Transcriptional Down-Regulation of Thromboxane A₂ Receptor Expression via Activation of MAPK ERK1/2, p38/NF-κB Pathways

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Key Words

Rat • Mesenteric artery • Vascular diseases • Thromboxane A₂ receptors

Abstract

Background: We have developed an in vitro model by organ culture of rat mesenteric arteries to imitate vascular smooth muscle cell (VSMC) receptor changes in cardiovascular disease. By using this model, alteration of VSMC thromboxane A₂ (TP) receptors was studied. **Methods and Results:** After organ culture of the arteries, VSMC TP receptors were studied by using myography, real-time PCR and immunohistochemistry. We observed that organ culture for 24 and 48 h resulted in depressed TP receptor-mediated contraction in the VSMC, in parallel with decreased TP receptor mRNA and protein expressions. Phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and nuclear factor-κB (NF-κB) was seen by Western blot within 1–3 h after organ culture. Inhibition of ERK1/2, p38 or NF-κB reversed depressed contraction as well as decreased receptor mRNA expression. Actinomycin D abolished decreased TP receptor-mediated contraction, while inhibition of translation, cyclooxygenase or nitric oxide synthase had no effect. TP receptor mRNA stability was unchanged during organ culture. **Conclusions:** The present study has demonstrated for the first time that organ culture of rat mesenteric arteries down-regulates VSMC TP receptor expression through activation of ERK1/2 and p38/NF-κB signal pathways.

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Introduction

Prostanoids are cyclooxygenase metabolites that include prostaglandin (PG) D₂, PGE₂, PGF_{2α}, PGI₂ and thromboxane A₂ [1]. In mammals, there are 8 subtypes of membrane prostanoid receptors that include PGD receptor (DP), 4 subtypes of PGE receptor (EP₁, EP₂, EP₃ and EP₄), PGF receptor (FP), PGI receptor (IP) and thromboxane A₂ receptor (TP) [2]. TP receptor is located in cardiovascular, reproductive, pulmonary and neural tissues [3]. In the vasculature, TP receptors are involved in blood platelet function, linked with hemostasis and thrombosis [4, 5], and play an important role in vascular cell proliferation and contraction [6, 7].

TP receptor density in vascular smooth muscle cells (VSMCs) is reported to be enhanced in cardiovascular diseases [8] and in hypertension [9]. However, TP receptor gene transcription is suppressed by peroxisome proliferator-activated receptor γ (PPARγ) and retinoids in VSMCs [10, 11]. Growth factors such as platelet-derived growth factor and basic fibroblast growth factor can modify TP receptor expression [12]. Interestingly, studies have shown that in streptozotocin-induced diabetic rats there is a decreased responsiveness of aortic segments to the selective TP receptor agonist U46619, which can be reversed by insulin therapy [13], indicating a down-regulation of TP receptors. In placentas from diabetic pregnancies, TP receptor affinity and vasoconstrictor responses to U46619 are reduced [14]. TP receptor gene

transcription is reported to be suppressed by PPAR γ and retinoids in VSMCs [10, 11]. Although ovariectomized rats exhibit enhanced vasoconstrictor responses to U46619, oestrogen replacement reduced TP receptor-mediated vasoconstriction [15]. Thus, it is important to obtain further knowledge about the molecular mechanisms responsible for alterations in TP receptor expression in VSMCs.

G protein-coupled receptor (GPCR)-mediated VSMC contraction and proliferation are key elements in the development of cardiovascular diseases [16]. Organ culture of arterial segments has been developed as a model for studying dynamic GPCR changes in VSMCs [17] which mimic receptor changes seen in vascular diseases [18]. We have revealed that organ culture of arteries results in up-regulation of 5-hydroxytryptamine (5-HT)_{1B/1D} [19–21] and endothelin B (ET_B) [22–24] receptors, and similar changes were observed in experimental stroke [25] as well as in patients [26]. De novo transcription and synthesis of new receptors through activation of intracellular mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1 and 2 (ERK1/2) are responsible for the up-regulation of these receptors [27, 28].

TP receptors are key elements in the development of cardiovascular diseases, which mediate VSMC proliferation and contraction, and platelet aggregation [29]. Therefore, the present study was designed to investigate the molecular mechanisms that are responsible for the regulation of TP receptors in VSMCs. We hypothesize that there is a transcriptional down-regulation of TP receptors in VSMCs which occurs through activation of MAPK/nuclear factor- κ B (NF- κ B) signal pathways. Our results demonstrate that the down-regulation of TP receptor during organ culture, which is seen in the vasculature of diabetic rats, could be reversed by inhibition of ERK1/2, p38/NF- κ B pathways. Thus, these intracellular signal events may provide new options for modulation of the vascular complications in diabetes.

Materials and Methods

Tissue Preparation and Organ Culture Procedure

Male Sprague-Dawley rats (weighing 300–350 g) were anaesthetized with CO₂ and exsanguinated. The superior mesenteric artery (about 1 mm in outer diameter) was removed gently, immersed into cold buffer solution (for composition, see below) and dissected free of adhering tissue under a microscope. The endothelium was denuded by perfusion of the vessel for 10 s with 0.1% Triton X-100 followed by another 10 s with a physiological buffer solution [17]. The removal of the endothelium was verified by no dilatory response to acetylcholine (10⁻⁶ M) on 5-HT (10⁻⁵ M) pre-

contracted segments, and confirmed by immunohistology. The vessels were then cut into 1-mm-long cylindrical segments, used directly (fresh group) or incubated at 37°C in humidified 5% CO₂ in air for different time points (organ culture group). The segments for organ culture were placed in a 24-well plate, 1 segment in each well, containing 1 ml Dulbecco's modified Eagle's medium containing L-glutamine (584 mg/l), supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml). For the inhibition experiments, different inhibitors were added to the culture medium. The same volume of vehicle served as control. The experimental protocol was approved by the Lund University Animal Ethics Committee (M217-03). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Buffer Solutions

Standard buffer solution (mM): NaCl 119; NaHCO₃ 15; KCl 4.6; MgCl₂ 1.2; NaH₂PO₄ 1.2; CaCl₂ 1.5; glucose 5.5. Analytical-grade chemicals and double-distilled water were used for preparing all solutions. Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Gibco BRL (Paisley, Scotland).

In vitro Pharmacology

Fresh or incubated segments were immersed in a temperature-controlled (37°C) myograph system (Organ Bath Model 700MO; J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution. The solution was continuously aerated with 5% CO₂ in O₂, resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension by the Chart software (AD Instruments, Hastings, UK). A resting tone of 2.5 mN was applied to each segment and the segments were allowed to stabilize at this tension for at least 1.5 h before being exposed to a potassium-rich (60 mM K⁺) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the segments were used only if potassium elicited reproducible responses over 1.0 mN. Concentration-response curves for vasoconstrictors were obtained by cumulative administration of the reagents.

Real-Time RT-PCR

Smooth muscle cells were isolated from fresh or cultured artery segments and homogenized in 1 ml of RNeasy lysis solution (Qiagen, Crawley, UK) by using a FastPrep instrument (Qiagen). The total RNA was extracted following a protocol from the FastRNA Pro kit supplier and purity was verified by a GeneQuant Pro (Amersham Biosciences, Cambridge, UK) spectrophotometer. Reverse transcription of total RNA to cDNA was carried out using TaqMan reverse transcription reagents (PE Applied Biosystems, Foster City, Calif., USA) in a Perkin-Elmer 2400 PCR machine (Perkin-Elmer, Waltham, Mass., USA) at 42°C for 30 min. Real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit in a GeneAmp 5700 sequence detection system (Perkin-Elmer). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification.

PCR was performed in a 50- μ l volume and started at a temperature of 50°C for 2 min, 95°C for 10 min and the following 40 PCR cycles were done with 95°C for 15 s and 60°C for 1 min. Dissociation curves were run after the real-time PCR to identify the specific PCR products. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by TAG Copenhagen A/S (Copenhagen, Denmark). Specific primers for the rat TP receptor (gene ID: NM_017054) were designed as follows:

TP receptor: forward: 5'-ATCTCCCATCTTGCCATAGTCC-3'; reverse: 5'-CCGATGATCCTTGAGCCTAAAG-3'.

Elongation factor-1 (EF-1, gene ID: BC072542) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, gene ID: NM_023964) mRNA were used as references, since they are the product of a housekeeping gene, continuously expressed to a constant amount in cells. The EF-1 and GAPDH primers were designed as follows:

EF-1: forward: 5'-GCAAGCCCATGTGTGTTGAA-3'; reverse: 5'-TGATGACACCCACAGCAACTG-3'.

GAPDH: forward: 5'-GGCCTTCCGTGTTCTACC-3'; reverse: 5'-CGGCATGTCAGATCCACAAC-3'.

Data were analysed with the comparative cycle threshold (C_T) method. To evaluate the amount of mRNA in a sample, EF-1 and GAPDH mRNA were assessed in the same sample simultaneously. The C_T values of EF-1 and GAPDH mRNA were used as references to quantify the relative amount of TP receptor mRNA. The relative amount of mRNA was calculated with the C_T values of TP receptor mRNA in relation to the C_T values of EF-1 or GAPDH mRNA in the sample.

TP Receptor mRNA Stability

To study if a post-transcriptional mechanism of mRNA stability was involved in the down-regulation of TP receptors after organ culture, the segments were incubated in the presence of actinomycin D (AcD) for 0.5, 6 and 24 h, so that de novo synthesis of receptor mRNA was inhibited. Real-time RT-PCR was used to determine the amount of TP receptor mRNA expression.

Immunohistochemistry

Fresh or cultured arterial segments were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C. After fixation, the specimens were dehydrated in 20% sucrose of phosphate buffer (0.1 M, pH 7.4) for 24 h at 4°C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and stored at -80°C. Sections were cut at 10- μ m thickness in a cryostat and mounted on SuperFrost Plus slides. Immunohistology staining with primary antibody against rat TP receptors from rabbits (Alexis Biochemicals, Lausen, Switzerland) and secondary antibody goat-anti-rabbit IgG conjugated to fluorescein isothiocyanate (Alexis Biochemicals) was used to demonstrate TP receptor proteins. Briefly, the sections were incubated with the primary antibody (1:150 dilution) overnight at 4°C, thereafter, the secondary antibody (1:200 dilution) was applied for 1 h at room temperature in the dark. In the control experiments, either the primary or the secondary antibody was omitted. The stained arterial segments were observed under a confocal microscope (C1plus; Nikon Instruments Inc., Melville, N.Y., USA) and analysed by the Image J software (<http://rsb.info.nih.gov/ij/>) [26]. The measurement was based on positive staining in the smooth muscle cells. For each

vessel, 6 sections were studied and the values obtained were mean fluorescence in the areas selected (there were 6 preset areas per section), and analysed blindly as to the treatment protocol.

Western Blot

After organ culture, vessels were collected and frozen in liquid nitrogen, and homogenized in denaturing cell extract buffer (BioSource, Carlsbad, Calif., USA) with phosphatase inhibitor cocktail and protease inhibitor cocktail (Sigma, St. Louis, Mo., USA). Protein concentration was measured by a Bio-Rad protein analysis kit and a Tecan Infinite M200 microplate reader. Proteins (40 μ g) were loaded and separated on 4–15% Ready Gel Precast Gels (BioRad, Hercules, Calif., USA) and followed by One-Step Complete Western Kit (GenScript Corporation, Piscataway, N.J., USA) according to the manual. Briefly, membranes were incubated with Pretreat Solution on shaker for 5 min at room temperature, and then with primary antibody overnight at 4°C. After washing 3 times for 10 min, the membranes were developed with Working Solution (LumiSensor A + B) for 3 min, visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Fujifilm, Stamford, Conn., USA), and then analysed with Image Gauge 4.0 (Fuji Photo Film Co. Ltd., Tokyo, Japan). The antibodies for phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-NF- κ B p65 (Ser536) and β -actin were from Cell Signaling Technology (Beverly, Mass., USA). All antibodies were used at 1:1,000 dilution and the experiments were repeated 3 times.

Intracellular Signal Inhibitors and Drugs

To link intracellular events to functional changes, a set of inhibitors was administered during organ culture. The MAPK pathway consists of 3 major members: ERK1/2, JNK and p38 [30]. NF- κ B, one of its downstream transcriptional factors, is a key inflammatory signal molecule [31, 32]. In the present investigation, inhibitors including PD98059 and U0126 (inhibitors of ERK1/2) [33, 34], SB203580 and SB239063 (inhibitors of p38) [35, 36], SP600125 (inhibitor of JNK) [37] and wedelolactone (inhibitor of NF- κ B) [38] were employed to examine the involvement of different MAPK subtypes and the NF- κ B pathway. AcD and cycloheximide (CHX) were used as general transcriptional and translational inhibitors, respectively. Cyclooxygenase (COX) inhibitor, indomethacin, and nitric oxide synthase (NOS) inhibitor, N^w-nitro-L-arginine methyl ester (L-NAME), were used separately or in combination to investigate whether COX and NOS were involved.

All drugs were purchased from Sigma. U46619 was dissolved in ethanol to a stock concentration of 10 mM and further diluted in distilled water; GR32191b (selective antagonist for TP receptors) and L-NAME were dissolved in distilled water; indomethacin was dissolved in 95% ethanol; the others were dissolved in dimethyl sulfoxide.

Data Analysis

All data are expressed as mean values $\pm$ SEM. The responses to U46619 are presented as percentage of contraction relative to 60 mM K⁺ or the absolute values (mN) in the inhibition experiments, since the inhibitors might affect the reference-induced contraction. The amount of receptor mRNA is expressed as percentage of control group. One-way or two-way ANOVA with Bonferroni post-test was used for comparisons of more than 2 data sets. $p < 0.05$ was considered to be significant.

Fig. 1. Attenuation of TP receptor-mediated contraction after organ culture. U46619-induced contraction of rat mesenteric arteries with/without endothelium of fresh and after organ culture for 24 h (**a**) and without endothelium after organ culture for 48 h (**b**). The contractile responses are presented as the percentage of contractile response induced by 60 mM K⁺. Data are shown as means $\pm$ SEM. Each data point is derived from 8–10 segments. Endo+ = With endothelium; Endo- = without endothelium.

Table 1. $E_{\max}$ to U46619 in endothelium-denuded rat mesenteric arteries of fresh controls or after organ culture for 24 h

	60 mM K ⁺ mN	U46619 $E_{\max}$ mN	U46619 $E_{\max}$ % of K ⁺
Fresh controls	2.58 $\pm$ 0.19	6.58 $\pm$ 0.40	259 $\pm$ 12
Organ culture	2.60 $\pm$ 0.20	6.66 $\pm$ 0.38	260 $\pm$ 10

Data are shown as means $\pm$ SEM. Each data point is derived from 8 segments.

Results

In vitro Pharmacology

Organ culture of rat mesenteric arterial segments for 24 h (fig. 1a) or 48 h (fig. 1b) resulted in significantly depressed contractile responses to the selective agonist for TP receptor U46619 [39], compared with fresh segments. For the endothelium-denuded group, the pEC₅₀ value was significantly decreased from 7.74 $\pm$ 0.03 (fresh) to 7.00 $\pm$ 0.05 (organ culture for 24 h; n = 8, p < 0.001). The

segments with intact endothelium had the same contractility and similar response to those without endothelium in both fresh and organ culture groups (fig. 1a).

Since the present study was designed to investigate alteration of VSMC TP receptor expression and in particular the molecular mechanisms involved, only endothelium-denuded segments were used for further studies. In the endothelium-denuded segments, the reference (60 mM K⁺)-induced contractions (absolute values, mN) and the maximal contractile response ($E_{\max}$, % of K⁺) to U46619 were not significantly changed within groups (n = 8, p > 0.05; table 1).

Pharmacological Characterization

In order to characterize the TP receptors in the VSMCs, the contractile response to U46619 in fresh segments was antagonized by GR32191b, a selective TP receptor antagonist [40]. GR32191b caused concentration-dependent, parallel rightward shift of the concentration-response curve to U46619 without significantly altering $E_{\max}$ (fig. 2a). Schild analysis gave a slope of 0.82 $\pm$ 0.21, and a pK_B value of 8.45 $\pm$ 0.06 was obtained when the slope was constrained to unity in fresh segments (fig. 2b). Sim-

Fig. 2. Pharmacological characterization of TP receptor. **a** U46619-induced contraction of rat mesenteric arteries without endothelium of fresh in the presence of vehicle or different concentrations of selective TP receptor antagonist, GR32191b. **b** Schild analysis of GR32191b. **c** Effect of GR32191b (10^{-8} M) on U46619 concentration-response curve of rat mesenteric arteries without endothelium after organ culture for 24 h. The contractile responses are presented as the percentage of contractile response induced by 60 mM K^+ . Data are shown as means $\pm$ SEM. Each data point is derived from 7 segments. GR = GR32191b.

ilar effects were seen with GR3219b in the cultured segments (fig. 2c), with an apparent pK_B value of 8.15 ± 0.09 , indicating the contractions in both fresh and organ-cultured segments were mediated by the same type of TP receptors.

TP Receptor mRNA Expression

Real-time RT-PCR was employed to investigate the alteration of TP receptor mRNA expression after organ

culture of rat mesenteric artery segments. In agreement with the functional results, organ culture for 24 h induced a significant decrease in TP receptor mRNA expression (fig. 3).

Immunohistochemistry

The TP receptor protein was visualized by the fluorescent immunohistology method and analysed by confocal microscopy with the Image J software. Collagen bands

Fig. 3. TP receptor mRNA expression in rat mesenteric artery in fresh segments and after organ culture for 24 h. The mRNA expression was decreased after organ culture, no matter if EF-1 or GAPDH was used as reference. Data are shown as means $\pm$ SEM. Each data point is derived from 6 experiments. *** $p < 0.001$ versus control (unpaired Student's *t* test).

(elastic fibres) appeared as orange autofluorescence and the TP receptor proteins were stained clearly in green colour between the collagen bands. There was only a strong TP receptor protein expression as shown by the bright green granules in the control group (fresh), located among the smooth muscle cells (fig. 4a). However, the TP receptor protein was markedly decreased in the organ culture group located on the smooth muscle cells (fig. 4b). Semi-quantitative measurement of TP receptor protein density by using Image J software showed that organ culture significantly depressed TP receptor protein expression (fig. 4c).

Western Blot

To explore the possible intracellular signal transductions which are invoked during the organ culture process, phosphorylated ERK1/2 (p-ERK1/2), p38 (p-p38) and NF- κ B (p-NF- κ B p65) proteins were examined by Western blotting in extracts of fresh/cultured arteries. The phosphorylation of these 3 proteins was markedly provoked within 1 or 3 h during organ culture (fig. 5), while the reference, β -actin, had constant protein expression in the tissue. Compared with the fresh segments, during the organ culture period, phosphorylation of the 3 proteins are significantly increased ($n = 3$, $p < 0.01$): p-ERK1/2 by $282.4 \pm 61.9\%$ at 1 h, $279.5 \pm 48.4\%$ at 3 h,

Fig. 4. Immunohistology demonstrates a down-regulation of TP receptor protein expression. Arteries of fresh controls (a) and after organ culture for 24 h (b) are shown. Arrows point to the TP receptor protein (a) and the collagen bands (b). Scale bar = 20 μ m. c Semi-quantitation of the receptor protein expression. Each data point is derived from 6 experiments. *** $p < 0.001$ versus controls (two-tailed unpaired Student's *t* test with Welch's correction).

$279.6 \pm 45.1\%$ at 6 h and $211.8 \pm 35.8\%$ at 24 h; p-p38 by $276.5 \pm 94.6\%$ at 1 h, $211.7 \pm 52.8\%$ at 3 h, $280.3 \pm 41.6\%$ at 6 h and $416.1 \pm 87.7\%$ at 24 h; p-NF- κ B by $166.0 \pm 20.3\%$ at 1 h, $188.3 \pm 18.3\%$ at 3 h, $374.1 \pm 51.8\%$ at 6 h

and $359.9 \pm 44.8\%$ at 24 h. Hence, organ culture induces the activation of intracellular MAPK ERK1/2, p38 and NF- κ B signal pathways.

Inhibition of Different MAPK and NF- κ B Pathways

In order to understand, in some detail, the molecular mechanisms involved in the reduction of TP receptor expression, specific inhibitors for various parts of signal transduction were tested: MAPK ERK1/2 (PD98059 10^{-5} M and U0126 $10^{-5.5}$ M), p38 (SB203580 $10^{-5.5}$ M and SB239063 $10^{-5.5}$ M), JNK (SP600125 $10^{-5.5}$ M) and the main inflammatory transcription factor NF- κ B (wedololactone $10^{-5.5}$ M). Co-incubation during organ culture with inhibitors of ERK1/2 (fig. 6a), p38 (fig. 6b) or NF- κ B (fig. 6c) significantly attenuated the organ culture-induced down-regulation of TP receptors. JNK inhibition had no effect (fig. 6d). In concert with the functional results, the decreased TP receptor mRNA expression induced by organ culture was significantly augmented in the presence of the above ERK1/2, p38 and NF- κ B inhibitors (fig. 7), but not by the JNK inhibitor (data not shown).

Inhibition of Transcription and Translation

A general transcriptional inhibitor, AcD (5 mg/l; fig. 8a), abolished the down-regulation of TP receptors, whereas CHX (10^{-5} M), a translational inhibitor, had no significant effect (fig. 8b). Thus, transcriptional and intracellular signal mechanisms are mainly responsible for the TP receptor down-regulation.

COX and NOS Inhibition

In order to examine if COX and NOS were involved in the down-regulation of TP receptor, the COX inhibitor indomethacin (10^{-5} M) or the NOS inhibitor L-NAME (10^{-4} M) was co-incubated with the segments, respectively, or in combination. The pEC₅₀ values for each group were: 6.92 ± 0.09 (control), 6.86 ± 0.07 (indomethacin), 6.97 ± 0.06 (L-NAME) and 6.94 ± 0.05 (indomethacin + L-NAME), showing that the U46619-induced contractile responses were not affected by these treatments ($n = 8$, $p > 0.05$ vs. control). Therefore, PGs and nitric oxide pathways are not involved in the down-regulation of TP receptors in the VSMC.

TP Receptor mRNA Stability

In order to study post-transcriptional mechanisms, arterial segments were co-cultured with AcD (5 mg/l) for 0.5, 6 and 24 h, and subsequently TP receptor mRNA degradation was assessed by real-time PCR. The results

Fig. 5. Western blot showed the activation of intracellular MAPK ERK1/2, p38 and NF- κ B in arterial segments during organ culture for various time points. The phosphorylation of the above 3 proteins was enhanced within 3 h. The protein expression of β -actin was constant in tissues. Experiments were repeated 3 times.

showed that, during organ culture of the arteries with AcD, the TP receptor mRNA expressions kept at a stable level from $100 \pm 0\%$ (0.5 h) to $106 \pm 7\%$ (6 h) and $99 \pm 7\%$ (24 h; $n = 6$, $p > 0.05$ vs. 0.5 h group). This indicates that no post-transcriptional mechanism of mRNA degradation is involved in the down-regulation of TP receptors after organ culture.

Discussion

This is the first study to demonstrate that organ culture of rat mesenteric arteries results in significantly depressed contractile responses mediated by TP receptors, as well as decreased receptor mRNA and protein expressions. The molecular mechanisms responsible for this down-regulation of TP receptor expression may involve transcriptional activities with intracellular MAPK ERK1/2 and p38/NF- κ B pathways.

TP receptors play important roles in the development of cardiovascular diseases and mediate VSMC proliferation and contraction [29]. It has been reported that TP receptor density is enhanced in cardiovascular diseases [8, 9]. However, in streptozotocin-induced diabetic rats there is a decreased responsiveness of aortic segments to the selective TP receptor agonist U46619, which can be reversed by insulin therapy [13], indicating a down-regulation of TP receptors. In placentas from diabetic pregnancies, TP receptor affinity and vasoconstrictor responses to U46619 are reduced [14]. TP receptor gene transcription is reported to be suppressed by PPAR γ and

Fig. 6. Co-incubation during organ culture abolished the down-regulation of TP receptor in rat mesenteric arterial segments: ERK1/2 (a), p38 (b) and NF- κ B (c); JNK inhibitor had no significant effect (d). Data are shown as means $\pm$ SEM. Statistical analysis was performed with two-way ANOVA with Bonferroni post-test. Each data point is derived from 8 segments. WDL = Wedelolactone.

retinoids in VSMCs [10, 11]. Although ovariectomized rats exhibit enhanced vasoconstrictor responses to U46619, oestrogen replacement reduced TP receptor-mediated vasoconstriction [15]. The present study dem-

onstrates that organ culture of rat mesenteric arteries down-regulates TP receptor expression in VSMC through activation of ERK1/2, p38/NF- κ B signal pathway. Intracellular molecule mechanisms might be relevant for the

Fig. 7. Inhibition of ERK1/2, p38 and NF- κ B significantly attenuated the down-regulation of TP receptor mRNA expression in rat mesenteric arterial segments after organ culture for 24 h. EF-1 and GAPDH were used as reference. The amount of TP receptor mRNA is relative to references (**a** EF-1; **b** GAPDH) expressed in percentage of control group (vehicle). Data are shown as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control (vehicle) group (unpaired Student's *t* test). Each data point is derived from 5 experiments.

Fig. 8. The general transcriptional inhibitor AcD abolished the organ culture-induced down-regulation of TP receptor (**a**), while the translational inhibitor CHX had no significant effect (**b**). Data are shown as means $\pm$ SEM. Each data point is derived from 8 segments.

abnormalities in the TP receptor-mediated VSMC tone in diabetes mellitus and the pharmacological effect of insulin, oestrogen, PPAR γ agonists and retinoids. Organ culture may provide a model to further study the underlying molecular mechanisms for TP receptor alteration in vasculature in diabetes and under the influence by insulin, estrogen, PPAR γ agonists and retinoids.

Pharmacological characterization of the receptors by using GR32191b, a selective TP receptor antagonist, resulted in a pK $_B$ value of 8.45 ± 0.06 in fresh segments and an apparent pK $_B$ value of 8.15 ± 0.09 in cultured segments. This agrees with studies by other groups [41] and thus demonstrates that the contraction is mediated by TP receptors in both fresh and cultured segments. It is important that we found the same dissociation constant in fresh and organ-cultured arterial segments, since this suggests the presence of the same type of TP receptor in the 2 experimental conditions. Therefore, the results suggest that the number of TP receptors was reduced after organ culture [42]. The rightward shift in the concentration-response curve, the reduced amount of TP receptor mRNA and protein expression support our view that the number of TP receptors was decreased after organ culture.

It is common that VSMCs change phenotypes from contractile to synthetic in tissue culture in the presence of serum and growth factors. However, our organ culture model is performed by only using serum-free medium. This means that the culture medium is without serum and growth factors. In smooth muscle cell culture in the absence of serum and growth factors, the smooth muscle cells alter the phenotype to contractile [43]. Although the contractile response to the TP receptor agonist is depressed in the present study, by using the same model we have demonstrated there are increased contractile responses to the agonists for ET $_B$ receptors [17] and 5-HT $_{2A}$ receptors [20] as well as during organ culture of the arterial segments. In addition, the response to potassium, a general indicator of smooth muscle contractility, is not affected by the organ culture. Taken together, this demonstrates that the change of the smooth muscle contractile response to the agonist is due to the altered receptor expression in the smooth muscle cells in the present study rather than the change of the smooth muscle cell phenotype to synthetic.

There are 3 main MAPK pathways: ERK 1/2, p38 and JNK [30, 44]. The downstream transcriptional factor NF- κ B is the key inflammatory signal molecule that can be activated by upstream MAPK [31, 32]. The stress induced by changing from a rich in vivo environment to poor se-

rum-free culture condition and in cerebral ischemic in vivo, there is activation of MAPK pathways [27, 45]. Our results showed that the phosphorylation of intracellular signal molecules including ERK1/2, p38 and NF- κ B were activated during organ culture in serum-free medium. This is in concert with our previous investigations [20, 27, 28, 46]. Furthermore, in order to understand if the above intracellular mechanisms are involved in the down-regulation of TP receptor expression in VSMC, specific inhibitors were administered during the organ culture procedure. The inhibition of ERK1/2, p38 and NF- κ B significantly attenuated the reduction of TP receptor-mediated contraction and TP receptor mRNA expression, while JNK inhibition had no effect. Hence, putatively the activation of intracellular ERK1/2, p38/NF- κ B pathways resulted in decreased TP receptor expression. The TP receptor-mediated contraction was reversed by the general transcriptional inhibitor AcD, together with the result that a decreased TP receptor mRNA expression in VSMCs was evoked by organ culture, suggesting a transcriptional mechanism. Thus, the present study agrees well with our previous findings that activation of intracellular signalling pathways is a key event in the process of receptor alterations [20, 22, 27, 28, 46–49].

In agreement with the present study, a depressed response to U46619 has been observed in porcine coronary artery after organ culture [50]. The down-regulation of contractile proteins like calponin has been proposed to correlate with the decrease in sensitivity to U46619. However, in our model, organ culture of rat mesenteric arteries does not significantly alter K $^{+}$ -induced contractile responses, and the maximum response to U46619 remained unchanged in both absolute (mN) and relative values (% of 60 mM K $^{+}$; table 1). These results failed to demonstrate that there is alteration of contractile proteins in our system. Moreover, in previous investigations, organ culture of rat mesenteric arteries has been revealed to up-regulate vascular receptors like ET $_B$ receptors [27, 51]. The enhanced expression of the ET $_B$ receptors occurs through de novo transcription and is again not the result of an increase in general vascular reactivity [28].

The production of prostacyclin [52] and nitric oxide [53] has been shown to suppress GPCR expression. However, COX and NOS inhibitors separately or in combination did not affect the depressed contractile responses mediated by TP receptors. This is further supported by the experiments that TP receptor-mediated contraction is independent of the integrity of endothelium both in fresh and cultured segments. In spite of this, it is possible that the activation of NF- κ B enhances transcription of

certain cytokines and growth factors which suppress TP receptor expression through transcription of the receptors. This phenomenon has also been demonstrated in mouse trachea smooth muscle cells in organ culture, in which interleukin-1 β induced down-regulation of ET_B receptor via transcriptional increase in the production of endothelin-1 (non-selective ET_A and ET_B agonist) that in turn resulted in down-regulation of the receptors [54]. In addition, MAPK pathways have been revealed to play key roles in the down-regulation of receptors in airway smooth muscle cells. This is in concert with the present view that transcriptional down-regulation of TP receptors occurred through MAPK pathways in VSMC.

In order to investigate if post-transcriptional mechanisms are involved, the effect of a general translational inhibitor, CHX, and TP receptor mRNA stability were studied. On one hand, the deficiency of CHX suggests that organ culture-induced down-regulation of TP receptor expression is not caused by translation. There are 2 isoforms of TP receptors, α and β [55], which are different in properties of receptor internalization. Studies demonstrated that enzymes like arrestin-3 and Rab-11 are required for agonist-stimulated receptor internalization of TP receptors, especially TP β [56]. However, such mechanisms are not presented in our system because if those degradation enzymes were inhibited by CHX, less receptors would be broken down and thus the contractile responses would be enhanced instead of being depressed. On the other hand, receptor mRNA is stable during organ culture for up to 24 h, further supporting that the degradation of TP receptor mRNA does not occur during organ culture. Hence, post-transcriptional mechanisms are not involved in the present study, that is, neither the degradation of receptor mRNA nor the internalization of receptor protein are responsible for the down-regulation of TP receptors.

Recently, we studied the up-regulation of TP receptors induced by stimulation of smoking particles in vitro and demonstrated that this up-regulation occurs via translation, which is distinguished from the transcriptional mechanism observed in the present investigation [57]. Enhanced thromboxane synthesis has been linked to cardiovascular diseases including acute myocardial ischemia [58], heart failure [59] and diabetes [60] in clinic. In contrast, the depressed TP receptor-mediated responsiveness in the aortic segments has been observed in streptozotocin-induced diabetic rats, while insulin therapy reversed the decreased contractile responses [13]. The down-regulation of TP receptors and suppressed contractile responses of vascular segments have been seen in

various types of arteries during organ culture and our study showed that the activation of MAPK/NF- κ B pathways is highly associated with this down-regulation. MAPK pathways are demonstrated to be required for the up-regulation of GPCRs like α -adrenoceptor, 5-HT and ET_B receptors [20, 27, 28, 46] and the down-regulation of TP receptors, therefore the latter might serve as a feedback mechanism in the pathogenesis of cardiovascular diseases. Similarly, in human coronary arteries, ET_B receptors are up-regulated, while angiotensin II type 1 receptors are down-regulated after myocardial ischemia [18]. The balance of up- and down-regulation of vascular contractile receptors determines vascular tone, proliferation and remodelling. Exploring the regulatory mechanisms for vascular receptor alteration might provide further knowledge about the pathogenesis and lead to new options to modulation of dysfunction of vasculature in diabetes.

In conclusion, organ culture of rat mesenteric artery induces a transcriptional down-regulation of TP receptor expression. This occurs through intracellular MAPK ERK1/2, p38/NF- κ B signal pathways, but not JNK, or by post-transcriptional mechanisms.

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References

- 1 Narumiya S, Sugimoto Y, Ushikubi F: Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999;79:1193–1226.
- 2 Narumiya S, FitzGerald GA: Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest* 2001;108:25–30.
- 3 Huang JS, Ramamurthy SK, Lin X, Le Breton GC: Cell signalling through thromboxane A2 receptors. *Cell Signal* 2004;16:521–533.
- 4 Thomas DW, Mannon RB, Mannon PJ, Lattour A, Oliver JA, Hoffman M, Smithies O, Koller BH, Coffman TM: Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest* 1998;102:1994–2001.
- 5 Radomski M: The biological role of thromboxane A2 in the process of hemostasis and thrombosis; pharmacology and perspectives of therapeutic use of thromboxane synthetase inhibitors and receptor PGH2/TXA2 antagonists. *Acta Physiol Pol* 1985;36:153–164.

- 6 Cockerham C, Webb ML, Hedberg SA, Runge MS: Regulation of the rat aortic smooth muscle cell thromboxane A₂ receptor. *Trans Assoc Am Physicians* 1991;104:173–180.
- 7 Sachinidis A, Flesch M, Ko Y, Schror K, Bohm M, Dusing R, Vetter H: Thromboxane A₂ and vascular smooth muscle cell proliferation. *Hypertension* 1995;26:771–780.
- 8 Katugampola SD, Davenport AP: Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT₁ receptor antagonist losartan. *Br J Pharmacol* 2001;134:1385–1392.
- 9 Geoffroy J, Benzoni D, Sassard J: Antihypertensive effect of thromboxane A₂ receptor blockade in genetically hypertensive rats of the Lyon strain. *J Hypertens Suppl* 1989;7:S272–S273.
- 10 Sugawara A, Uruno A, Kudo M, Ikeda Y, Sato K, Taniyama Y, Ito S, Takeuchi K: Transcription suppression of thromboxane receptor gene by peroxisome proliferator-activated receptor- γ via an interaction with spl1 in vascular smooth muscle cells. *J Biol Chem* 2002;277:9676–9683.
- 11 Uruno A, Sugawara A, Kudo M, Sato M, Sato K, Ito S, Takeuchi K: Transcription suppression of thromboxane receptor gene expression by retinoids in vascular smooth muscle cells. *Hypertens Res* 2003;26:815–821.
- 12 Dorn GW 2nd, Becker MW: Growth factors downregulate vascular smooth muscle thromboxane receptors independent of cell growth. *Am J Physiol* 1992;262:C927–C933.
- 13 Morinelli TA, Tempel GE, Jaffa AA, Silva RH, Naka M, Folger W, Halushka PV: Thromboxane A₂/prostaglandin H₂ receptors in streptozotocin-induced diabetes: effects of insulin therapy in the rat. *Prostaglandins* 1993;45:427–438.
- 14 Wilkes BM, Mento PF, Hollander AM: Reduced thromboxane receptor affinity and vasoconstrictor responses in placentae from diabetic pregnancies. *Placenta* 1994;15:845–855.
- 15 Davidge ST, Zhang Y: Estrogen replacement suppresses a prostaglandin h synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circ Res* 1998;83:388–395.
- 16 Raines EW, Ross R: Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *Br Heart J* 1993;69:S30–S37.
- 17 Adner M, Cantera L, Ehlert F, Nilsson L, Edvinsson L: Plasticity of contractile endothelin-B receptors in human arteries after organ culture. *Br J Pharmacol* 1996;119:1159–1166.
- 18 Wackenfors A, Emilson M, Ingemansson R, Hortobagyi T, Szok D, Tajti J, Vecsei L, Edvinsson L, Malmstro M: Ischemic heart disease induces upregulation of endothelin receptor mRNA in human coronary arteries. *Eur J Pharmacol* 2004;484:103–109.
- 19 Luo G, Xu CB, Cao YX, Edvinsson L: Transcriptional up-regulation in expression of 5-hydroxytryptamine 2A and transcriptional down-regulation of angiotensin II type 1 receptors during organ culture of rat mesenteric artery. *Basic Clin Pharmacol Toxicol* 2004;95:280–287.
- 20 Cao YX, He LC, Xu CB, Luo GG, Edvinsson L: Enhanced transcription of contractile 5-hydroxytryptamine 2A receptors via extracellular signal-regulated kinase 1/2 after organ culture of rat mesenteric artery. *Basic Clin Pharmacol Toxicol* 2005;96:282–288.
- 21 Hoel NL, Hansen-Schwartz J, Edvinsson L: Selective up-regulation of 5-HT_{1B/1D} receptors during organ culture of cerebral arteries. *Neuroreport* 2001;12:1605–1608.
- 22 Hansen-Schwartz J, Svensson CL, Xu CB, Edvinsson L: Protein kinase mediated up-regulation of endothelin A, endothelin B and 5-hydroxytryptamine 1B/1D receptors during organ culture in rat basilar artery. *Br J Pharmacol* 2002;137:118–126.
- 23 Moller S, Uddman E, Welsh N, Edvinsson L, Adner M: Analysis of the time course for organ culture-induced endothelin ET_B receptor upregulation in rat mesenteric arteries. *Eur J Pharmacol* 2002;454:209–215.
- 24 Eskesen K, Edvinsson L: Upregulation of endothelin ET_B receptor-mediated vasoconstriction in rat coronary artery after organ culture. *Eur J Pharmacol* 2006;539:192–194.
- 25 Stenman E, Malmstro M, Uddman E, Gido G, Wieloch T, Edvinsson L: Cerebral ischemia upregulates vascular endothelin ET_B receptors in rat. *Stroke* 2002;33:2311–2316.
- 26 Vikman P, Edvinsson L: Gene expression profiling in the human middle cerebral artery after cerebral ischemia. *Eur J Neurol* 2006;13:1324–1332.
- 27 Uddman E, Henriksson M, Eskesen K, Edvinsson L: Role of mitogen-activated protein kinases in endothelin ET_B receptor up-regulation after organ culture of rat mesenteric artery. *Eur J Pharmacol* 2003;482:39–47.
- 28 Henriksson M, Xu CB, Edvinsson L: Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries. *Br J Pharmacol* 2004;142:1155–1161.
- 29 De Clerck F, Janssen PA: 5-Hydroxytryptamine and thromboxane A₂ in ischaemic heart disease. *Blood Coagul Fibrinolysis* 1990;1:201–209.
- 30 Lewis TS, Shapiro PS, Ahn NG: Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998;74:49–139.
- 31 Karin M: Mitogen activated protein kinases as targets for development of novel anti-inflammatory drugs. *Ann Rheum Dis* 2004;63(suppl 2):ii62–ii64.
- 32 Surh YJ, Na HK, Lee SS: Transcription factors and mitogen-activated protein kinases as molecular targets for chemoprevention with anti-inflammatory phytochemicals. *Biofactors* 2004;21:103–108.
- 33 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* 1995;270:27489–27494.
- 34 Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feese WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM: Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623–18632.
- 35 Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC: SB 203580 is a specific inhibitor of a map kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;364:229–233.
- 36 Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW, Griswold DE: SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J Pharmacol Exp Ther* 2000;293:281–288.
- 37 Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW: SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001;98:13681–13686.
- 38 Kobori M, Yang Z, Gong D, Heissmeyer V, Zhu H, Jung YK, Gakidis MA, Rao A, Sekine T, Ikegami F, Yuan C, Yuan J: Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. *Cell Death Differ* 2004;11:123–130.
- 39 Malmstro C: Some biological effects of prostaglandin endoperoxide analogs. *Life Sci* 1976;18:169–176.
- 40 Lumley P, White BP, Humphrey PP: GR32191, a highly potent and specific thromboxane A₂ receptor blocking drug on platelets and vascular and airways smooth muscle in vitro. *Br J Pharmacol* 1989;97:783–794.
- 41 He GW, Yang CQ: Inhibition of vasoconstriction by the thromboxane A₂ antagonist GR32191B in the human radial artery. *Br J Clin Pharmacol* 1999;48:207–215.
- 42 Black JW, Leff P: Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* 1983;220:141–162.
- 43 Poliseño L, Cecchetti A, Mariani L, Evangelista M, Ricci F, Giorgi F, Citti L, Rainaldi G: Resting smooth muscle cells as a model for studying vascular cell activation. *Tissue Cell* 2006;38:111–120.
- 44 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001;22:153–183.

- 45 Henriksson M, Stenman E, Vikman P, Edvinsson L: MEK1/2 inhibition attenuates vascular ET_A and ET_B receptor alterations after cerebral ischaemia. *Exp Brain Res* 2007; 178:470–476.
- 46 Cao YX, Xu CB, Luo GG, Edvinsson L: Up-regulation of α_{1A} -adrenoceptors in rat mesenteric artery involves intracellular signal pathways. *Basic Clin Pharmacol Toxicol* 2006;98:61–67.
- 47 Uddman E, Adner M, Edvinsson L: Protein kinase C inhibitors decrease endothelin ET_B receptor mRNA expression and contraction during organ culture of rat mesenteric artery. *Eur J Pharmacol* 2002;452:215–222.
- 48 Henriksson M, Stenman E, Edvinsson L: Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. *Stroke* 2003;34: 1479–1483.
- 49 Jamali R, Edvinsson L: Involvement of protein kinases on the upregulation of endothelin receptors in rat basilar and mesenteric arteries. *Exp Biol Med (Maywood)* 2006;231: 403–411.
- 50 Thorne GD, Hilliard GM, Paul RJ: Vascular oxygen sensing: detection of novel candidates by proteomics and organ culture. *J Appl Physiol* 2004;96:802–808; discussion 792.
- 51 Luo G, Jamali R, Cao YX, Edvinsson L, Xu CB: Vascular endothelin ET_B receptor-mediated contraction requires phosphorylation of ERK1/2 proteins. *Eur J Pharmacol* 2006; 538:124–131.
- 52 Dusting GJ, Mullins EM, Nolan RD: Prostacyclin (PGI₂) release accompanying angiotensin conversion in rat mesenteric vasculature. *Eur J Pharmacol* 1981;70:129–137.
- 53 Bucher M, Hobbhahn J, Kurtz A: Nitric oxide-dependent down-regulation of angiotensin II type 2 receptors during experimental sepsis. *Crit Care Med* 2001;29: 1750–1755.
- 54 Zhang Y, Adner M, Cardell LO: Interleukin-1 β attenuates endothelin B receptor-mediated airway contractions in a murine in vitro model of asthma: roles of endothelin converting enzyme and mitogen-activated protein kinase pathways. *Clin Exp Allergy* 2004; 34:1480–1487.
- 55 Parent JL, Labrecque P, Orsini MJ, Benovic JL: Internalization of the TXA₂ receptor α and β isoforms: role of the differentially spliced cooh terminus in agonist-promoted receptor internalization. *J Biol Chem* 1999; 274:8941–8948.
- 56 Laroche G, Rochdi MD, Laporte SA, Parent JL: Involvement of actin in agonist-induced endocytosis of the G protein-coupled receptor for thromboxane A₂: overcoming of actin disruption by arrestin-3 but not arrestin-2. *J Biol Chem* 2005;280:23215–23224.
- 57 Zhang W, Zhang Y, Edvinsson L, Xu CB: Up-regulation of thromboxane A₂ receptor expression by lipid soluble smoking particles through post-transcriptional mechanisms. *Atherosclerosis* 2008;196:608–616.
- 58 Oates JA, FitzGerald GA, Branch RA, Jackson EK, Knapp HR, Roberts LJ 2nd: Clinical implications of prostaglandin and thromboxane A₂ formation (1). *N Engl J Med* 1988; 319:689–698.
- 59 Castellani S, Paladini B, Paniccia R, Di Serio C, Vallotti B, Ungar A, Fumagalli S, Cantini C, Poggessi L, Neri Serneri GG: Increased renal formation of thromboxane A₂ and prostaglandin F₂ α in heart failure. *Am Heart J* 1997;133:94–100.
- 60 Konieczkowski M, Skrinska VA: Increased synthesis of thromboxane A₂ and expression of procoagulant activity by monocytes in response to arachidonic acid in diabetes mellitus. *Prostaglandins Leukot Essent Fatty Acids* 2001;65:133–138.