhibited the mmLDL-induced enhancement of the contractile response to S6c, and decreased  $E_{\max}$  from  $264\% \pm 20\%$  in the mmLDL group to  $204\% \pm 19\%$  ( $p < 0.01$ ;  $n=8$ ). Staurosporine also markedly reduced the culture-alone-induced enhancement of the contractile response to S6c, with decreasing  $E_{\max}$  from  $163\% \pm 15\%$  in the organ culture group to  $110\% \pm 10\%$  ( $p < 0.01$ ,  $n=8$ ) (Fig. 2A). The percentage decrease staurosporine caused in the mmLDL-induced enhancement of contractile response was 22.7%, and the percentage decrease staurosporine caused in the organ-culture-induced enhancement of contractile response was 32.5%.

The  $ET_B$  receptor mRNA and protein expression in the coronary arterial smooth muscle cells was examined after culture with staurosporine. The results showed that staurosporine inhibited both the organ culture- and mmLDL-culture-induced elevation of the  $ET_B$  receptors mRNA (Fig. 2B) and protein levels (Fig. 3). The percentage decrease staurosporine caused in both the mmLDL-enhanced and organculture-enhanced mRNA expression was 27.3%. The percentage decreases staurosporine caused on the mmLDL-enhanced and organ culture-enhanced protein levels were 35.6% and 30.9%, respectively (Fig. 3).

### 3.4. Inhibition of MAPK on mmLDL-upregulated ET<sub>B</sub> receptors

Culture with mmLDL and specific inhibitors for ERK1/2 (SB386023 and U0126) totally attenuated the mmLDL-enhanced contractile responses and partly attenuated the organ-culture-enhanced contractile responses of the coronary artery to S6c. SB386023 and U0126 depressed the  $E_{\max}$  of the mmLDL-cultured artery from  $264\% \pm 20\%$  to  $106\% \pm 10\%$  and  $121\% \pm 12\%$  ( $p < 0.01$ ,  $n=8$ ) (Fig. 4A–B). However, the JNK inhibitor SP600125 and p38 inhibitor SB203580 had no obvious effects on the mmLDL-enhanced contractile responses to S6c (Fig. 4C–D).

SB386023 and U0126 markedly reduced the organ-culture-enhanced contractile response to S6c, exhibiting a decreased  $E_{\max}$  (SB386023  $77\% \pm 6\%$ , U0126  $80\% \pm 9\%$ ;  $p < 0.01$ ;  $n=8$ ) (Fig. 4A–B). SP600125 and SB203580 had no significant inhibitory effects on the organ-culture-enhanced contractile response to S6c ( $E_{\max}$ : SP600125  $235\% \pm 20\%$ , SB203580  $222\% \pm 19\%$ ;  $p > 0.05$ ;  $n=8$ ) (Fig. 4C–D).

The ET<sub>B</sub> receptor mRNA and protein expression in the coronary arteries after culture with MAPK inhibitors was measured using real-time PCR and Western blot analysis. The results showed that the inhibitors for ERK1/2 (SB386023 and U0126) reduced the increases in ET<sub>B</sub> receptor mRNA expression induced by culture with or without mmLDL, and the decreased degrees were similar in both groups (Fig. 4E). Also, SB386023 and U0126 totally abolished the mmLDL-increased ET<sub>B</sub> receptor protein expression and partly attenuated the organ-culture-increased ET<sub>B</sub> receptor protein expression (Fig. 3). In the presence of inhibitors, the reduction of ET receptor-mediated contraction paralleled the reduced expressions of the ET<sub>B</sub> receptor mRNA and protein. Although both the JNK inhibitor SP600125 and p38 MAPK

inhibitor SB203580 slightly reduced the increased expression of ET<sub>B</sub> receptor mRNA (Fig. 4E) and protein (Fig. 3) induced by culture with or without mmLDL groups, there was no significant difference between with or without inhibitors.

### **3.5. Inhibition of NF- $\kappa$ B on mmLDL-upregulated ET<sub>B</sub> receptors**

Wedelolactone, a specific NF- $\kappa$ B pathway inhibitor, shifted the mmLDL-enhanced concentration–contraction curve of the coronary artery induced by S6c toward the right, decreasing the E<sub>max</sub> to 111% $\pm$ 9% from 264% $\pm$ 20% and the pEC<sub>50</sub> to 8.31 $\pm$ 0.09 from 8.75 $\pm$ 0.10 in the mmLDL group ( $p < 0.01$ ,  $n = 8$ ). Wedelolactone also markedly decreased the culture-alone-enhanced contractile response to S6c, decreasing the E<sub>max</sub> to 79% $\pm$ 8% compared to that of the culture alone (163% $\pm$ 16%,  $p < 0.01$ ,  $n = 8$ ) (Fig. 5A). Wedelolactone reduced the mmLDL-enhanced and organ-culture-enhanced contractile responses by 58.0% and 51.5%, respectively.

The mRNA and protein expression levels in the coronary artery showed that wedelolactone almost totally inhibited the mmLDL-increased expression of ET<sub>B</sub> receptors and partly attenuated the organ-culture-increased expression of the ET<sub>B</sub> receptors (Fig. 5B; Fig. 3). In the presence of wedelolactone, the reduction of the ET<sub>B</sub> receptor-mediated contraction paralleled the reduced expression of the ET<sub>B</sub> receptor mRNA and protein.

Wedelolactone inhibits NF- $\kappa$ B-mediated gene transcription in cells by blocking the phosphorylation and degradation of I $\kappa$ B $\alpha$ . In the present study, we examined the level of I $\kappa$ B $\alpha$  protein. The results showed that organ culture decreased the I $\kappa$ B $\alpha$  protein level compared to that of the fresh group, and this decrease was further enhanced by addition of mmLDL (Fig. 5C). In the presence of wedelolactone, the decreased I $\kappa$ B $\alpha$  protein levels were significantly

increased in these two groups ( $p < 0.05$ ,  $p < 0.05$ , Fig. 5C), and the percentage decreases were 49.7% in the mmLDL group and 31.9% in the culture alone group.

#### **4. Discussion**

The upregulation of ET<sub>B</sub> receptors plays an important role in the development of coronary heart disease [8,9,11,33,34]. Organ culture of mesenteric or cerebral arteries results in ET<sub>B</sub> receptor upregulation that is similar to that seen in vivo. Organ culture provides a model for studies of the mechanisms involved in enhanced expression of ET<sub>B</sub> receptors and the underlying molecular mechanisms. The present study showed that organ culture increased the S6c-induced contraction of coronary artery and the expression levels of ET<sub>B</sub> receptor mRNA and protein, suggesting that organ culture upregulates the ET<sub>B</sub> receptors of the rat coronary artery, which agrees well with previous studies [26,27]. The present study also showed that LDL did not significantly increase the contractility of coronary arterial segments, suggesting that LDL may not affect ET<sub>B</sub> receptor regulation. It should be pointed out that the aim of the present study was to investigate the effects of mmLDL, and the organ culture group was used as a control. We further demonstrated that culture with mmLDL induced a higher contractile response to S6c in coronary arterial segments and a greater increase in ET<sub>B</sub> receptor mRNA and protein expression in coronary artery than did the organ culture group. These findings show that mmLDL upregulates ET<sub>B</sub> receptors in the rat coronary artery. The upregulation of ET<sub>B</sub> receptors has been shown in experimental ischemic stroke, coronary artery infarction and in human atherosclerotic plaque [7,11].

It is well known that there is a significant relationship between receptor upregulation and its stimulating factors. PKC takes part in signal transduction events in response to stimuli. In the present study, the PKC inhibitor staurosporine had significant effects on both the organ

culture-induced and mmLDL-induced increases in ET<sub>B</sub> receptor contractile function and expression. The percentage decrease caused by staurosporine in the mmLDL-enhanced contractile response was not higher than that of the culture alone-enhanced contractile response. The percentage decrease in the mmLDL-increased mRNA expression caused by staurosporine was the same as that of organ culture. Here, the results showed that the PKC pathway was involved in organ culture upregulation of ET<sub>B</sub> receptors, which agreed well with previous studies [26,35–37], but did not suggest that the PKC pathway was involved in the mmLDL upregulation of the ET<sub>B</sub> receptors.

By using different MAPK inhibitors, the present study demonstrated that the ERK1/2 inhibitors SB386023 and U0126 almost completely abolished the mmLDL-increased contractile function and protein expression of ET<sub>B</sub> receptors and partially attenuated the organ culture-increased contractile function and expressions of ET<sub>B</sub> receptors. In the presence of inhibitors, the percentage decrease in the contractile effects, mRNA and protein levels in the mmLDL group was more potent than that in the organ culture group. The results suggest that the mmLDL-induced upregulation of ET<sub>B</sub> receptors is related to the ERK1/2 pathway.

However, JNK inhibitor SP600125 and p38 inhibitor SB203580 had no obvious effect on organ culture-induced and mmLDL-induced increases of contraction function, mRNA and protein expression of ET<sub>B</sub> receptors. In some previous studies, the p38 MAPK was not involved in organ culture-induced elevated contractile effects of ET<sub>B</sub> receptors in rat middle cerebral arteries and porcine coronary arteries [12,26], and JNK pathway was not involved in DSP-induced elevated contractile effects and expressions of ET<sub>B</sub> receptor in rat basilar artery [38]. Therefore, it suggested that JNK and p38 MAPK signaling pathways didn't appear to be involved in the upregulation of ET<sub>B</sub> receptors induced by mmLDL.



The NF- $\kappa$ B pathway plays a pivotal role in controlling the inducible expression of genes that contribute to a variety of biological functions, including cell survival, cell proliferation and contraction [39]. The classic form of NF- $\kappa$ B is composed of a heterodimer of the p65 and p50 subunits, preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the I $\kappa$ B family. The canonical pathway of NF- $\kappa$ B activation involves the removal of I $\kappa$ Bs under a variety of stimuli. Following I $\kappa$ B degradation, the p65–p50 heterodimer is released from the I $\kappa$ B–NF- $\kappa$ B complex and translocates into the nucleus where specific binding sites are located within target gene promoters [32,40]. Wedelolactone, one inhibitor of I $\kappa$ B kinase, can inhibit the NF- $\kappa$ B pathway by blocking the phosphorylation and degradation of I $\kappa$ B $\alpha$ . In the present study, wedelolactone attenuated the organ culture- and mmLDL-induced increases in contraction function and in expression of ET<sub>B</sub> receptor mRNA and protein. The decrease was greater in the mmLDL group than in the organ culture group, suggesting that NF- $\kappa$ B may be involved in the process of mmLDL-induced upregulation of ET<sub>B</sub> receptors. Benes recently pointed out that wedelolactone can act as growth suppressor of the cell cycle, in addition to activating the NF- $\kappa$ B pathway [41]. Organ culture significantly decreased the I $\kappa$ B $\alpha$  protein level compared to that of the fresh group, and this decrease was further enhanced by addition of mmLDL. When wedelolactone was added, the decreased I $\kappa$ B $\alpha$  protein levels were significantly increased in these two groups, and the percentage increase was higher in the mmLDL group than in the organ culture group. The results suggested that organ culture with mmLDL activates the NF- $\kappa$ B pathway by degradation of the I $\kappa$ B $\alpha$  protein.

Our findings are supported by a study in porcine [26] coronary arteries demonstrating that MAPK-related pathways appear to be involved in organ culture upregulation of ET<sub>B</sub> receptors. In human ischemic heart disease, there is upregulation of endothelin receptor expression [11]

in the coronary arteries. Culture of human internal mammary arteries induces PKC- and MAPK-dependent upregulation of ET<sub>B</sub> receptors in the smooth muscle cells [42]. The results agree well with our previous studies demonstrating that organ culture of rat mesenteric and cerebral arteries induces upregulation of ET<sub>B</sub> receptors in the smooth muscle cells, and this upregulation occurs via activation of NF-κB [14] and ERK1/2 MAPK [29,43]. When LPS is added to the culture, the upregulation of ET<sub>B</sub> receptors can be further increased in the coronary arteries [44]. Similar results are seen in rat mesenteric arteries when lipid-soluble smoke particles are presented in the process of organ culture. The increased expression of ET<sub>B</sub> receptors caused by the smoke particles in the vascular smooth muscle cells occurs via activation of the MAPK and NF-κB signaling pathways [15]. Taken together, these data demonstrate that when risk factors (mmLDL, LPS and smoke particles), organ culture, and ischemia induce damage to the arteries, there is activation of intracellular signaling molecules, such as MAPK and NF-κB, and subsequent upregulation of ET<sub>B</sub> receptors. This phenomenon is seen in the process of cardiovascular disease.

In conclusion, mmLDL upregulates the ET<sub>B</sub> receptors in coronary arterial smooth muscle cells via activation of the ERK1/2 MAPK and the transcription factor NF-κB. Understanding the underlying molecular mechanisms may provide new options for treating coronary heart disease.

### **Acknowledgements**

This work was supported by the National Natural Science Foundation of China (30772566, 81173059).

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## Figure legends

### Figure 1

The mmLDL up-regulated ET<sub>B</sub> receptors in rat coronary artery. After coronary artery rings were cultured with mmLDL (10 µg/mL) or natural LDL (10 µg/mL) for 24 h, the concentration–contraction curves of the artery segments were induced by sarafotoxin 6c. Organ culture shifted the curves of the fresh artery induced by sarafotoxin 6c toward the left, which was further shifted toward the left by culture with mmLDL, but not natural LDL (A, n=8 artery ring segments). The mRNA (B, n=5–6 coronary arteries) and protein levels (C–D, n=4 samples, each sample being a pool of 4 coronary arteries) of the ET<sub>B</sub> receptors in the coronary artery were measured after 24 h of culture with mmLDL. Data are presented as the mean±S.E.M. \*\*p<0.01 vs. fresh group, ##p<0.01 vs. culture group and LDL group.

### Figure 2

The PKC pathway involved in organ culture-induced the increase in contractile function and mRNA levels of ET<sub>B</sub> receptors in rat coronary artery. After the coronary artery rings were cultured with or without mmLDL (10 µg/mL) in the presence of or absence of PKC inhibitor staurosporine (0.1 µM) for 24 h, the concentration–contraction curves of the rings mediated by ET<sub>B</sub> receptors (A, n=8 ring segments) and the mRNA levels of the ET<sub>B</sub> receptors (B, n=5–6 coronary arteries) were showed. Staurosporine inhibited both the organ-culture-induced and mmLDL-induced increases in ET<sub>B</sub> receptor contractile function and expression. Data are presented as the mean±S.E.M. \*p<0.05, \*\*p<0.01 vs. mmLDL, #p<0.05, ##p<0.01 vs. culture.

### Figure 3

The mmLDL increased the ET<sub>B</sub> receptor protein expression levels in the coronary artery and the inhibitors affected the increased expression. Rat coronary arteries were cultured with or

without mmLDL (10  $\mu$ g/mL) in the presence of or absence of PKC inhibitor staurosporine, ERK1/2 inhibitors SB386023, U0126, JNK inhibitor SP600125, p38 inhibitor SB203580, NK- $\kappa$ B inhibitor wedelolactone for 24 h. The protein levels of the ET<sub>B</sub> receptors were determined by Western blot. The results were expressed as mean $\pm$ S.E.M. relative to  $\beta$ -actin levels, n=3–4 coronary arteries. \*\*p<0.01 vs. mmLDL, ##p<0.01 vs. culture (without mmLDL).

#### **Figure 4**

The MAPK pathway involved in mmLDL-induced the increase in contractile function and mRNA levels of ET<sub>B</sub> receptors in the coronary artery. After rat coronary arteries were cultured with or without mmLDL (10  $\mu$ g/mL) in the presence of or absence of MAPK inhibitors for 24 h, the ERK1/2 inhibitors SB386023(A), U0126(B), JNK inhibitor SP600125(C), and p38 inhibitor SB203580 (D) on the S6c-induced concentration–contraction curves were constructed (n=8 ring segments). Effects of the MAPK inhibitors on the mRNA levels of the ET<sub>B</sub> receptors were shown (E, n=5–6 coronary arteries). Data are presented as the mean $\pm$ S.E.M. \*\*p<0.01 vs. mmLDL, ##p<0.01 vs. culture (without mmLDL).

#### **Figure 5**

The NF- $\kappa$ B pathway involved in mmLDL-induced upregulation of ET<sub>B</sub> receptors in the coronary artery. Rat coronary artery segments were cultured with or without mmLDL (10  $\mu$ g/mL) in the presence of or absence of the NF- $\kappa$ B inhibitor wedelolactone (10  $\mu$ M) for 24 h. The concentration–contraction curves mediated by ET<sub>B</sub> receptors (A, n=8 segments), the mRNA levels of the ET<sub>B</sub> receptors (B, n=6 coronary arteries) and the protein levels of the I $\kappa$ B $\alpha$  (C, n=3 samples, each sample being a pool of 4 coronary arteries) were shown. Data are presented as the mean $\pm$ S.E.M. \*p<0.05, \*\*p<0.01 vs. mmLDL; #p<0.05, ##p<0.01 vs. culture.

Figure 1

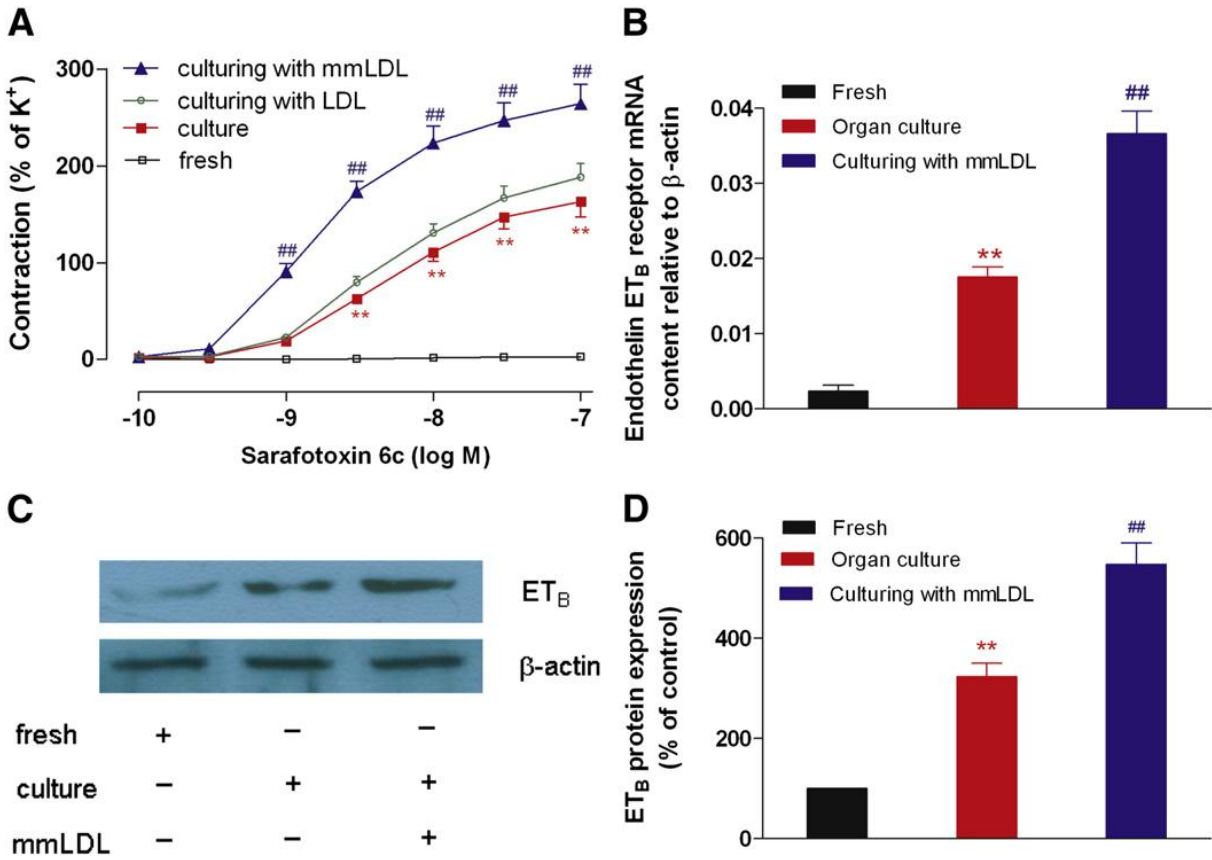


Figure 2

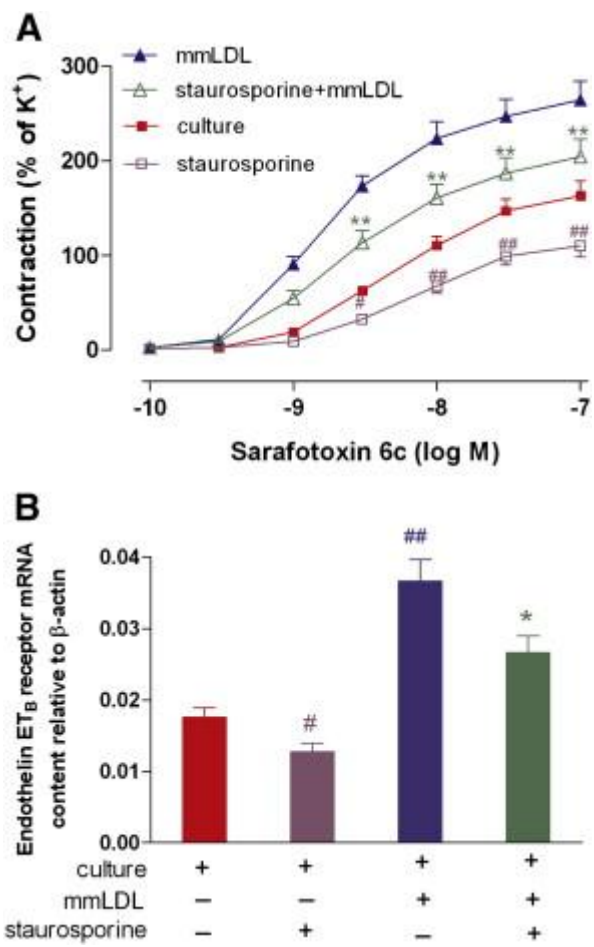


Figure 3

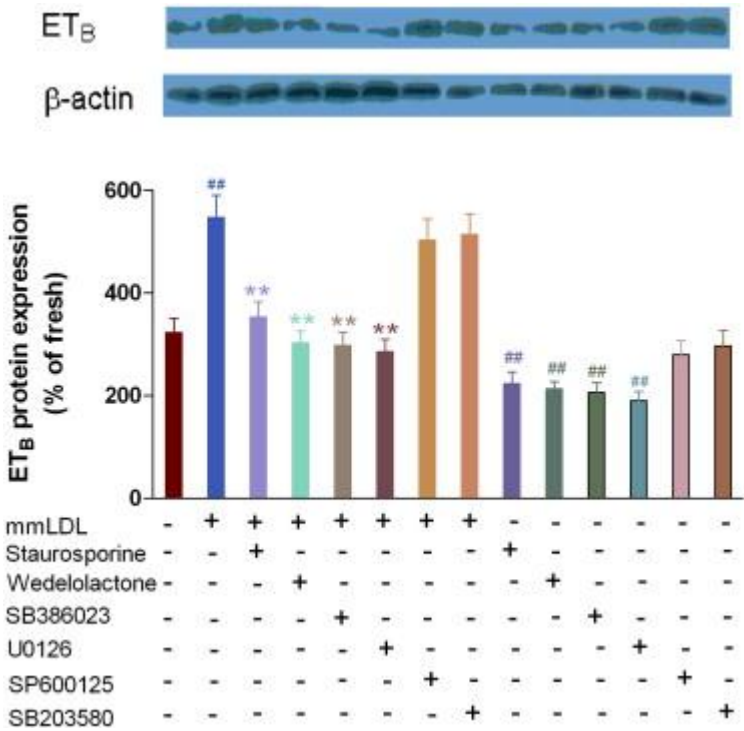


Figure 4

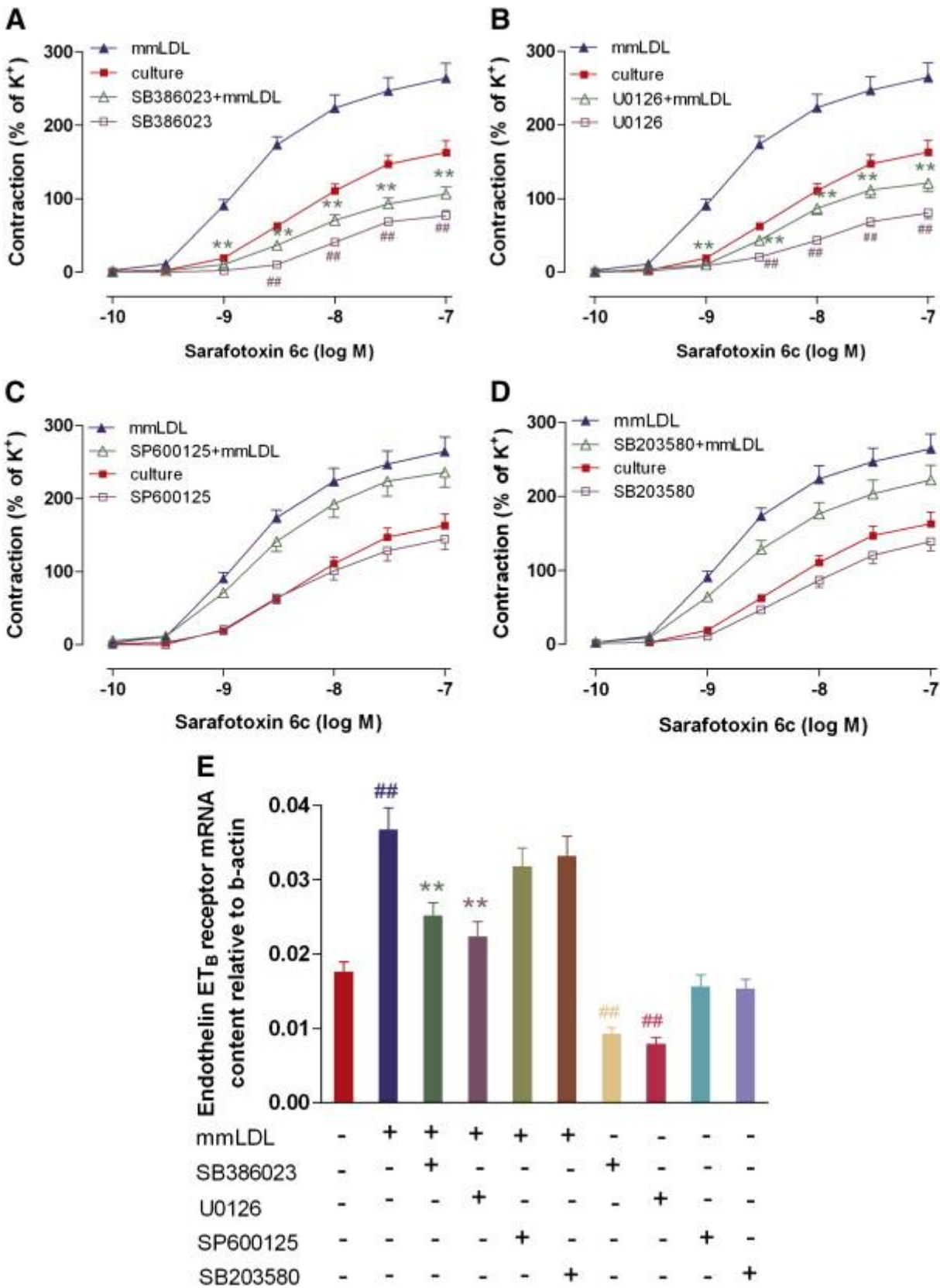


Figure 5

