



LUND UNIVERSITY
Faculty of Medicine

LU:research

Institutional Repository of Lund University

This is an author produced version of a paper published in *Atherosclerosis*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Zhang, Wei and Zhang, Yaping and Edvinsson, Lars and Xu, Cang-Bao.

"Up-regulation of thromboxane A₂ receptor expression by lipid soluble smoking particles through post-transcriptional mechanisms."

Atherosclerosis, 2007, Aug 10 [Epub ahead of print]

<http://dx.doi.org/10.1016/j.atherosclerosis.2007.06.031>

Access to the published version may
require journal subscription.
Published with permission from: Elsevier

**Up-regulation of Thromboxane A₂ receptor expression by lipid soluble
smoking particles through post-transcriptional mechanisms**

Wei Zhang^{a,b,c}, Yaping Zhang^{a,b}, Lars Edvinsson^{a,b} and Cang-Bao Xu^{a,b,*}

^a Division of Experimental Vascular Research, Institute of Clinical Science in Lund, Lund University, Lund, Sweden.

^b Department of Clinical Experimental Research, University Hospital of Glostrup, Copenhagen University, Copenhagen, Denmark.

^c Department of Pharmacology, Xi'an Jiaotong University School of Medicine, Ministry of Education, Xi'an, P. R. China.

* Correspondence should be addressed to:

Cang-Bao Xu, MD, PhD

Division of Experimental Vascular Research, BMC A13, WNC

Institute of Clinical Science in Lund, Lund University

SE-221 84 Lund, Sweden.

Phone: +46-46-2220825

Fax: +46-46-2220616

Email: Cang-Bao.Xu@med.lu.se

Total words count: 5591

Abstract

Atherosclerosis is a key factor in vascular disease and cigarette smoking is a well-known risk factor that may induce an inflammatory response and enhance plaque formation in arteries. Thromboxane (Tx) is one key inflammatory mediator involved in the pathogenesis of cardiovascular disease. The present study was designed to test if lipid soluble smoking particles (DSP) enhance TxA₂ receptor (TP) expression in rat mesenteric arteries, and if intracellular mitogen-activated protein kinase (MAPK) pathways play a role. Organ culture of rat mesenteric arteries in the presence of DSP (0.2 µl/ml for 24 hrs) resulted in markedly elevated contractile responses to the Tx analog U46619, compared with the control DMSO. There was no increase in TP receptor mRNA expression, while the protein expression was significantly enhanced. This up-regulation was not affected by a general transcriptional inhibitor actinomycin D, but was almost completely abolished by cycloheximide, a general translational inhibitor. Dexamethasone, a glucocorticoid, manifested a potent inhibitory effect as well. These results suggest that the up-regulation of TP receptor occurs via post-transcriptional events, and mainly translation. This is supported by experiments with specific inhibitors for c-Jun-NH₂-terminal kinase (SP600125), extracellular signal-regulated kinase 1 and 2 (PD98059 and U0126) and p38 (SB203580) that had no inhibitory effect on the up-regulation of TP receptors. Collectively, the results show that MAPK pathways are not involved in TP receptor up-regulation. Study on TP receptor mRNA stability showed that during organ culture, the TP receptor mRNA was stable in both DMSO and DSP group, but the latter elicited a tendency to stabilize the TP receptor mRNA at higher level. Thus, post-transcriptional mechanisms are responsible for the up-regulation of TP receptor by DSP, in which enhanced translation is the major cause of the elevated protein expression and the enhanced contraction.

Key Words: smoking; thromboxane; receptor; post-transcription; atherosclerosis; mesenteric; artery; rat.

Introduction

Cigarette smoking and second hand smoking (SHS), well-known risk factors, are associated with atherosclerosis (AS), coronary heart disease (CHD), stroke, myocardial infarction, aortic aneurysm, peripheral vascular disease and other cardiovascular diseases [1]. Smoking per se has been reported to result in 5 million premature deaths per year worldwide, quite interestingly the majority of these are due to a cardiovascular event [2]. It is considered that smoking particles cause damage to the arterial wall with localized dysfunction of the endothelium and enhanced plaque formation [3]. However, the molecular mechanisms involved in this phenomenon are still largely unknown.

Lipid soluble smoking particles (DSP) cause damage to vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) [4]; reduce the formation and release of prostacyclin from VEC [5, 6], increase ¹²⁵I-low density lipoprotein (LDL) uptake by VSMC; and together with LDL DSP increase the release of growth factors and endothelin-1 (ET-1) [7]. Furthermore, DSP may have a direct toxic effect on VEC and reduce endothelium-dependent dilatation in rat mesenteric arteries and human middle cerebral arteries [8]. As one putative mechanism, DSP result in up-regulation of endothelin A and B (ET_A and ET_B) receptors in rat bronchi via a translational mechanism [9].

Thromboxane (Tx) is widely known as a key inflammatory substance, and mediates VSMC contraction and proliferation via the thromboxane A₂ (TP) receptor [10, 11]. Tx is involved in blood platelet function and linked with hemostasis and thrombosis [12, 13]. The TP receptors density has been reported to be enhanced in cardiovascular disease [14] and in hypertension [15]. Previous experimental and clinical data have demonstrated that treatment with a TP receptor antagonist (e.g. Z-335, BM-573, Ramatroban (Bay U 3405), S18886)

provides positive effects as shown both in vitro and in vivo [16-20]. Furthermore, injury-induced vascular proliferation and platelet activation are depressed in mice genetically deficient in the TP receptor or treated with a TP antagonist [21].

The present study was designed to examine if DSP up-regulates TP receptors in VSMC and molecular mechanisms bind this event. We show for the first time that DSP result in up-regulation of TP receptors in rat mesenteric artery SMC via a translational mechanism, while mitogen-activated protein kinase (MAPK) pathways are not involved.

Materials and Methods

Tissue preparation and organ culture procedure

Sprague-Dawley rats (body weight 300-350g) were anaesthetized with CO₂ and exsanguinated. The superior mesenteric artery was gently removed, immersed into cold buffer solution (for composition, see below) and freed of adhering tissue under a dissection microscope. The endothelium was denuded by perfusion of the vessel for 10 seconds with 0.1% Triton X-100 followed by another 10 seconds with a physiologic buffer solution [22]. The removal of the endothelium was verified by no dilatatory response to acetylcholine (10⁻⁶M) on 5-hydroxytryptamine (10⁻⁵M) pre-contracted segments, and confirmed by immunohistology. The vessels were then cut into 1 mm long cylindrical segments, incubated at 37 °C in humidified 5 % CO₂ in air. Culture was carried out in a 24-well plate, two segments in each well, containing 1 ml Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (584 mg/L) and supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) [23]. The experiments were performed in organ culture of the arterial segments with DSP or dimethyl sulphoxide (DMSO). The experimental protocol was approved by Lund University Animal Ethic's Committee (M217-03).

Buffer solutions

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

In vitro pharmacology

Arterial segments were immersed in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution. The solution was continuously aerated with 5% CO₂ in O₂ resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension by the Chart software (ADInstruments, Hastings, UK). A resting tone of 2.5 mN was applied to each segment and the segments were allowed to stabilize at this tension for at least 1.5 hrs 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 [23].

Intracellular signal inhibitions and drugs

To link intracellular events to functional changes, a set of inhibitors were administered with DSP during organ culture. The MAPK pathway consists of three major members: c-Jun-

NH₂-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 [24]. Their specific inhibitors including: SP600125 (10⁻⁵M, inhibitor of JNK) [25], PD98059 and U0126 (10⁻⁵M, inhibitors of ERK1/2) [26, 27] and SB203580 (10⁻⁵M, inhibitor of p38) [28] were employed to examine the involvement of different MAPK subtypes. Actinomycin D (AcD, 5 mg/L) and cycloheximide (CHX, 10⁻⁵M) were used as general transcriptional and translational inhibitors, respectively. The inhibitory effect of dexamethasone (DEX, 10⁻⁶M), a potent anti-inflammatory glucocorticoid, was tested as well. The concentrations of these inhibitors were determined by recommendations from product information sheet and literatures.

All drugs were purchased from Sigma (St. Louis, USA). U46619 was diluted in ethanol to a stock concentration of 10 mM and diluted further in distilled water; the other reagents were dissolved in DMSO and stored according to the product information sheet and preparation guide.

Real-time reverse transcription (RT)-PCR

Smooth muscle cells were isolated from fresh or cultured artery segments and homogenized in 1 ml of the RNAPro solution (Q-BIOgene, CA, USA) by using a FastPrep instrument (Q-BIOgene, CA, USA). The total RNA was extracted following a protocol from the FastRNA Pro kit supplier, and checked by 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, CA, USA) in a Perkin-Elmer 2400 PCR (Perkin-Elmer, MA, USA) machine at 42 °C for 30 min. The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit in a GeneAmp 5700 sequence detection system (Perkin-Elmer, MA, USA). 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.

The PCR reaction was performed in a 50 µl volume and started at a temperature of 50 °C for 2 min, 95 °C for 10 min and the following 40 PCR cycles with 95 °C for 15 sec 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, CA, USA) and synthesized by TAG Copenhagen A/S (Copenhagen, Denmark). Specific primers for the rat TP receptor mRNA (GenBank accession no. [NM_017054](#)) was designed as follows:

TP receptor Forward: 5'- ATCTCCCATCTTGCCATAGTCC -3'
Reverse: 5'- CCGATGATCCTTGAGCCTAAAG -3'

Elongation factor-1 (EF-1, GenBank accession no. [BC072542](#)) and glyceraldehyde 3-phosphate dehydrogenase (GADPH, GenBank accession no. [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 analyzed with the comparative cycle threshold (C_T) method. To evaluate the amount 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 a reference 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 role of mRNA stability was involved in the up-regulation of TP receptors after organ culture with DSP, the segments were incubated with either DMSO or DSP (0.2 μ l/ml) in the presence of AcD for ½, 3, 6 and 24 hrs, respectively. The administration of AcD resulted in inhibition of any de novo synthesis of receptor mRNA was inhibited. Real-time RT-PCR was used to determine the amount of TP receptor mRNA expression.

Immunohistochemistry

The arterial segments after organ culture in presence of DMSO or DSP (0.2 μ l/ml) were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 3 hrs at 4 °C. After fixation, the specimens were dehydrated in 20% sucrose of phosphate buffer (0.1M, pH 7.4) for 24 hrs at 4 °C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and stored at -80 °C. Sections were cut at 10 μ m thickness in a cryostat and mounted on SupperFrost Plus slides. Immunohistology staining with primary antibody against rat TP receptors from rabbits (Alexis Biochemicals, Lausen, Switzerland) and secondary antibody goat-anti-rabbits IgG conjugated to fluorescein isothiocyanate (FITC) (Alexis Biochemicals, Lausen, Switzerland) 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 hr at room temperature in dark. In the control experiments, either the primary antibody or the secondary antibody were omitted. The stained arterial segments were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments Inc. NY, USA) and analysed by Image J software (<http://rsb.info.nih.gov/ij>) [29]. The measurement was done on positive staining on 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 analyzed blindly as to the treatment protocol.

Extraction of DSP

DSP were extracted as previously described [8]. Briefly, three cigarettes (Marlboro, 0.8 mg nicotine per cigarette) were "smoked" by a water aspirator and the smoke was directed through a cotton wool filter. The retained smoke particles in the filter were dissolved in 1 ml DMSO and finally diluted by DMSO to standard nicotine content of 0.1 mg/L [9].

Data analysis

All data are expressed as mean \pm SEM. The amount of receptor mRNA are expressed as percentage of housekeeping gene mRNA, or percentage of control group. Unpaired Student's t-test was used to compare two sets of data and one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni post-test for comparisons of more than two data sets. A *P* value less than 0.05 was considered to be significant.

Results

In vitro pharmacology

The viability and general contractility of the arteries during organ culture in presence and absence of DMSO and DSP were examined by their contractile response to 60 mM K⁺. The contractile responses to K⁺ were not significantly changed among the groups (fresh 2.58 ± 0.19 mN vs. organ culture 2.60 ± 0.20 mN, n=8, P > 0.05, and organ culture in the presence of DMSO 2.54 ± 0.29 mN vs. organ culture in the presence of DSP 2.47 ± 0.16 mN, n=8, P > 0.05). Organ culture of rat mesenteric arterial segments for 24 hrs with DSP 0.1 µl/ml did not affect the contractile responses to U46619 compared to the control group (Fig 1A). However, a higher DSP concentration (0.2 µl/ml) resulted in markedly elevated contractions compared with the DMSO control group (Fig 1B).

Removal of the endothelium did not affect the contractile responses to U46619 in both DMSO and DSP groups (U46619 Emax: DMSO with endothelium 7.25 ± 0.63 mN vs. DMSO without endothelium 7.15 ± 0.48 mN, n = 8, P > 0.05; U46619 pEC₅₀: DSP with endothelium 8.34 ± 0.46 vs. DSP without endothelium 8.66 ± 0.60, n = 8, P > 0.05), indicating that the endothelium did not modify the reactions. In addition, organ culture itself induced a reduction in sensitivity of the smooth muscle cells to the thromboxane agonist (U46619 pEC₅₀: fresh 7.74 ± 0.03 vs. organ culture 7.00 ± 0.05, n = 8, P < 0.05), while the maximal contractile response to U46619 remained the same (U46619 Emax: fresh 6.58 ± 0.40 mN vs. organ culture 6.66 ± 0.38 mN, n = 8, P > 0.05).

The contractile responses to U46619 were shifted towards right by 10⁻⁸M of the selective TP receptor antagonist, GR32191b, suggesting that U46619-induced contraction is mediated by TP receptor (data not shown).

Nicotine is an important substance in DSP. After organ culture for 24 hrs of the segments, the presence of nicotine (0.02 µg/ml, equivalent amount contained in DSP 0.2 µl/ml) did not result in any altered TP receptor-mediated contractile responses (Fig 1C).

Receptor mRNA expression and mRNA stability

TP receptor mRNA expression in VSMC was detected by real-time RT-PCR. After co-culture with DSP 0.2 µl/ml for 24 hrs it was not increased (Fig 2A, $P > 0.05$). In mRNA stability tests AcD was added to both DMSO and DSP groups to inhibit de novo synthesis of receptor mRNA for different time points of organ culture. The mRNA appeared to be quite stable in both groups. In parallel with the non-inhibited situation, DSP tended to stabilize mRNA and resulted in a slightly higher mRNA expression after 6 hrs though not significant (Fig 2B, $P > 0.05$).

Immunohistochemistry

The TP receptor protein was visualized by the fluorescent immunohistology method and analysed by confocal microscopy with the Image J software. Collagen bands (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 weak TP receptor protein expression in the DMSO group, localized among the smooth muscle cells (Figure 3A). However, the TP receptor protein was strongly enhanced in the DSP treated group as shown by the bright green granules localized on the SMC (Figure 3B). Semi-quantitative measurement of TP receptor protein density showed that DSP significantly enhanced TP receptor protein expression by 2 fold ($n = 6$, $P < 0.001$), compared with the control (DMSO) group (Figure 3C).

Inhibitor studies

The general transcriptional inhibitor AcD did not affect the elevated TP receptor-mediated responses elicited by DSP. However, the translational inhibitor CHX almost completely abolished the DSP-induced up-regulation of TP receptors. DEX, a glucocorticoid which shows extensive anti-inflammation effects, attenuated the up-regulation of TP receptor significantly (Fig 4A). Inhibition experiments of several intracellular pathways were carried out by using a set of specific inhibitors of various MAPK subtypes: JNK (SP600125), ERK1/2 (PD98059 and U0126) and p38 (SB203580). However none of these presented a significant effect (Fig 4B). Thus, a post-transcriptional mechanism is involved in the DSP-induced up-regulation of TP receptor expression.

Discussion

It is known that Tx and TxA₂ receptors are involved in the atherosclerotic process; enhanced contraction and proliferation of various cell types involved in the plaque formation [30]. Smoking presents an elevated risk factor enhancing this process. The present study is the first to demonstrate that organ culture of rat mesenteric arteries in the presence of DSP results in markedly elevated contractile responses to the thromboxane analog U46619 (selective agonist for TP receptors). The up-regulation of TP receptors after co-culture with DSP was shown also at the receptor protein level and localized to the smooth muscle cells in the vessel wall. A general transcriptional inhibitor AcD or subtype specific MAPK inhibitors did not affect the up-regulation of TP receptor induced by DSP, while CHX, a general translational inhibitor abolished the up-regulation. This suggests that post-transcription events are involved in the TP receptor up-regulation.

Studies have demonstrated that organ culture of rat mesenteric artery induces up-regulation of endothelin B (ET_B) receptor expression in the SMC. This occurs through de novo transcription and translation of the receptors via activation of MAPK pathways [31-33]. A set of inhibitors, previously tested, were used in the present study to examine if MAPK pathways are involved in DSP-induced up-regulation of TP receptors. The results showed that, specific inhibitors of JNK (SP600125), ERK1/2 (PD98059 and U0126) and p38 (SB203580) had no inhibitory effect on the DSP-induced up-regulation of TP receptors. These results agree with the inefficiency of AcD and further prove that the up-regulation of TP receptors does not occur via MAPK activated transcription. In contrast, we found that organ culture per se induces a decrease in sensitivity of the VSMC in response to selective TP receptor agonist U46619 via a transcription mechanism rather than translation (unpublished data).

In order to examine if the observation that there was no change in TP receptor mRNA was due to an artifact, mRNA stability experiments were carried out. Under the condition that de novo synthesis of receptor mRNA was inhibited, the TP receptor mRNA expression was stable at different time points of the organ culture progress. Despite of the observed nonsignificant differences, DSP showed a tendency to stabilize TP receptor mRNA expression compared with DMSO group. However, the marked increase in receptor protein expression indicates that a translational mechanism is the major cause of TP receptor up-regulation. In agreement with the present study, we have previously reported that DSP enhances ET_A and ET_B receptor expression through a translational mechanism in rat bronchioles [9]. The up-regulation of TP receptors may also occur through inhibition of the receptor protein break down mechanisms. However, CHX is a well-known general translational inhibitor that acts on both the synthesis of TP receptor protein and degrading enzymes for the TP receptors. If the degrading enzymes are inhibited by CHX, it could lead to

more TP receptor proteins and enhanced contractile responses to U46619. The present study showed that CHX had an inhibitory effect on the up-regulation of TP receptors. This suggests that CHX specifically acts on the translation process of TP receptors rather than degrading enzymes for TP receptors.

Previous investigations have revealed that TxA₂ generation is increased in aortic segments of cholesterol-fed atherosclerotic rabbits [34]; and that in two transgenic murine models of atherogenesis the Tx biosynthesis is elevated [35]. In addition, it has been reported that in coronary artery smooth muscle cells, thrombin-induced proliferation is markedly enhanced by TxA₂ and this involves up-regulation of TP receptor mRNA [36]. Thus, there exist data supporting that the TP receptor is an important target in prevention and treatment of atherosclerosis. Antagonists for TP receptors have achieved positive effects in experimental and clinical trials [16-20], and antagonism or deletion of TP receptor expression retards atherogenesis in apolipoprotein E knockout mice [37]. In our study, we have described a novel method for studies of the up-regulation of TP receptor expression induced by smoking particles which also suggests a possible role for active or passive cigarette smoking to promote atherogenesis and vascular diseases.

As a member of glucocorticoids family DEX exhibits non-specific anti-inflammation effects through several mechanisms. Firstly, it suppresses gene expression at the transcriptional level via inhibition of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) [38, 39]; secondly, post-transcriptional mechanisms account for the effects of DEX by depressing protein expression, including destabilization and translational inhibition of mRNA [40, 41]. In the present study, the inefficiency of the transcriptional inhibitor AcD as well as the specific MAPK inhibitors indicate that

transcription does not account for the up-regulation of TP receptors. Therefore, it is suggested that DEX has effect via a post-transcriptional process rather than a transcriptional mechanism. Glucocorticoids have been used in the treatment of experimental atherosclerosis [42, 43] and restenosis after coronary artery angioplasty [44], showing some anti-atherogenic effects. In human aortic smooth muscle cells, DEX may attenuate intimal proliferation at the site of vascular injury during atherogenesis [45].

In the present setup, DSP was quantified and standardized with nicotine content as an internal quantitative control for the DSP. The DSP preparations were analyzed by gas chromatography and then diluted by DMSO to standard nicotine content (0.1 mg/ml) [8, 9]. It has been reported that in smokers, the plasma concentration of nicotine varies between 4 - 72 ng/ml, and that the average concentration is 33 ng/ml [46]. In the present study, we used DSP 0.2 µl/ml which contains 20 ng/ml of nicotine. The used concentration is therefore slightly below the average plasma concentration in smokers, and thus our findings are relevant to *in vivo* both for active and passive smoking.

Nicotine, one of the key substances in smoking particles, was also examined in the present study. Equivalent amounts of nicotine as occur in DSP did not affect the contractile responses to U46619 after organ culture, suggesting that in our model nicotine is unrelated to the up-regulation of TP receptors. This finding is in concert with a previous study in which nicotine did not contribute to the increased production of TxA₂ following cigarette smoking in man no matter if it was studied *in vitro* or *in vivo* [47]. In a smoke exposed guinea pig model, the second-phase response of the bronchoconstriction characterized by the release of TxA₂, prostaglandin D₂, and prostaglandin F_{2α}, which act on TP receptors in airway smooth muscles, was shown to be evoked by non-nicotine smoking particulates [48].

As a complex, smoking particles consist of more than 4000 different substances. It is difficult to verify which substances are mainly responsible for the up-regulation of TP receptors. Despite that the involvement of nicotine has been excluded here, reports have demonstrated that polycyclic aromatic hydrocarbons (dimethylbenzanthracene and benzpyren) and oxidants like free radicals are contained in DSP. These particles may result in dysfunction of the arterial cells [7, 49, 50] in aspects to cell proliferation, release of vasoactive substances as well as alteration of vascular receptors.

In conclusion, the present study is the first to conclusively demonstrate that smoking particles provoke up-regulation of TP receptors, with increased receptor expression and enhanced contractility in VSMC. This up-regulation occurs through a translational mechanism. The elevated TP receptor expression may contribute to the development of atherosclerosis and cardiovascular diseases.

Acknowledgments

This study was supported by grant from the Swedish Research Council (No 5958), the Heart-Lung Foundation, the Council for Swedish Medical Tobacco Research and Flight Attendant Medical Research Institute (FAMRI), USA.

References

- [1] Ezzati M, Henley SJ, Thun MJ and Lopez AD. Role of smoking in global and regional cardiovascular mortality. *Circulation* 2005;112:489-97.
- [2] Lightwood J. The economics of smoking and cardiovascular disease. *Prog Cardiovasc Dis* 2003;46:39-78.
- [3] Holden WE, Kishiyama SS, Dong SP and Osborne ML. Endothelium-dependent effects of cigarette smoke components on tone of porcine intrapulmonary arteries in vitro. *Toxicol Appl Pharmacol* 1990;104:191-9.
- [4] Xu CB, Pessah-Rasmussen H and Stavenow L. Interactions between cultured bovine arterial endothelial and smooth muscle cells: effects of injury on the release of growth stimulating and growth inhibiting substances. *Pharmacol Toxicol* 1991;69:195-200.
- [5] Xu CB, Stavenow L and Pessah-Rasmussen H. Interactions between cultured bovine arterial endothelial and smooth muscle cells; studies on the release of prostacyclin by endothelial cells. *Artery* 1992;19:94-111.
- [6] Xu CB, Stavenow L and Pessah-Rasmussen H. Interactions between cultured bovine arterial endothelial and smooth muscle cells; further studies on the effects of injury and modification of the consequences of injury. *Artery* 1993;20:163-79.
- [7] Xu CB, Stavenow L and Pessah-Rasmussen H. Interactions between cultured bovine arterial endothelial and smooth muscle cells; effects of modulated low density lipoproteins on cell proliferation and prostacyclin release. *Scand J Clin Lab Invest* 1994;54:191-8.
- [8] Zhang JY, Cao YX, Xu CB and Edvinsson L. Lipid-soluble smoke particles damage endothelial cells and reduce endothelium-dependent dilatation in rat and man. *BMC Cardiovasc Disord* 2006;6:3.
- [9] Granstrom BW, Xu CB, Nilsson E, Vikman P and Edvinsson L. Smoking particles enhance endothelin A and endothelin B receptor-mediated contractions by enhancing translation in rat bronchi. *BMC Pulm Med* 2006;6:6.
- [10] Cockerham C, Webb ML, Hedberg SA and Runge MS. Regulation of the rat aortic smooth muscle cell thromboxane A2 receptor. *Trans Assoc Am Physicians* 1991;104:173-80.
- [11] Sachinidis A, Flesch M, Ko Y, Schror K, Bohm M, Dusing R and Vetter H. Thromboxane A2 and vascular smooth muscle cell proliferation. *Hypertension* 1995;26:771-80.
- [12] Radomski M. The biological role of thromboxane A2 in the process of hemostasis and thrombosis; pharmacology and perspectives of therapeutical use of thromboxane synthetase inhibitors and receptor PGH2/TXA2 antagonists. *Acta Physiol Pol* 1985;36:153-64.
- [13] Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA, Hoffman M, Smithies O, Koller BH and Coffman TM. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest* 1998;102:1994-2001.

- [14] Katugampola SD and Davenport AP. Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT(1) receptor antagonist losartan. *Br J Pharmacol* 2001;134:1385-92.
- [15] Geoffroy J, Benzoni D and Sassard J. Antihypertensive effect of thromboxane A2 receptor blockade in genetically hypertensive rats of the Lyon strain. *J Hypertens Suppl* 1989;7:S272-3.
- [16] Tanaka T, Ito S, Higashino R, Fukuta Y, Fukuda Y, Takei M, Kurimoto T and Tamaki H. A new thromboxane receptor antagonist, Z-335, ameliorates experimental thrombosis without prolonging the rat tail bleeding time. *Thromb Res* 1998;91:229-35.
- [17] Rolin S, Petein M, Tchana-Sato V, Dogne JM, Benoit P, Lambermont B, Ghuysen A, Kolh P and Masereel B. BM-573, a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, prevents pig myocardial infarction induced by coronary thrombosis. *J Pharmacol Exp Ther* 2003;306:59-65.
- [18] Fiedler VB, Seuter F and Perzborn E. Effects of the novel thromboxane antagonist Bay U 3405 on experimental coronary artery disease. *Stroke* 1990;21:IV149-51.
- [19] Pieters H, Roodt JP, Badenhorst PN, van Wyk V, Schall R, Lotter MG, Hundt HK and Nel CJ. Antithrombotic activity of Bay u3405, a thromboxane A2-antagonist, in patients with Dacron aortic grafts: a random controlled clinical trial. *Thromb Haemost* 1993;70:903-8.
- [20] Belhassen L, Pelle G, Dubois-Rande JL and Adnot S. Improved endothelial function by the thromboxane A2 receptor antagonist S 18886 in patients with coronary artery disease treated with aspirin. *J Am Coll Cardiol* 2003;41:1198-204.
- [21] Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA and FitzGerald GA. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 2002;296:539-41.
- [22] Hamel E, Assumel-Lurdin C, Edvinsson L, Fage D and MacKenzie ET. Neuronal versus endothelial origin of vasoactive acetylcholine in pial vessels. *Brain Res* 1987;420:391-6.
- [23] Adner M, Cantera L, Ehlert F, Nilsson L and Edvinsson L. Plasticity of contractile endothelin-B receptors in human arteries after organ culture. *Br J Pharmacol* 1996;119:1159-66.
- [24] Lewis TS, Shapiro PS and Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998;74:49-139.
- [25] 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 and Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* 2001;98:13681-6.
- [26] Alessi DR, Cuenda A, Cohen P, Dudley DT and 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;270:27489-94.
- [27] Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA

and Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623-32.

[28] Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR and 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;364:229-33.

[29] Vikman P, Beg S, Khurana T, Hansen-Schwartz J and Edvinsson L. Gene expression and molecular changes in cerebral arteries following subarachnoid hemorrhage in the rat. *J Neurosurg* 2006;105:438-44.

[30] Das UN. Atherosclerosis and prostaglandins. *Int J Tissue React* 1982;4:127-32.

[31] Uddman E, Henriksson M, Eskesen K and Edvinsson L. Role of mitogen-activated protein kinases in endothelin ETB receptor up-regulation after organ culture of rat mesenteric artery. *Eur J Pharmacol* 2003;482:39-47.

[32] Henriksson M, Xu CB and Edvinsson L. Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries. *Br J Pharmacol* 2004;142:1155-61.

[33] Luo G, Jamali R, Cao YX, Edvinsson L and Xu CB. Vascular endothelin ET(B) receptor-mediated contraction requires phosphorylation of ERK1/2 proteins. *Eur J Pharmacol* 2006;538:124-31.

[34] Mehta JL, Lawson D, Mehta P and Saldeen T. Increased prostacyclin and thromboxane A2 biosynthesis in atherosclerosis. *Proc Natl Acad Sci U S A* 1988;85:4511-5.

[35] Pratico D, Cyrus T, Li H and FitzGerald GA. Endogenous biosynthesis of thromboxane and prostacyclin in 2 distinct murine models of atherosclerosis. *Blood* 2000;96:3823-6.

[36] Zucker TP, Bonisch D, Muck S, Weber AA, Bretschneider E, Glusa E and Schror K. Thrombin-induced mitogenesis in coronary artery smooth muscle cells is potentiated by thromboxane A2 and involves upregulation of thromboxane receptor mRNA. *Circulation* 1998;97:589-95.

[37] Egan KM, Wang M, Fries S, Lucitt MB, Zukas AM, Pure E, Lawson JA and FitzGerald GA. Cyclooxygenases, thromboxane, and atherosclerosis: plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism. *Circulation* 2005;111:334-42.

[38] Mierendorf RC, Jr. and Mueller GC. Role of dexamethasone in globin gene expression in differentiating Friend cells. *J Biol Chem* 1981;256:6736-41.

[39] Jeon YJ, Han SH, Lee YW, Lee M, Yang KH and Kim HM. Dexamethasone inhibits IL-1 beta gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kappa B/Rel and AP-1 activation. *Immunopharmacology* 2000;48:173-83.

[40] Mukaida N, Zachariae CC, Gusella GL and Matsushima K. Dexamethasone inhibits the induction of monocyte chemotactic-activating factor production by IL-1 or tumor necrosis factor. *J Immunol* 1991;146:1212-5.

- [41] Papaconstantinou J, Stewart JA, Rabek JP, McClintock PR and Wong EY. Glucocorticoids inhibit the coordinated translation of alpha- and beta-globin mRNAs in Friend erythroleukemia cells. *Arch Biochem Biophys* 1983;227:542-51.
- [42] Bailey JM and Butler J. Anti-inflammatory drugs in experimental atherosclerosis. I. Relative potencies for inhibiting plaque formation. *Atherosclerosis* 1973;17:515-22.
- [43] Naito M, Yasue M, Asai K, Yamada K, Hayashi T, Kuzuya M, Funaki C, Yoshimine N and Kuzuya F. Effects of dexamethasone on experimental atherosclerosis in cholesterol-fed rabbits. *J Nutr Sci Vitaminol (Tokyo)* 1992;38:255-64.
- [44] Macdonald RG, Panush RS and Pepine CJ. Rationale for use of glucocorticoids in modification of restenosis after percutaneous transluminal coronary angioplasty. *Am J Cardiol* 1987;60:56B-60B.
- [45] Nakano T, Raines EW, Abraham JA, Wenzel FGt, Higashiyama S, Klagsbrun M and Ross R. Glucocorticoid inhibits thrombin-induced expression of platelet-derived growth factor A-chain and heparin-binding epidermal growth factor-like growth factor in human aortic smooth muscle cells. *J Biol Chem* 1993;268:22941-7.
- [46] Russell MA, Jarvis M, Iyer R, Feyerabend C. Relation of nicotine yield of cigarettes to blood nicotine concentrations in smokers. *Br Med J.* 1980;280:972-6.
- [47] Toivanen J, Ylikorkala O and Viinikka L. Effects of smoking and nicotine on human prostacyclin and thromboxane production in vivo and in vitro. *Toxicol Appl Pharmacol* 1986;82:301-6.
- [48] Hong JL and Lee LY. Cigarette smoke-induced bronchoconstriction: causative agents and role of thromboxane receptors. *J Appl Physiol* 1996;81:2053-9.
- [49] Pessah-Rasmussen H, Stavenow L, Xu CB and Berglund A. Increased smooth muscle cell proliferation by dimethylbenzanthracene is correlated to variations in activity of ornithine decarboxylase but not arylhydrocarbonhydroxylase. *Artery* 1991;18:240-55.
- [50] Stavenow L and Pessah-Rasmussen H. Effects of polycyclic aromatic hydrocarbons on proliferation, collagen secretion and viability of arterial smooth muscle cells in culture. *Artery* 1988;15:94-108.

Figure Captions

Fig 1: U46619-induced contraction of rat mesenteric arteries after organ culture for 24 hrs in the presence of DMSO / DSP: (A) 0.1 $\mu\text{l/ml}$ and (B) 0.2 $\mu\text{l/ml}$. Data are shown as the mean \pm SEM. Each data point is derived from 8 segments. (C) TP receptor-mediated contraction of rat mesenteric arteries after organ culture for 24 hrs in the presence of nicotine 0.02 $\mu\text{g/ml}$ (equivalent to DSP 0.2 $\mu\text{l/ml}$). In contrast with DSP, nicotine didn't elevate the TP receptor-mediated contractile response. Data are shown as the mean \pm SEM. Each data point is derived from 7-8 segments.

Fig 2: (A) TP receptor mRNA expression in rat mesenteric artery after organ culture with DMSO or DSP (0.2 $\mu\text{l/ml}$) for 24 hrs. Data are expressed as mean \pm SEM. Unpaired student's t-test, $P > 0.05$ vs. control group. Each data point is derived from 6 experiments. (B) TP receptor mRNA stability after co-culture with DMSO or DSP (0.2 $\mu\text{l/ml}$) for $\frac{1}{2}$, 3, 6 and 24 hrs in the presence of AcD (5 mg/L). Data are expressed as mean \pm SEM. Unpaired student's t-test, $P > 0.05$ vs. $\frac{1}{2}$ hr group. Each data point is derived from 5 experiments.

Fig 3: Immunohistology demonstrates an up-regulation of TP receptor protein expression. Arteries were cultured for 24 hrs in the presence of: (A) DMSO and (B) DSP (0.2 $\mu\text{l/ml}$). Arrows point to: (A) the collagen bands and (B) the TP receptor protein. The size bar corresponds to 10 μm . (C) semi-quantitation of the receptor protein expression. Each data point is derived from 6 experiments. Two-tailed unpaired Student's t-test with Welch's correction, *** $P < 0.001$ compared with control (DMSO).

Fig 4: (A) DSP-induced up-regulation of TP receptor was not affected by a transcriptional inhibitor AcD. General translation inhibitor CHX almost completely abolished the up-

regulation. DEX, a glucocorticoid showed a potent inhibitory effect. Data are shown as the mean \pm SEM. Each data point is derived from 6-12 segments. (B) Inhibition of JNK (SP600125), ERK1/2 (PD98059 and U0126) and p38 (SB203580) had no effect on DSP-induced up-regulation of TP receptor. Data are shown as the mean \pm SEM. Each data point is derived from 7-10 segments.

Figures

Fig 1

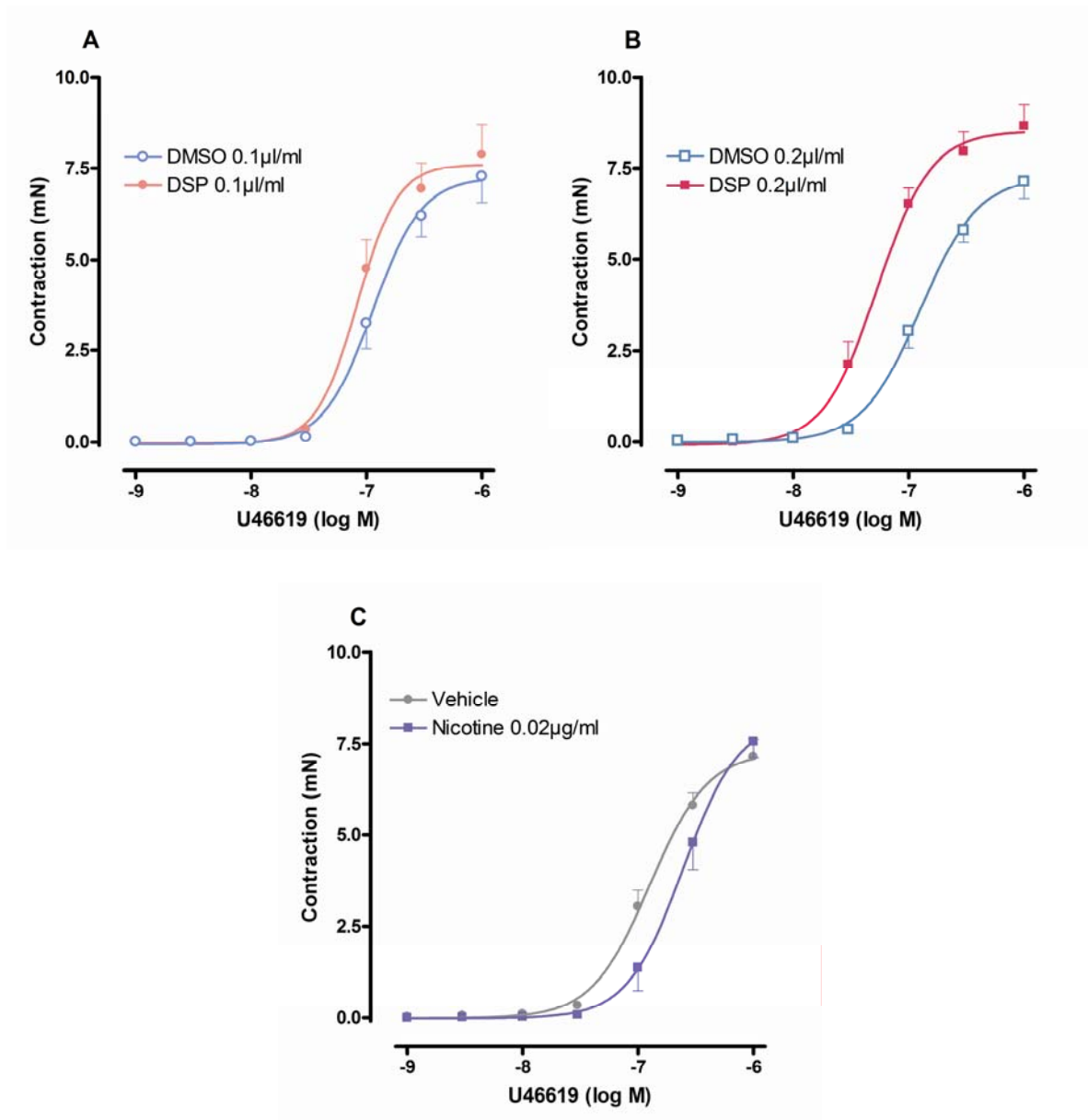


Fig 2

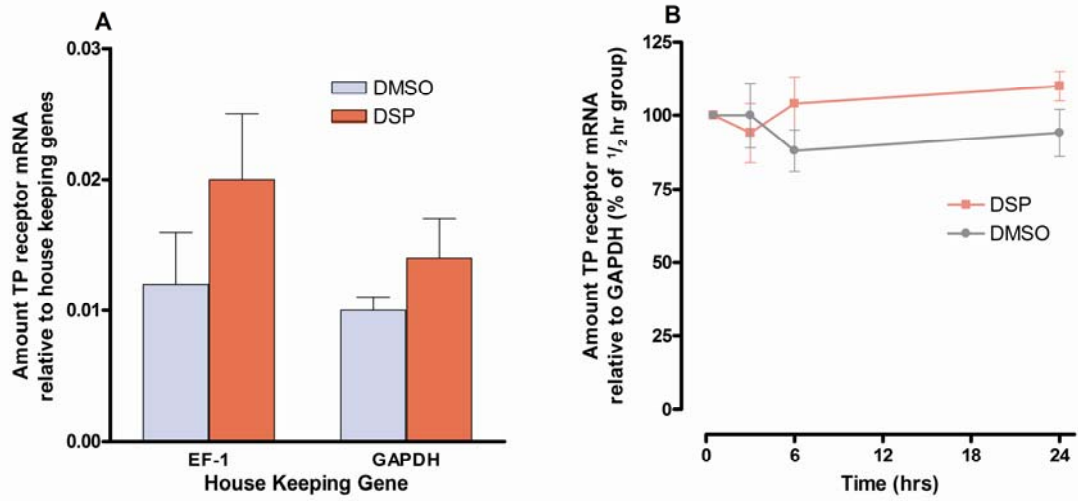


Fig 3

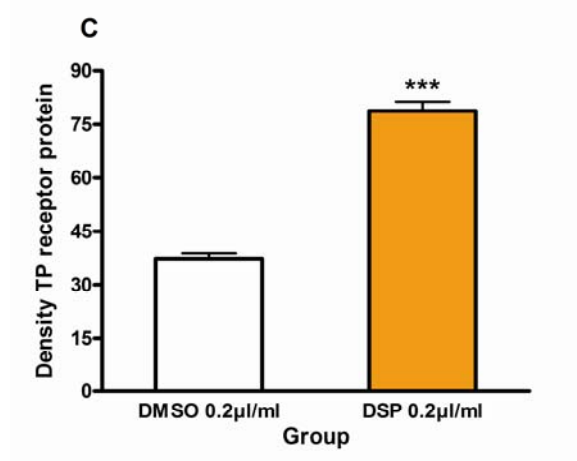
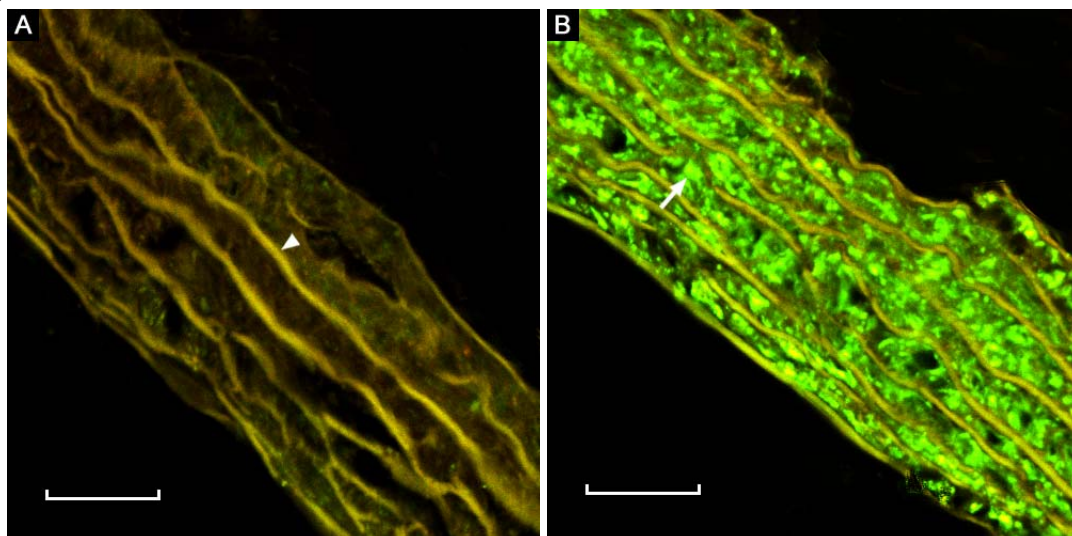


Fig 4

