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# Activation of nuclear factor- $\kappa$ B pathway is responsible for tumor necrosis factor- $\alpha$ -induced up-regulation of endothelin B2 receptor expression in vascular smooth muscle cells in vitro.

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## Abstract

The endothelin B2 (ETB2) receptors are induced in vascular smooth muscle cells (VSMCs) in cardiovascular diseases. We tested if in vitro short-term exposure to the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) could up-regulate ETB2 receptors in rat mesenteric arteries, and if this effect is through activation of intracellular nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway. The mesenteric arteries were dissected from male Sprague-Dawley rats and the endothelium was removed. The arteries were co-incubated with TNF- $\alpha$  in serum-free Dulbecco's modified Eagle's medium. Real-time reverse transcription-PCR, Western blot and immunohistochemical staining were employed to assess the mRNA/protein expression of ETB2 receptors and activation of NF- $\kappa$ B pathway. The results showed that, during organ culture, TNF- $\alpha$  concentration-dependently enhanced ETB2 receptors expression at both mRNA and protein levels, paralleled with activation of NF- $\kappa$ B pathway in VSMC. The up-regulated ETB2 receptor expression and NF- $\kappa$ B activation could be effectively suppressed by general transcriptional inhibitor actinomycin D, or either of the selective I $\kappa$ B kinase inhibitors wedelolactone and IMD-0354. Conclusively, the activation of intracellular NF- $\kappa$ B pathway is responsible for the up-regulation of ETB2 receptors induced by short-term exposure to TNF- $\alpha$ . This could partly explain the toxic effects of TNF- $\alpha$  on VSMCs that account for cardiovascular diseases.

## Highlights

We examined the mechanism of TNF- $\alpha$  on regulating ETB2 receptors during organ culture. TNF- $\alpha$  augmented ETB2 receptor expression and activated NF- $\kappa$ B pathway in VSMC. The inhibition of transcription or NF- $\kappa$ B abolished the ETB2 receptor up-regulation. NF- $\kappa$ B pathway is responsible for the ETB2 receptor up-regulation by TNF- $\alpha$ .

## Keywords

Tumor necrosis factor- $\alpha$ ; Endothelin B2 receptor; Nuclear factor- $\kappa$ B; Vascular smooth muscle cell.

## 1. Introduction

The endothelin system plays important roles in vasomotion and proliferation of the vascular smooth muscle cells (VSMCs), and its disorders are involved in pathogenesis of atherosclerosis and other cardiovascular diseases (CVDs). Endothelin receptors have so far been classified as subtypes A, B and C (ETA, ETB and ETC), respectively (Arai et al., 1990, Douglas et al., 1995 and Karne et al., 1993). The ETA receptors are located on VSMC and mediate vasoconstriction and proliferation through binding with endogenous ligands like endothelin-1 and -2 (ET-1 and ET-2) (Davenport, 2002 and Janakidevi et al., 1992). Whereas the ETB receptors are primarily expressed in vascular endothelial cells (VECs) and mediate vasodilatation of VSMC through the release of nitric oxide (NO) and prostacyclin I<sub>2</sub> (PGI<sub>2</sub>) (Marasciulo et al., 2006 and Schneider et al., 2007).

However, the role of ETB receptors may switch from vasodilatation (relaxing phenotype, termed ETB1) to vasoconstriction (contractile phenotype, termed ETB2) under certain pathophysiological conditions (Stenman et al., 2002 and Xu et al., 2010). The different subpopulations of ETB receptors exert distinct biological effects. The ETB2 receptors are not expressed in VSMC under physiological conditions, but under pathophysiological conditions, they could be induced and mediate vasoconstriction and proliferation, which is closely associated with atherosclerotic CVDs (Janakidevi et al., 1992). The up-regulation of ETB2 receptors in VSMC was observed in animal models with subarachnoid hemorrhage (Hansen-Schwartz et al., 2003), cerebral ischemia (Stenman et al., 2002), hypertension (Fink et al., 2007) and diabetes (Kelly-Cobbs et al., 2011), as well as in patients with peripheral artery disease (Lind et al., 1999), severe pulmonary hypertension (Bauer et al., 2002), subarachnoid hemorrhage (Hansen-Schwartz et al., 2003), ischemic heart disease (Dimitrijevic et al., 2009) and after cerebral ischemic stroke (Stenman et al., 2002). Interestingly, up-regulation of ETB2 receptors was also found in in vitro organ culture model of rat mesenteric arteries after long-term exposure (1–5 days) to serum-free medium (Adner et al., 1998). Recent studies demonstrated that addition of lipid-soluble cigarette smoke particles (Huang et al., 2010 and Xu et al., 2008), or low-density lipoprotein (Xu et al., 2010) led to increased ETB2 receptors expression and/or enhanced ETB2 receptor-mediated vasoconstriction. These in vitro findings expanded the knowledge regarding the importance of ETB2 receptor up-regulation in the development of atherosclerotic CVDs. The up-regulated expression of ETB2 receptors resulted in potent vasoconstriction mediated by selective ETB receptor agonist sarafotoxin 6C or endogenous non-selective ETA and ETB receptor agonist ET-1, and thus modulated vascular tone and accelerated the proliferation of the VSMCs, which account for the pathogenesis of CVDs (Xu et al., 2010).

Inflammatory progress has been considered to be an important cause of atherosclerotic CVDs (Ross, 1999), accounting for the whole pathogenesis of atherosclerosis. The elevated serum levels of pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are one of the typical characteristics of inflammation. Cytokine-induced activation of intracellular signals lead to biological effects that are critical for pathophysiological changes in the VSMC. It was reported that, cytokines resulted in contractility changes involving ETB2 receptor-mediated vasoconstriction in rat mesenteric arteries, indicating that the receptor expression was increased (Uddman et al., 1999). However, the up-regulation of ETB2 receptor expression induced by TNF- $\alpha$  has not been clearly demonstrated and the underlying mechanisms remained unclear. Hence we hypothesized that TNF- $\alpha$  might exert toxic effects to the VSMC via up-regulating ETB2 receptors through activation of its classic down-stream intracellular signal, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, because NF- $\kappa$ B is very critical for inflammatory response and regulation of gene expression (Wang et al., 2009). By using the in vitro organ culture model of rat mesenteric arteries, we assessed the ETB2 receptor expression and intracellular NF- $\kappa$ B pathway activation in VSMC after short-term exposure to TNF- $\alpha$ , and the results

supported our view that the activation of NF- $\kappa$ B pathway is responsible for the TNF- $\alpha$ -induced ETB2 receptor up-regulation.

## 2. Materials and methods

### 2.1. Medium and reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Gibco BRL (Life Technologies, Gaithersburg, MD, USA). Recombinant rat TNF- $\alpha$  was obtained from Life Technologies (Camarillo, CA, USA). IMD-0354 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Wedelolactone (WDL) and actinomycin D (AcD) were obtained from Calbiochem (San Diego, CA, USA). TNF- $\alpha$  was reconstituted in double distilled water. All other reagents were dissolved in dimethyl sulphoxide.

### 2.2. Tissue preparation and organ culture procedure

The mesenteric arterial segments from healthy rats were dissected and cultured as described previously (Zhang et al., 2008). Briefly, the superior mesenteric artery was gently removed, immersed into cold standard buffer solution and freed of adhering tissue under a dissection microscope. The endothelium was denuded by the vessel perfusion in 0.1% Triton X-100 for 10 s, followed in a standard buffer solution for another 10 s. The vessels were then cut into 1 mm long cylindrical segments and incubated in 24-well plates in DMEM medium containing l-glutamine (584 mg/L) and supplemented with penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL) at 37 °C in 5% CO<sub>2</sub>. TNF- $\alpha$  (1–100  $\mu$ g/L) or equivalent volume of vehicle (water) was added to the culture medium. Ethics approval was obtained from the First Affiliated Hospital of Xiamen University.

### 2.3. Real-time reverse transcription (RT)-PCR

Total RNA was extracted from arterial segments by using E.Z.N.A. Tissue RNA Kit (Omega Bio-Tek, Doraville, GA, USA). Reverse transcription of total RNA to cDNA was carried out with ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction.

Real-time quantitative PCR was performed with LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) in a Light Cycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany). PCR reaction was performed in a 20  $\mu$ L volume in 96 LightCycler 480 Multiwell Plate (Roche Diagnostics GmbH, Mannheim, Germany) and the cycling protocol was: 95 °C for 5 min, followed by 45 PCR cycles with 95 °C for 5 s, 60 °C for 15 s and 72 °C for 20 s. Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) were used for designing the PCR primers. The primers for rat ETB receptor (GenBank accession no. NM017333) mRNA were: forward: 5'-GATACGACAACCTCCGCTCCA-3' and reverse: 5'-GTCCACGATGAGGACAATGAG-3'. Elongation factor-1 (EF-1, GenBank accession no. NM175838) mRNA was used as reference. The primers were: forward: 5'-CGGTCAGGTCATCACTATCG-3' and reverse: 5'-TTCCATACCCAGGAAGGAAG-3'.

### 2.4. Western blot

Fresh or cultured arterial segments were frozen in liquid nitrogen, and homogenized in Tissue Extraction Reagent I (Invitrogen, Carlsbad, CA, USA) supplemented with Protease Inhibitor Cocktail

(Sigma, St. Louis, MO, USA) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany). Protein concentration was measured using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (15 µg) were loaded and separated in 10% polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was immersed in methanol for 15 s and air-dried, followed by incubation with blocking buffer (phosphate buffered saline containing 0.05% Tween-20 and 3% non-fat milk) on a shaker at room temperature for 1 h, and then with primary antibody at 4 °C overnight followed by secondary antibody at room temperature for 1 h. The membrane was finally visualized by using the ECL reagent (Lulong Biotech, Xiamen, P.R. China), exposed to Kodak X-OMAT BT film (Kodak, Xiamen, PR China) and developed. The antibodies for ETB receptor (1:500), NF-κB p65 (1:500), phospho-IKKα (Thr23, 1:500), and total-IKKα (1:500) were purchased from Abcam (Cambridge, UK). The antibodies for phospho-IκBα (Ser32/36, 1:1000) and total-IκBα (1:1000) were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody for β-actin (1:400) was from Boster (Wuhan, PR China). Horseradish peroxidase conjugated secondary antibodies (goat anti-mouse, 1:10,000, and goat anti-rabbit, 1:10,000) were from Pierce (Rockford, IL, USA). The experiments were repeated three times independently. Image J software (National Institutes of Health, Bethesda, MD, USA) was used for semi-quantitative analysis.

## 2.5. Immunohistochemistry

Immunohistochemical studies were performed on paraformaldehyde-fixed and paraffin-embedded tissue sections. The sections were deparaffinized with xylene and stepwise rehydrated with serial dilutions of ethanol. For epitope retrieval, slides were incubated in 0.01 M citric acid buffer (pH 6.0) at 95 °C for 20 min, and incubated with primary antibody against ETB receptors (Abcam, Cambridge, UK, 1:200) at 4 °C overnight. After being washed, the specifically bound antibodies were detected with the EliVision Plus Kit (Maixin Biotechnology, Fuzhou, PR China) according to the manufacturer's instruction. All sections were counterstained with haematoxylin. Non-immune rabbit serum (10%) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for negative controls. No detectable staining was observed in all negative control slides.

## 2.6. Inhibition of NF-κB signaling pathway

To examine the involvement of intracellular NF-κB activation, specific IκB kinase (IKK) inhibitors including WDL and IMD-0354 were administered during organ culture. The specificities of the inhibitors were described previously (Kobori et al., 2004 and Tanaka et al., 2005). AcD was used as general transcriptional inhibitor.

## 2.7. ETB2 receptor mRNA stability

The mRNA stability of receptors during organ culture in the presence of TNF-α was examined as described before (Zhang et al., 2008). Briefly, the segments were incubated with either vehicle (water) or TNF-α (30 µg/L) in the presence of AcD for different time points from 0.5 to 24 h, respectively. The administration of AcD resulted in inhibition of any de novo synthesis of receptor mRNA. Real-time RT-PCR was employed to determine the amount of ETB2 receptor mRNA expression.

## 2.8. Data analysis

All data are expressed as mean ± S.E.M. The amount of receptor mRNA is expressed in relative to house-keeping gene mRNA, normalized with control group. Unpaired Student's t-test was used to

compare two sets of data, and one-way analysis of variance (ANOVA) with Dunnett 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. TNF- $\alpha$ up-regulates ETB2 receptor expression in VSMCs during organ culture

Organ culture of rat mesenteric arterial segment in the presence of TNF- $\alpha$  for 6 h resulted in increased ETB2 receptor mRNA expression (Fig. 1A) and protein expression (Fig. 1B and C) in a concentration-dependent manner compared to that of control (vehicle), demonstrated by real-time RT-PCR and Western blot results, respectively. The enhanced protein expression of ETB2 receptors in VSMC is also confirmed by immunohistological staining (Fig. 2) after co-incubation with TNF- $\alpha$  (30  $\mu$ g/L).

#### 3.2. Activation of NF- $\kappa$ B signals is involved in TNF- $\alpha$ exposure during organ culture

After exposure to TNF- $\alpha$ , NF- $\kappa$ B p65 in VSMC was activated (Fig. 3A). When the arterial segments were co-incubated with TNF- $\alpha$  at 10  $\mu$ g/L, NF- $\kappa$ B p65 activation was observed at 3 h and further enhanced at 6 h, whereas TNF- $\alpha$  at 30  $\mu$ g/L led to remarkable activation of NF- $\kappa$ B p65 at 3 h and remained high until 6 h. Semi-quantitation results showed that NF- $\kappa$ B p65 protein expression is significantly induced by TNF- $\alpha$  (Fig. 3B).

#### 3.3. Inhibition of transcription and NF- $\kappa$ B signals abolished TNF- $\alpha$ -induced ETB2 receptor up-regulation

Two specific inhibitors for NF- $\kappa$ B pathway, WDL and IMD, were applied in the present study. After co-incubation for 6 h, both WDL and IMD exerted potent inhibitory effects on the mRNA expression of ETB2 receptors (Fig. 4A), and the expression of ETB2 receptor and NF- $\kappa$ B p65, as well as the phosphorylation of IKK $\alpha$  and the downstream I $\kappa$ B $\alpha$  (Fig. 4B and C).

The augmented expression of ETB2 receptor mRNA induced by TNF- $\alpha$  was also abolished by non-selective transcription inhibitor AcD (Fig. 4A). Additionally, the ETB2 receptor mRNA stability was not modified after TNF- $\alpha$  stimulation during organ culture (data not shown).

### 4. Discussion

By using the organ culture model, we have for the first time demonstrated that short-term (6 h) incubation with TNF- $\alpha$  could concentration-dependently up-regulate contractile ETB2 receptors in VSMC of rat mesenteric arterial segment at both mRNA and protein levels. The up-regulation of ETB2 receptors has been observed in VSMC of patients with various atherosclerotic CVDs, for example, peripheral artery disease (Lind et al., 1999), severe pulmonary hypertension (Bauer et al., 2002), subarachnoidal hemorrhage (Hansen-Schwartz et al., 2003), ischemic heart disease (Dimitrijevic et al., 2009) and after cerebral ischemic stroke (Stenman et al., 2002), as well as in animal models with subarachnoid hemorrhage (Hansen-Schwartz et al., 2003), cerebral ischemia (Stenman et al., 2002), hypertension (Fink et al., 2007) and diabetes (Kelly-Cobbs et al., 2011). The increased expression of ETB2 receptors in VSMC has also been found in atherosclerotic plaques and neointimas (Iwasa et al., 1999). Additionally, the mixed endothelin receptor antagonist bosentan was evaluated for the treatment of mildly symptomatic pulmonary arterial hypertension in a clinical trial (Galie et al., 2008). The up-regulation of contractile ETB2 receptors may result in abnormal VSMC tone and enhanced VSMC proliferation, which are closely associated with atherosclerotic CVD.

TNF- $\alpha$  is one of the most important pro-inflammatory cytokines. Previously it gained much attention for its role of inducing apoptosis of cells via activation of NF- $\kappa$ B and caspase pathways (MacFarlane, 2003). However, TNF- $\alpha$  could elicit complicated biological effects through binding to its receptors followed by activation of numerous intracellular signals ( Caminero et al., 2011, Rosenblum and Amital, 2011, Veerappan et al., 2011 and Vujanovic, 2011). Since ETB2 receptors are not expressed in VSMC under normal conditions, but was impressively induced by short-term exposure to TNF- $\alpha$  during organ culture, which is also observed in patients with CVD, we were interested in the underlying mechanisms that may extend our knowledge of the toxic effects of TNF- $\alpha$ . We aimed to find out the key intracellular molecules that account for the alteration of ETB2 receptors.

We targeted on NF- $\kappa$ B, because TNF- $\alpha$  is known to be one of the most potent physiological inducers of NF- $\kappa$ B (Schutze et al., 1995). NF- $\kappa$ B is activated in all cells, where it regulates expression of diverse target genes that promote cell proliferation, regulate immune and inflammatory response, and contribute to pathogenesis of various diseases (Wang et al., 2009). Principally, the binding of a ligand to a cell surface receptor (like TNF-receptor) results in phosphorylation of IKK complex, which phosphorylates I $\kappa$ B and subsequently leads to its ubiquitination and degradation by the proteasome, releasing NF- $\kappa$ B to the nucleus to turn on target genes (Baldwin, 1996). In the present study we firstly observed the activation of the key molecules of NF- $\kappa$ B pathway, including NF- $\kappa$ B p65 and phosphorylation of IKK $\alpha$  and I $\kappa$ B $\alpha$ , in the VSMC after short-term exposure to TNF- $\alpha$ . We subsequently revealed the importance of NF- $\kappa$ B in the up-regulation of ETB2 receptors with the results that, when specific IKK inhibitors (WDL and IMD-0354) were administered during organ culture, the TNF- $\alpha$ -induced activation of NF- $\kappa$ B pathway was effectively suppressed along with the decreased expression of ETB2 receptor mRNA and protein. This is supported by recent studies in which NF- $\kappa$ B activation is involved in the up-regulation of ETB2 receptors induced by lipid-soluble smoke particles ( Huang et al., 2010 and Xu et al., 2008).

Besides the ETB2 receptor, the alteration of G-protein coupled receptors (GPCRs) like thromboxane A2 receptors (Zhang et al., 2008) and  $\alpha$ 1A-adrenoceptors (Zhang et al., 2007) in the VSMC has been studied under pathophysiological conditions. These findings along with additional clinical and experimental data provided the importance of the alteration of GPCRs in the pathogenesis of atherosclerotic CVDs, for their capabilities in regulating vascular tone and VSMC proliferation, which result in structure changes in the vascular wall associated with adverse remodeling, plaque formation, and in clinical sequelae such as myocardial infarction, stroke, and ischemia. Specific blockage of these receptors and the intracellular signaling pathways, which account for the abnormal GPCR alteration, for instance, the NF- $\kappa$ B pathway, might be potential targets for treatment of atherosclerosis and other CVDs in future.

Taken together, the present study demonstrated that the activation of intracellular NF- $\kappa$ B pathway is responsible for the up-regulation of ETB2 receptors induced by in vitro administration of TNF- $\alpha$  in VSMC. This could at least partly explain the toxicological effects of TNF- $\alpha$  on VSMC, which are associated with CVDs like atherosclerosis, and could extend the current knowledge on inflammation-related diseases in the vasculature.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1

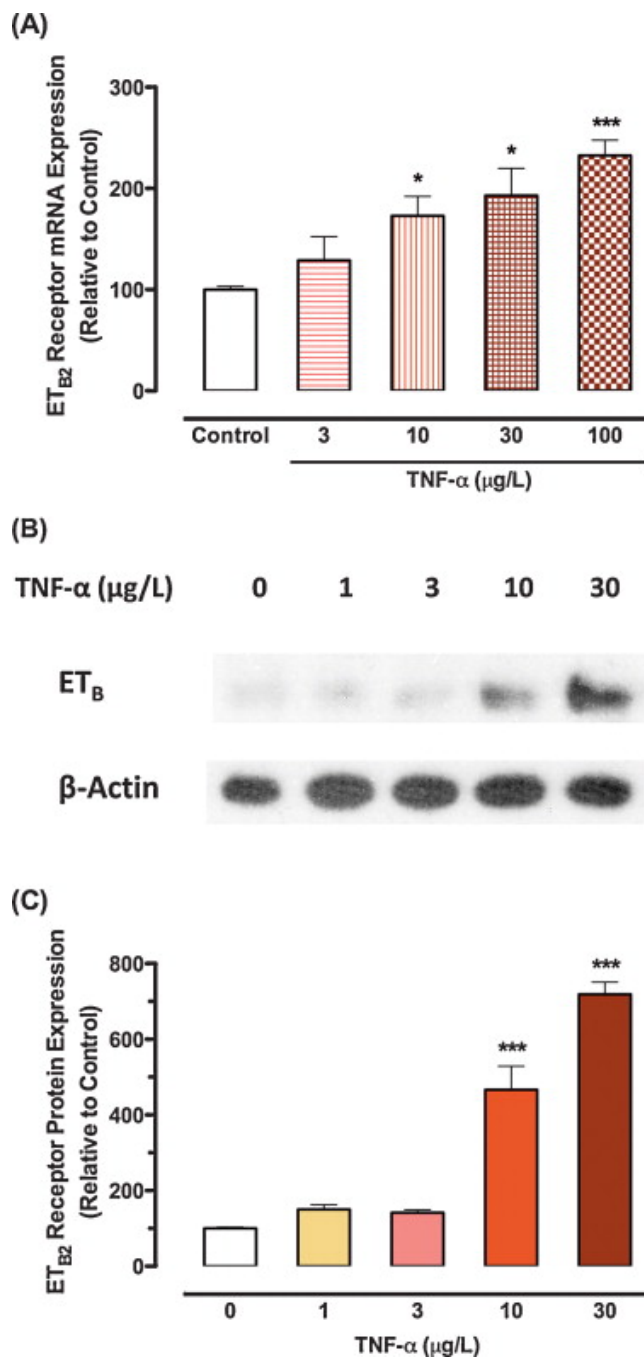


Fig. 1. (A) During organ culture, the exposure to TNF- $\alpha$  (3–100  $\mu\text{g/L}$ ) for 6 h increased mRNA expression of ET<sub>B2</sub> receptors in VSMC of rat mesenteric arteries in a concentration-dependent manner. Data are expressed as mean  $\pm$  S.E.M. One-way ANOVA, \* $P$  < 0.05, \*\*\* $P$  < 0.001 versus control (vehicle).  $n$  = 5. (B) Exposure to TNF- $\alpha$  (0–30  $\mu\text{g/L}$ ) resulted in concentration-dependently increased protein expression of ET<sub>B2</sub> receptors after organ culture for 6 h, demonstrated by Western blot. (C) Semi-quantitation of ET<sub>B2</sub> receptor protein density for Western blot results by using ImageJ software. Data are expressed as mean  $\pm$  S.E.M. One-way ANOVA, \*\*\* $P$  < 0.001 versus control (vehicle).  $n$  = 3.

Figure 2

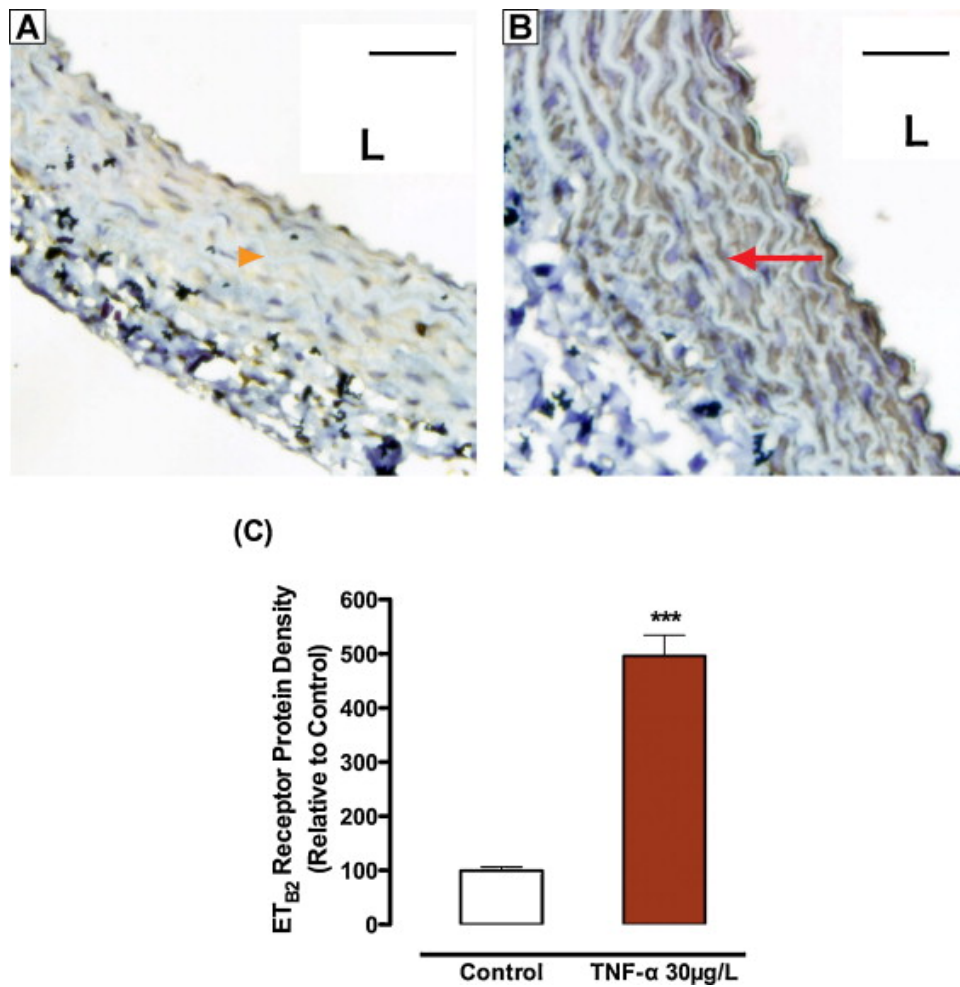


Fig. 2. Immunohistochemical staining on paraffin-embedded tissue sections showed up-regulation of ETB<sub>2</sub> receptors after TNF- $\alpha$ . Arteries (without endothelium) were incubated with: (A) vehicle and (B) TNF- $\alpha$  (30  $\mu$ g/L) for 6 h during organ culture. The arrowhead points to the collagen bands (A), and the arrows point to the positive staining of ETB<sub>2</sub> receptor protein (B). L: lumen. The size bar corresponds to 50  $\mu$ m. Zoom: 400 $\times$ . Similar results were obtained from three independent experiments. (C) Semi-quantitation of ETB<sub>2</sub> receptor protein density for immunohistochemistry results by using ImageJ software. Data are expressed as mean  $\pm$  S.E.M. Unpaired Student's t-test, \*\*\*P < 0.001 versus control (vehicle). n = 10.

Figure 3

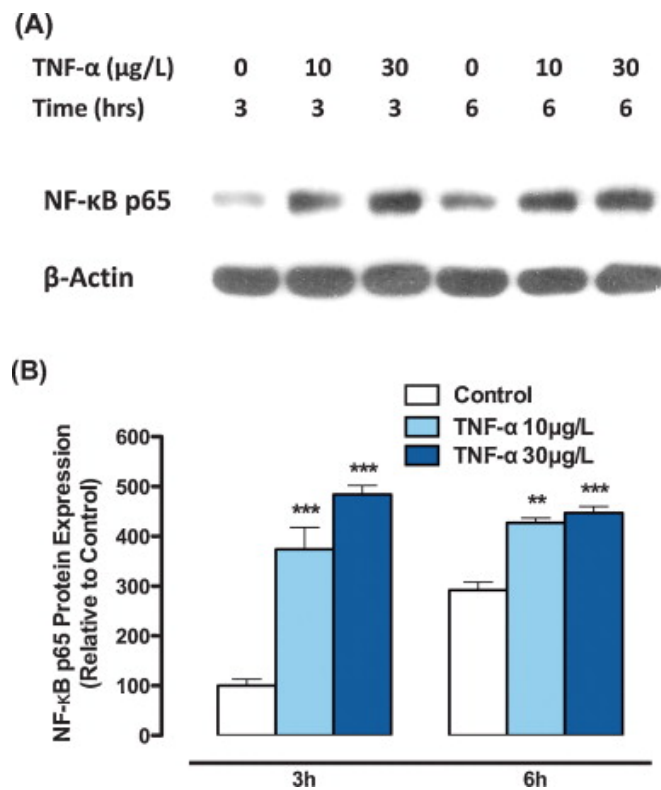


Fig. 3. (A) Activation of NF- $\kappa\text{B}$  p65 occurred during organ culture, and markedly enhanced at 3 h after exposure to TNF- $\alpha$  (30  $\mu\text{g/L}$ ). At longer incubation time point (6 h), very strong signal of NF- $\kappa\text{B}$  p65 could be detected after co-incubation with lower TNF- $\alpha$  concentration (10  $\mu\text{g/L}$ ). (B) Semi-quantitation of NF- $\kappa\text{B}$  p65 protein density for Western blot results by using ImageJ software. Data are expressed as mean  $\pm$  S.E.M. One-way ANOVA with Dunnett post-test, \*\*\* $P < 0.001$  versus control (vehicle).  $n = 3$ .

Figure 4

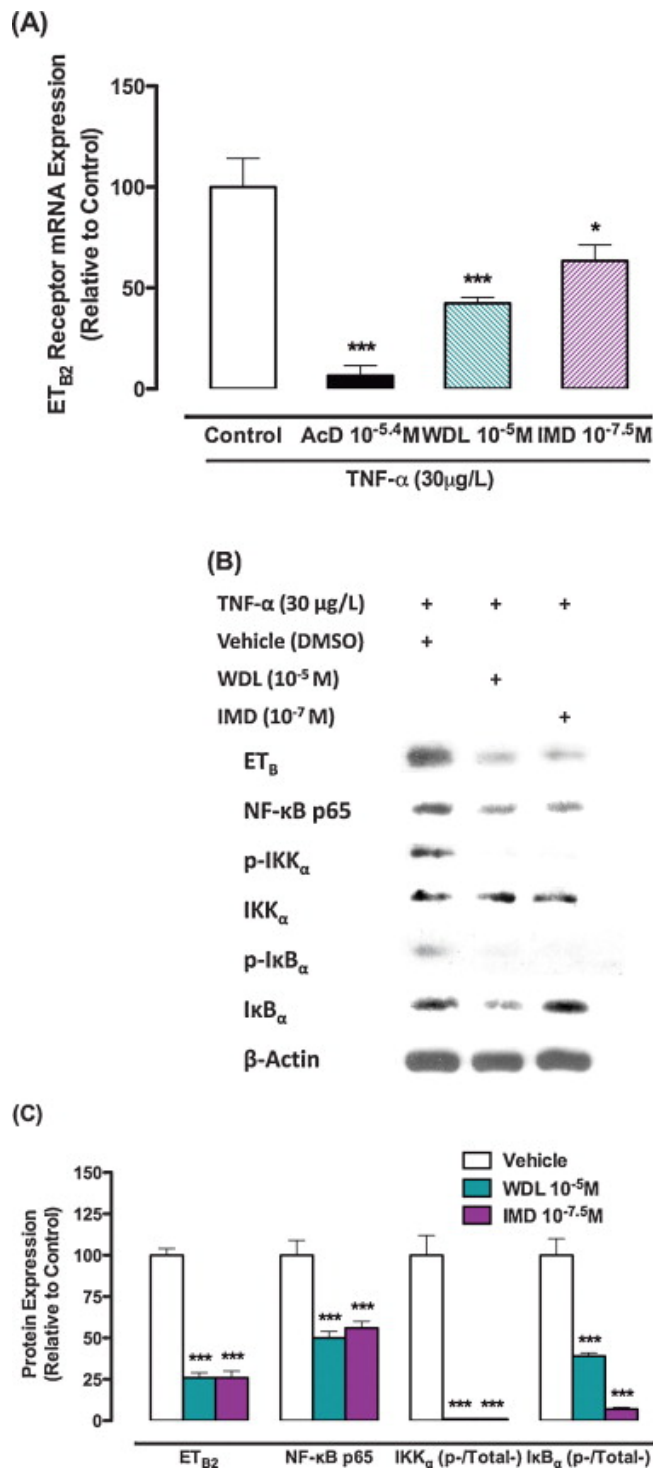


Fig. 4. (A) Non-selective transcription inhibitor AcD (10<sup>-5.4</sup> M) and selective NF- $\kappa$ B inhibitors WDL (10<sup>-5</sup> M) and IMD (10<sup>-7.5</sup> M) markedly abolished TNF- $\alpha$ -augmented ET<sub>B2</sub> receptor mRNA expression, respectively. Data are expressed as mean  $\pm$  S.E.M. One-way ANOVA with Dunnett post-test, \*P < 0.05, \*\*\*P < 0.001 versus control. n = 5–6. (B) Exposure to either WDL or IMD potently

suppressed TNF- $\alpha$ -augmented ETB2 receptor protein expression, paralleled with inhibition on NF- $\kappa$ B p65 activation and phosphorylation of IKK $\alpha$  and I $\kappa$ B $\alpha$ . (C) Semi-quantitation for Western blot results by using ImageJ software. Data are expressed as mean  $\pm$  S.E.M. One-way ANOVA with Dunnett post-test, \*\*\*P < 0.001 versus control (vehicle). n = 3.