Basic & Clinical Pharmacology & Toxicology, **107**, 803–812 Doi: 10.1111/j.1742-7843.2010.00585.x

Up-Regulation of G-Protein-Coupled Receptors for Endothelin and Thromboxane by Lipid-Soluble Smoke Particles in Renal Artery of Rat

Yan-hua Xie[1], Si-wang Wang[1], Yaping Zhang[2], Lars Edvinsson[2] and Cang-Bao Xu[2]

[1]Institute of Materia Medica, School of Pharmacy, Fourth Military Medical University, Xi'an, Shaanxi, China and [2]Division of Experimental Vascular Research, Institute of Clinical Science in Lund, Lund University, Lund, Sweden

(Received 13 October 2009; Accepted 15 February 2010)

Abstract: Up-regulation of G-protein-coupled receptors (GPCR) plays key roles in renal hypertension and cardiovascular disease pathogenesis. The present study was designed to examine if lipid-soluble cigarette smoking particles (DSP), nicotine and endotoxin (LPS), induce GPCR up-regulation for thromboxane A_2 (TP), endothelin type A (ET_A) and type B (ET_B) receptors in renal artery, and if intracellular signal mechanisms are involved. Renal artery segments of rats were exposed to DSP, nicotine or LPS, in organ culture for up to 24 hr. The GPCR-mediated contractions were recorded by using a myograph system. Expression of the GPCR was examined by real-time PCR and immunohistochemistry at mRNA and protein levels. Sarafatoxin 6c (S6c, selective ET_B receptor agonist), endothelin-1 (ET-1, non-selective ET_A and ET_B receptor agonist) and 9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F_{2a} (U46619, a TP receptor agonist) induced contractions were significantly increased after the arterial segments exposed to DSP in a concentration-dependent (0.1–0.4 μl/ml) manner, and S6c also induced a time-dependent contraction, compared to control (dimethyl sulfoxide). This was in parallel with enhanced mRNA expression for ET_B receptor but not ET_A and TP receptors, while increased protein expression for ET_A, ET_B and TP receptors was seen. The specific nuclear factor-kappa B (NF-κB) signal pathway inhibitor BMS345541 was applied to link DSP effects to the GPCR up-regulation. It totally abolished ET_B receptor up-regulation, but not ET_A and TP receptor up-regulations. Our results suggest that DSP transcriptionally up-regulated ET_B receptor expression in rat renal artery via NF-κB signal pathways, whereas up-regulation of ET_A and TP receptor-mediated contraction may involve post-transcriptional mechanisms.

Both active and passive cigarette smoking is a well-known risk factor for hypertension, atherosclerosis (AS), coronary heart disease (CHD), stroke, myocardial infarction, aortic aneurysm, peripheral vascular disease and other cardiovascular diseases [1]. In a previous study, we demonstrated that lipid-soluble cigarette smoking particles (DSP) cause damage to vascular endothelial cells and vascular smooth muscle cells [2] reduce the formation and release of prostacyclin from [3,4]. DSP may also have a direct toxic effect on vascular endothelial cells and reduce endothelium-dependent dilatation in rat mesenteric arteries and human middle cerebral arteries [5]. As a complex, DSP consist of more than 4000 different substances. Among them, nicotine and endotoxin (LPS) are important substances contained in cigarette smoke, which may induce damage to the cardiovascular system. The up-regulation of G-protein-coupled receptors (GPCR) for endothelin type B (ET_B) receptors in arteries is suggested to be one of key mechanisms that cigarette smoke leads to cardiovascular disease [6]. The intracellular signal pathways activated by DSP involve activation of mitogen-activated protein kinase and the downstream transcriptional factor nuclear factor-kappa B (NF-κB) [6]. However, there is limited knowledge about how cigarette smoke exposure results in renal vascular GPCR up-regulation and subsequently leads to hypertension and cardiovascular diseases.

The renal blood flow is a key factor affecting the kidney's ability to regulate plasma volume and blood pressure [7–9]. Endothelin-1 (ET-1) and thromboxane A_2 (TP) are strong vasoconstrictors that control renal blood flow. Increased ET-1 and TP levels in renal blood may induce renal ischaemia via activation of their respective GPCR in the smooth muscle cells [10]. We hypothesize that cigarette smoke may cause damage to renal arteries, which results in renal vascular GPCR alteration for endothelin and thromboxane via NF-κB intracellular signal mechanisms. The presence of structural and functional changes in pre-glomerular and afferent arterioles before the development of hypertension and the persistence of structural changes, despite normalization of blood pressure, suggest that these renal vascular changes are not a mere consequence of elevated blood pressure, but may be involved in the pathogenesis of hypertension [10].

The present study was designed to examine if DSP up-regulates TP, ET_A and ET_B receptors in renal arteries, if LPS in DSP are involved in the DSP effects and if blockage NF-κB

Author for correspondence: Si-wang Wang, Institute of Materia Medica, School of Pharmacy, Fourth Military Medical University, Xi'an, Shaanxi, China (fax +86-29-84774748, e-mail wangsiw58@gmail.com).

signal pathways can abolish the DSP effects. Understanding the intracellular signal mechanisms behind cigarette smoke-induced renal arterial dysfunction and damage may provide a new target for developing therapies for hypertension and cardiovascular disease.

Materials and Methods

Tissue preparation and organ culture procedure. Male Sprague–Dawley rats (weighing 300–350 g) were anaesthetized with CO_2 and exsanguinated. The renal artery was gently removed, immersed into cold buffer solution (for composition, see below) and dissected free of adhering tissue under a microscope. As this study was designed to investigate alteration of vascular smooth muscle cells GPCR expression and the molecular mechanisms involved, only endothelium-denudated segments were used for the studies. The endothelium was denuded by perfusion of the vessel for 10 sec. with 0.1% Triton X-100 followed by another 10 sec. with a physiological buffer solution [11]. The removal of the endothelium was verified by the absence of dilatory response to acetylcholine (10^{-6} M) in 5-hydroxytryptamine (10^{-5} M) pre-contracted segments, and confirmed by immunohistology (fig. 1). Then, the vessels were cut into 1∼1.5 mm-long cylindrical segments, used incubated at 37°C in humidified 5% CO_2 in O_2 for different time points (organ culture group) [12]. Segments for organ culture were placed in a 24-well plate, one 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 μg/ml).

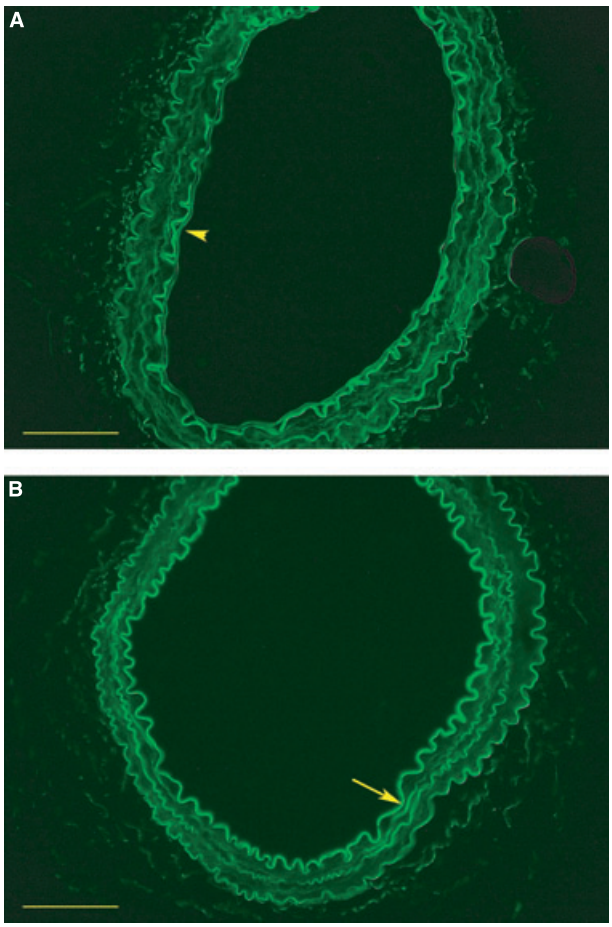


Fig. 1. Immunohistological demonstration of renal artery with and without endothelium. Renal artery with endothelium (A) and without endothelium (B), Endothelium cell (arrow head) and autofluorescent (arrow) is easily distinguished. The scale bar = 100 μm.

Experiments were performed in an organ culture of arterial segments in the presence of different concentrations of DSP or dimethyl sulfoxide (DMSO, control). For concentration effects, DSP 0.1 ∼0.4 μl/ml were used, and for time-course effects only DSP 0.2 μl/ml was applied. The strongest effect in all groups is DSP 0.4 μl/ml and therefore this concentration was selected for intracellular signal mechanism studies. For inhibition experiments, 4(2′-aminoethyl)amino-1,8-dimethylimidazo(1,2-a) quinoxaline (BMS345541, 1–25 μM), the specific blocker for NF-κB signal pathway was 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 (M161-07).

Standard buffer solution (mM): NaCl 119; $NaHCO_3$ 15; KCl 4.6; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.5; glucose 5.5. 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. Analytical-grade chemicals and double-distilled water were used for preparing all solutions.

Extraction of cigarette smoke. DSP were extracted as previously described: three cigarettes (Marlboro, 0.8 mg nicotine per cigarette) were 'smoked' by a water aspirator, the smoke being directed through a cotton-wool filter. The smoke particles retained in the filter were dissolved in 1 ml DMSO [5].

In vitro pharmacology. 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% CO_2 in O_2, 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 2.0 mN was applied to each segment and the segments were allowed to stabilize at this tension for 1.5 hr before being exposed to potassium-rich buffer solution. 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 PCR. The smooth muscle was isolated mechanically on an ice tray under a microscope. After removal of epithelium, the smooth muscle was rinsed with cold PBS and stored in the RNAlater™ (QIAGEN, Hilden, Germany) at −80°C until extraction of RNA. The total RNA was extracted by using the RNeasy Mini following the kit instructions (QIAGEN). The purity of total RNA was checked by a spectrophotometer, and wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 in all preparations. Total RNA that extracted from the cultured segments was reversely transcribed to cDNA. The real-time PCR was performed in a GeneAmp 7300 Sequence Detection system (Perkin-Elmer, Applied Biosystems, Wellesley, MA, USA), using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) with a 25-μl reaction volume. The PCR reaction 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. β-Actin was used as housekeeping gene. The gene expressions were normalized *versus* the housekeeping gene to account for differences in the starting material and in the cDNA reaction efficiency. 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. Data were analysed with the comparative cycle threshold method. To evaluate the amount of mRNA in a sample, β-Actin mRNA was assessed in the same sample simultaneously. The cycle threshold values of β-Actin mRNA were used as reference to quantify the relative amount of the studied receptor mRNA. The relative amount of mRNA was calculated with the cycle

Table 1.
Accession numbers and primer sequence for the genes that were investigated.

Gene name	Abbreviation	Accession number	Primer sequence
ET_A receptor	ET_A	NM_012550.2	Fwd: 5′-ATTGCCCTCAGGGAACAC-3′ Rev: 5′-CAACCAAGCAGAAAGACGGTC-3′
ET_B receptor	ET_B	NM_017333.1	Fwd: 5′-GATACGACAACTTCCGCTCCA-3′ Rev: 5′-GTCCACGATGAGGACAATGAG-3′
Thromboxane A_2 receptor	TP	NM_017054.1	Fwd: 5′-ATCTCCCATCTTGCCATAGTCC-3′ Rev: 5′-CCGATGATCCTTGAGCCTAAAG-3′
β-Actin	ACTB	NM_031144.2	Fwd: 5′-GTAGCCATCCAGGCTGTGTTG-3′ Rev: 5′-TGCCAGTGGTACGACCAGAG-3′

threshold value of the studied receptor mRNA in relation to the cycle threshold value of β-Actin mRNA in the sample.

All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by TAG Copenhagen A/S (Copenhagen, Denmark). Total gene specificity of the nucleotide sequences chosen for primers and probes was confirmed by results of BLAST searches (GenBank database sequences). The nucleotide sequences of the primers used in the investigation were as shown in table 1.

Immunohistochemistry. Arterial segments were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 hr at 4°C. After fixation, the specimens were dehydrated in 20% sucrose of phosphate buffer (0.1 M, pH 7.4) for 24 hr at 4°C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and stored at −80°C [13]. Sections were cut at 10 μm thickness in a cryostat and mounted on Super-Frost Plus slides. The sections were incubated overnight with goat anti-human ET_B (sc-21196, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:150, rabbit anti-mouse ET_A (sc-33536, Santa Cruz Biotechnology) 1:100, rabbit anti-human TP (Cayman, 10004452, Ann Arbor, MI, USA) 1:200 in PBS with 10% foetal calf serum. The secondary antibodies used were donkey anti-goat Cy™2 conjugated (JacksonImmunoResearch, 705-225-003, West Grove, PA, USA) 1:200, donkey anti-rabbit Cy™2 conjugated (JacksonImmunoResearch, 711-225-152) 1:200, donkey anti-rabbit IgG FITC conjugated (JacksonImmunoResearch, 711-095-152) 1:200 in PBS. As control, either the primary or the secondary antibody was omitted. The stained arterial segments were observed under a confocal microscope (C1plus; Nikon Instruments Inc., Melville, NY, USA) and analysed by the Image J software (http://rsb.info.nih.gov/ij) [14]. The measurement was based on positive staining in the smooth muscle cells. For each vessel, six sections were studied and the values obtained were mean fluorescence in the areas selected (there were six pre-set areas per section) and analysed blindly as to the treatment protocol.

Drugs. All drugs were purchased from Sigma. 9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F_{2a} (U46619, a TP receptor agonist) was dissolved in ethanol to a stock concentration of 10 mM and further diluted in distilled water; others were dissolved in distilled water.

Data analysis. Calculations and statistics were performed using Microsoft Office Excel 2003 and GraphPad Prism 4.0 software (La Jolla, CA, USA).. The experiments were repeated 6–8 times. All data are expressed as mean values ± S.E.M. Statistical significance was accepted when $p < 0.05$, using ANOVA Bonferroni post-test or two-tailed unpaired t-test with Welch's correction. The results of contractile responses were analysed by ANOVA/Bonferoni, the mRNA amount and the density of protein were analysed by t-test. The maximum contraction (E_{max}) was calculated as percentage of the contractile capacity of 60.0 mM potassium. The negative logarithm of the concentration that elicited 50% contraction (pEC_{50}) was determined by linear regression analysis using the values immediately above and below half-maximum response.

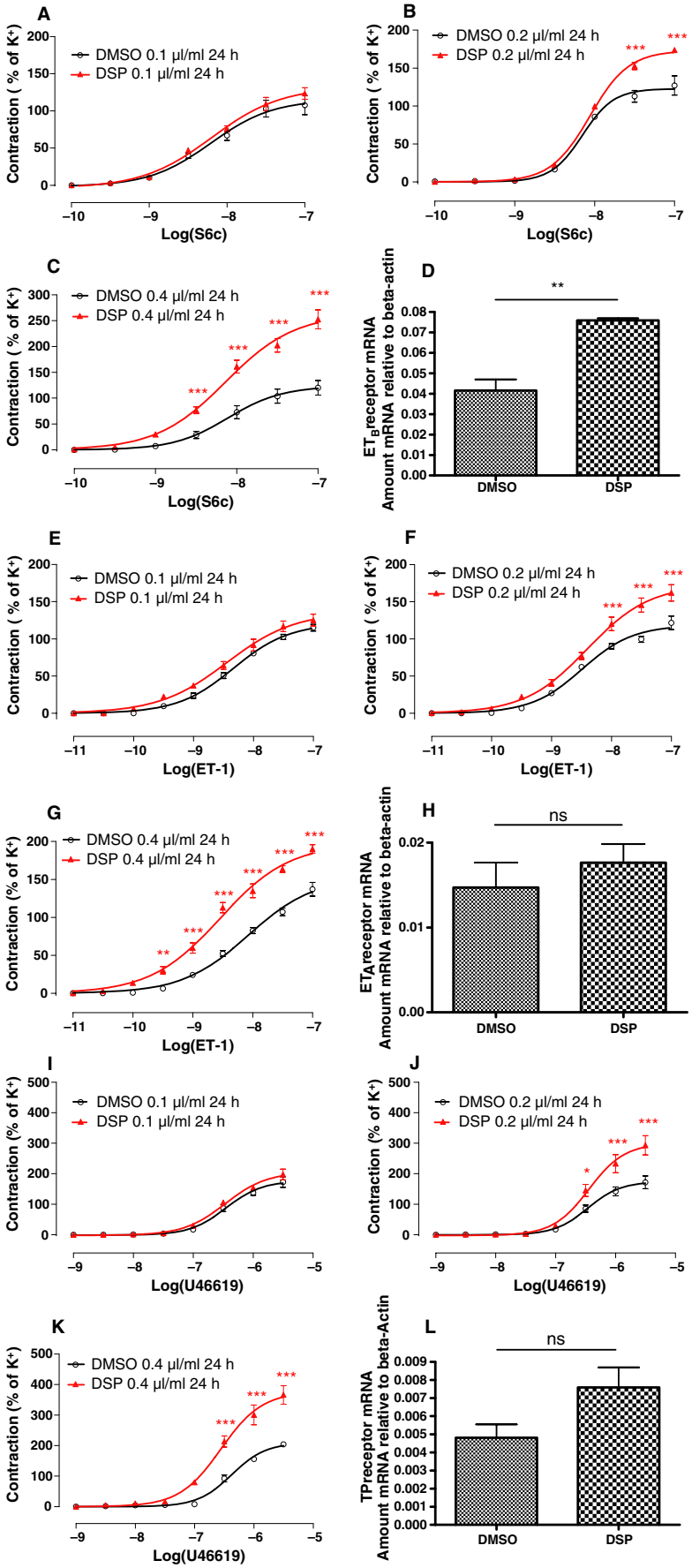
Results

Effects of DSP on ET_B receptor-mediated contraction.
Rat renal artery ring segments organ cultured in presence of DSP 0.2 μl/ml for 12 hr or 24 hr exhibited increased contractile response to sarafatoxin 6c (S6c, selective ET_B receptor agonist). S6c-induced contraction is diminished after 24-hr incubation with DSP in comparison to 12-hr incubation. The E_{max} to S6c at 12 hr increased from 56.0 ± 2.9% to 151.5 ± 21.1% and at 24 hr from 122.7 ± 4.6% to 172.9 ± 2.6% (table 2). Lower concentration (0.1 μl/ml, 24 hr) of DSP had no significant effects compared with control DMSO (fig. 2A). Similar results were obtained in ET_A and TP receptor-mediated contractions (figs 2E and I). Organ culture of the segments with 0.4 μl/ml of DSP for 24 hr, caused stronger contractile response to S6c (E_{max} from 123.1 ± 11.5% to 262.5 ± 16.7%, fig. 2C), compared with

Table 2.
Time-course effects of DSP on contractile responses to S6c, ET-1 and U46619.

	S6c		ET-1		U46619	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Fresh	2.4 ± 0.5	–	82.2 ± 5.3	8.20 ± 0.04	158.2 ± 6.6	6.48 ± 0.04
DMSO 0.2 μl/ml, 12 hr	56.0 ± 2.9	8.69 ± 0.08	131.3 ± 5.8	8.33 ± 0.07	159.5 ± 6.0	6.46 ± 0.07
DSP 0.2 μl/ml, 12 hr	151.5 ± 21.1[1]	7.97 ± 0.19	181.2 ± 11.9[1]	8.25 ± 0.10	217.4 ± 9.8[1]	6.44 ± 0.08
DMSO 0.2 μl/ml, 24 hr	122.7 ± 4.6	8.15 ± 0.04	118.5 ± 4.7	8.49 ± 0.06	173.8 ± 12.3	6.46 ± 0.08
DSP 0.2 μl/ml, 24 hr	172.9 ± 2.6[1]	8.06 ± 0.02	172.4 ± 9.3[1]	8.41 ± 0.09	297.8 ± 23.1[1]	6.45 ± 0.09

Time-course effects of DSP on contractile responses to S6c, ET-1 and U46619. Organ culture of rat renal artery segments in presence of DSP 0.2 μl/ml for 12 and 24 hr resulted in stronger contractile response to S6c, ET-1 and U46619.
[1]$p < 0.05$ when cultured with DSP, compared to DMSO. E_{max} are expressed as percent of 60 mM K^+-induced contraction. pEC_{50} values mean negative logarithm of the molar concentration that produced half maximum contraction. Data are shown as mean ± S.E.M., n = 6.



0.2 μl/ml of DSP (E_{max} from 122.7 ± 4.6% to 172.9 ± 2.6%, fig. 2B), but the leftwards shift of the concentration effect curves was not seen (figs 2B and C). However, 0.8 μl/ml of DSP (co-cultured for 24 hr) did not cause a further up-regulation of S6c-induced contraction and similar phenomenon was seen in ET_A and TP receptor-mediated contractions (data not shown).

Effects of DSP on ET_A receptor-mediated contraction.

Endothelin-1 (non-selective ET_A and ET_B receptor agonist administered after desensitization of the ET_B receptors) was used. When ET_B receptor is desensitized by S6c (10^{-7} M), ET-1 only excites ET_A receptor. The desensitization of the ET_B receptors was achieved by performing S6c concentration effect curves and waiting for the curve to return to basic level. Thereafter, a higher dose of S6c ($10^{-6.5}$ M) was used to verify the absence of ET_B receptor. ET-1-induced concentration-effect curves were obtained following the verification and S6c was present together during the whole process of ET-1 administration. Previous experiments have shown that under these conditions, ET-1 only activates ET_A receptor [15,16]. Our results showed that rat renal artery ring segments co-cultured with 0.2 μl/ml DSP for 12 hr or 24 hr resulted in markedly enhanced contractions induced by ET-1 (table 2), while the maximal contractions were reached at 12 hr and no further increase in contraction induced by ET-1 after 24-hr DSP incubation compared to 12 hr. The E_{max} mediated by ET_A receptor after incubation with 0.4 μl/ml DSP for 24 hr (196.7 ± 8.4%, fig. 2G) was larger than 0.2 μl/ml DSP (172.4 ± 9.3%, fig. 2F).

Effects of DSP on TP receptor-mediated contraction.

Compared to the control group, organ culture of rat renal arterial segments for 12 or 24 hr with DSP 0.2 μl/ml also enhanced the contractile responses to U46619 and the increase in contraction to U46619 at 24 hr is almost as same as that of 12 hr (table 2). In addition, organ culture of the segments with 0.4 μl/ml of DSP for 24 hr, caused stronger contractile response to U46619 (E_{max} from 209.3 ± 7.8% to 376.8 ± 24.2%, fig. 2K), compared with 0.2 μl/ml of DSP (E_{max} from 173.8 ± 12.3% to 297.8 ± 23.1%, fig. 2J).

Effects of NF-κB inhibitor, BMS345541 on DSP-enhanced ET_A, ET_B and TP receptor-mediated contraction.

In order to examine if the transcriptional mechanism is involved in the up-regulation of the studied receptors, BMS345541 was used. BMS345541 was a selective inhibitor of the catalytic subunits of IKK. The compound selectively inhibited the stimulated phosphorylation of IκB, as well as mitogen-activated protein kinase-activated protein kinases activation in cells [17]. The results revealed that BMS345541 abolished the increase in the ET_B receptor-mediated contraction in a concentration-dependent manner, the contractions in BMS345541 (25, 5 and 1 μM) treated segments returned to the basic line (fig. 3A). However, BMS345541 had no effects on contractions induced by the ET_A (fig. 3B) and TP receptor (fig. 3C).

Effects of DSP on ET_A, ET_B and TP receptor mRNA expression.

Endothelin type B, ET_A and TP receptor mRNA expression were assessed using real-time PCR. Organ culture of vessel segments in the presence of DSP (0.4 μl/ml) for 24 hr significantly increased ET_B receptor mRNA expression ($p < 0.01$ *versus* DMSO, fig. 2D). In contrast to the ET_B receptor mRNA expression, organ culture of vessel segments in the presence of DSP did not significantly affect the mRNA expressions of the ET_A and TP receptors ($p > 0.05$ *versus* DMSO, figs 2H and L). In concert with the functional results, these results confirm that the up-regulation of ET_B receptor mainly involves in a transcriptional mechanism and the ET_A and TP receptor up-regulations may be involved in a post-transcriptional mechanism.

Effects of DSP on ET_A, ET_B and TP receptor protein expression.

The images (figs 4A, D, and G) showed that there were expressions of ET_B, ET_A and TP receptors in the smooth muscle cells, bright green granules in the control group located among the smooth muscle cells (control, 0.4 μl/ml DMSO). There were almost no expressions of ET_B, ET_A and TP receptors in the primary (figs 5A, C, and E) and secondary negative controls (figs 5B, D, and F). Organ culture of vessel segments in the presence of DSP (0.4 μl/ml) for 24 hr resulted in enhanced ET_B, ET_A and TP receptor protein expressions in the smooth muscle cells (figs 4B, E, and H).

Effects of nicotine or LPS on ET_B, ET_A and TP receptor-mediated contraction.

Nicotine and LPS are important substances in cigarette smoke, so we studied their effects on rat renal arterial segments. We found that co-cultured with 100 ng/ml of nicotine for 24 hr has no effects on the ET_B, ET_A and TP receptor-mediated contractions, but co-cultured with 0.01 mg/ml LPS for 24 hr induced significant up-regulations of the ET_B and ET_A receptor-mediated contractions (for ET_B, E_{max} from 117.4 ± 5.7% to 187.3 ± 17.9%, n = 8 in each group and $p < 0.05$, pEC_{50} from 8.11 ± 0.05 to 8.08 ± 0.14, $p > 0.05$, fig. 6A. For ET_A, E_{max} from 173.3 ± 17.0% to 317.9 ± 44.5%, n = 10 in each group and $p < 0.05$, pEC_{50} from 7.92 ± 0.14 to 8.00 ± 0.21, $p > 0.05$, fig. 6B), while the TP receptor-mediated contraction has no change (fig. 6C).

Fig. 2. Concentration effects of DSP on contractile responses to S6c, ET-1 and U46619. Contractile responses induced by S6c (A∼C), ET-1 (E∼G) and U46619 (I∼K) in rat renal arterial segments cultured for 24 hr in the presence of 0.1, 0.2 and 0.4 μl/ml DSP and the mRNA expression of ET_B(D), ET_A(H) and TP receptor (L). The contractile responses are presented as a percentage of contractile response induced by 60 mM K^+. Data are shown as means ± S.E.M. Each data point is derived from six to eight experiments. $^{**}p < 0.01$ *versus* DMSO.

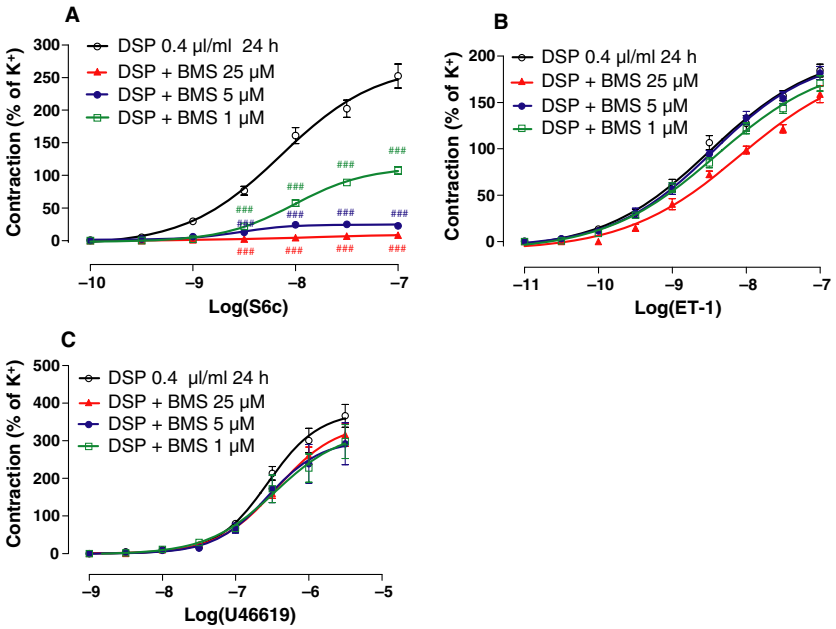


Fig. 3. Effects of NF-κB pathway inhibitor BMS345541 on S6c (A), ET-1 (B) and U46619 (C) induced contraction. Renal artery segments were organ-cultured for 24 hr in the presence of 0.4 µl/ml DSP and 25, 5, 1 µM of BMS345541. The contractile responses are presented as the percentage of contractile response induced by 60 mM K^+. Data are shown as means ± S.E.M. Each data point is derived from six to eight experiments. BMS = BMS345541.

Discussion

Cigarette smoke exposure strongly associates with the development of hypertension and cardiovascular disease. However, the underlying molecular mechanisms that cigarette smoke leads to hypertension and cardiovascular disease are not fully understood. The present study has demonstrated that DSP, lipid-soluble cigarette smoke particles, induced alteration of renal vascular GPCR expression for thromboxane and endothelin at functional contraction of the renal artery, mRNA and/or protein levels. The ET_B-, ET_A- and TP-mediated contractions were significantly increased after DSP exposure in a concentration-dependent manner, though the dose-effect was only present within a relatively narrow concentration window (0.1–0.4 µl/ml), while ET_B-mediated contraction also increased in a time-dependent manner. The mRNA expression for ET_B, but not ET_A and TP receptor, was significantly increased. The enhanced protein expressions of ET_A, ET_B and TP receptors were seen in the arterial smooth muscle. This suggests that both transcriptional and post-transcriptional mechanisms were involved in the DSP effects. Further exploring the intracellular signal mechanisms showed that the up-regulation of ET_B receptor was totally abolished by BMS345541, while the up-regulations of TP and ET_A receptors were not affected by BMS345541. In addition, LPS, but not nicotine, enhanced S6c- and ET-1-induced contraction, while LPS and nicotine did not affect TP receptor-mediated contraction. The present findings well agree with our previous study on rat mesenteric arteries that showed DSP-induced activation of mitogen-activated protein kinase and the downstream NF-κB pathways [6] and subsequent up-regulation of ET_B receptor, while the increased TP receptor was independent on mitogen-activated protein kinase activation [13]. Nicotine was not involved in the DSP effects [5,6,13].

Studies showed that cigarette smoking acutely increases aortic stiffness and blood pressure in the smokers and the effects persist longer in smokers with hypertension than in the smokers without hypertension [18]. However, the role of nicotine in the development of cardiovascular disease is still being debated, as nicotine replacement by chewing tobacco or using moist snuff does not appear to have as many associated cardiovascular risks as smoking does [19]. It has been reported that in smokers, the plasma concentration of nicotine varies between 4 and 72 ng/ml, and that the average concentration is 33 ng/ml [20]. In the present study, we used DSP 0.4 µl/ml which contains 40 ng/ml of nicotine [21]. In our experiments, three doses of nicotine (1000, 100, 10 ng/ml, data not shown, except 100 ng/ml) did not affect the contractile responses to S6c, ET-1 and U46619 after organ culture, suggesting that nicotine was not involved in the up-regulation of endothelin and TP receptors. This finding is in concert with a previous study in which nicotine did not contribute to the increased production of TP following cigarette smoking in man no matter if it was studied *in vitro* or *in vivo* [22].

LPS is another key substance in DSP. It has essential component of the bacterial wall of pathogenic gram negative bacteria and it is known that LPS is an important mediator in septic shock secondary to infection [23]. It is known that LPS mediates its vasodilator effects, in part, by altering the contractile function of smooth muscle via up-regulation of

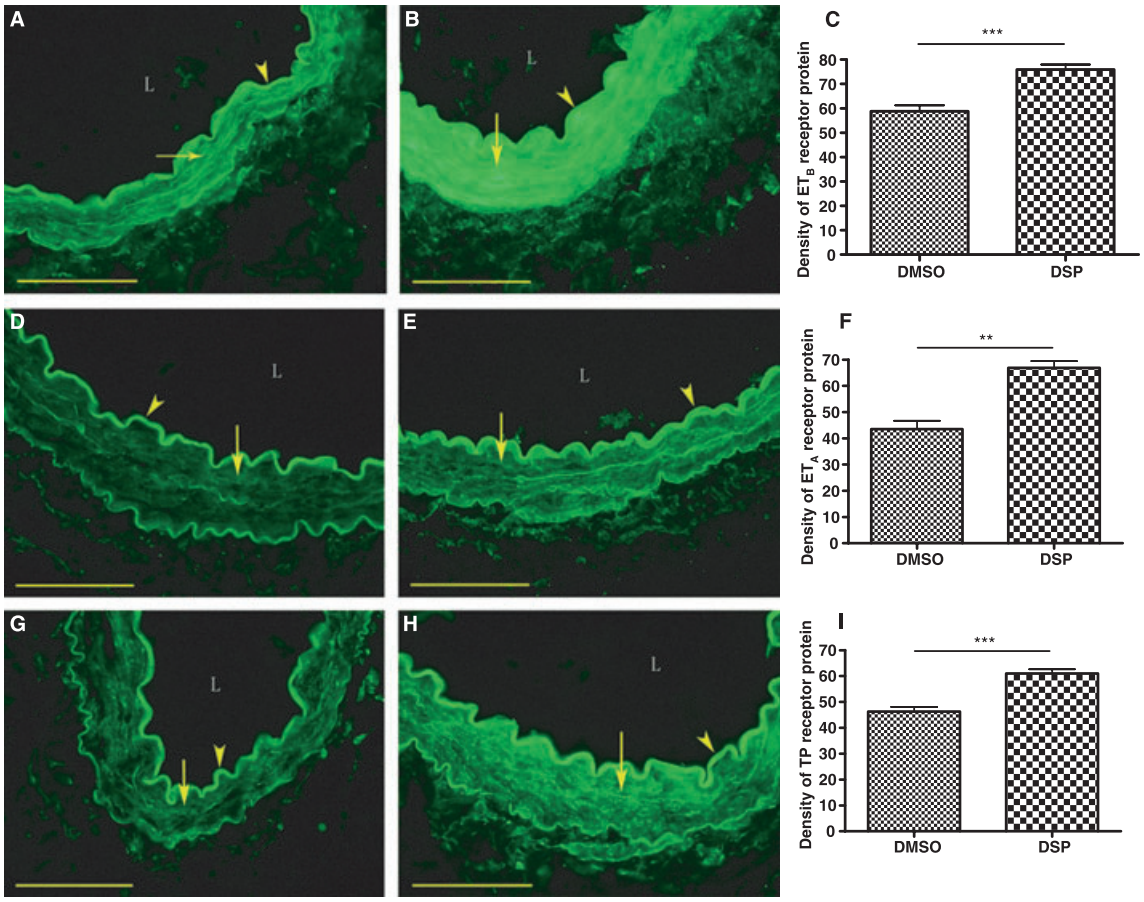


Fig. 4. Immunohistological demonstration of ET_B (A–C), ET_A (D–F) and TP (G–I) receptors protein expression (DMSO controls, A, D and G *versus* after organ culture in the presence of 0.4 μl/ml DSP for 24 hr; B, E, and H are shown, respectively). The vessels were denuded of endothelium; the innermost elastic membrane is easily depicted and autofluorescent (arrow head). The smooth muscle cell protein expression is homogenously distributed (arrow). L indicates lumen. The scale bar = 100 μm. The protein expression was quantified using the program Image J software. Statistical results of protein expression of DMSO controls and after organ-cultured for 24 hr in the presence of 0.4 μl/ml DSP are shown (C, F, I). Each data point is derived from eight experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* DMSO.

inducible nitric oxide (NO) synthase [24,25] and prostaglandins E2/I2 production [26,27]. The up-regulate endothelin receptors by LPS indicates a role in the development of hypertension and cardiovascular disease. However, the concentration of LSP in tobacco smoke is much lower [28] than the concentration effects in the present study, in which the concentrations of LPS were referenced from [29]. It is unlikely that LPS was responsible for the DSP effects.

Thromboxane is widely known as a key inflammatory substance that mediates vascular smooth muscle cells contraction and proliferation via the TP receptor [30,31]. The TP receptor density has been reported to be enhanced in cardiovascular disease [32] and in hypertension [33]. Previous investigations have revealed that TP generation is increased in aortic segments of cholesterol-fed atherosclerotic rabbits [34], and that in two transgenic murine models of atherogenesis the TP biosynthesis is elevated [35]. In addition, it has been reported that in coronary artery smooth muscle cells, thrombin-induced proliferation is markedly enhanced by TP and this involves up-regulation of TP receptor mRNA [36]. The up-regulation of TP receptor in rat mesenteric arteries occurs through intracellular mitogen-activated protein kinase ERK1/2, p38 signal pathways, but not JNK pathway [13]. In agreement with this, the up-regulation of TP receptors in renal artery induced by DSP was not inhibited by specific NF-κB inhibitor BMS345541 in the present study, suggesting that the post-transcriptional mechanisms might be involved.

Endothelin-1 is a strong vasoconstrictor of renal activates, produced locally in the endothelial cells and renal disorders are characterized by increased renal vascular resistance, including acute ischaemic renal failure, calcineurin inhibitor toxicity, endotoxaemia, hepatorenal syndrome and others [10]. In mammals, ET-1 mediates its actions via two GPCR subtypes: ET_A and ET_B receptors [37,38]. In the vasculature, ET_A receptors are found in smooth muscle cells; they are the dominant receptor subtype on vascular smooth muscle cells and mediate contraction, while ET_B receptors are localized on endothelial cells (ET_{B1}) and in smooth muscle cells (ET_{B2}), ET_{B1} and ET_{B2} receptor stimulation promote opposite effects on vascular tone: ET_{B1} receptors induce vasodilatation through the release of NO, whereas ET_{B2} receptors

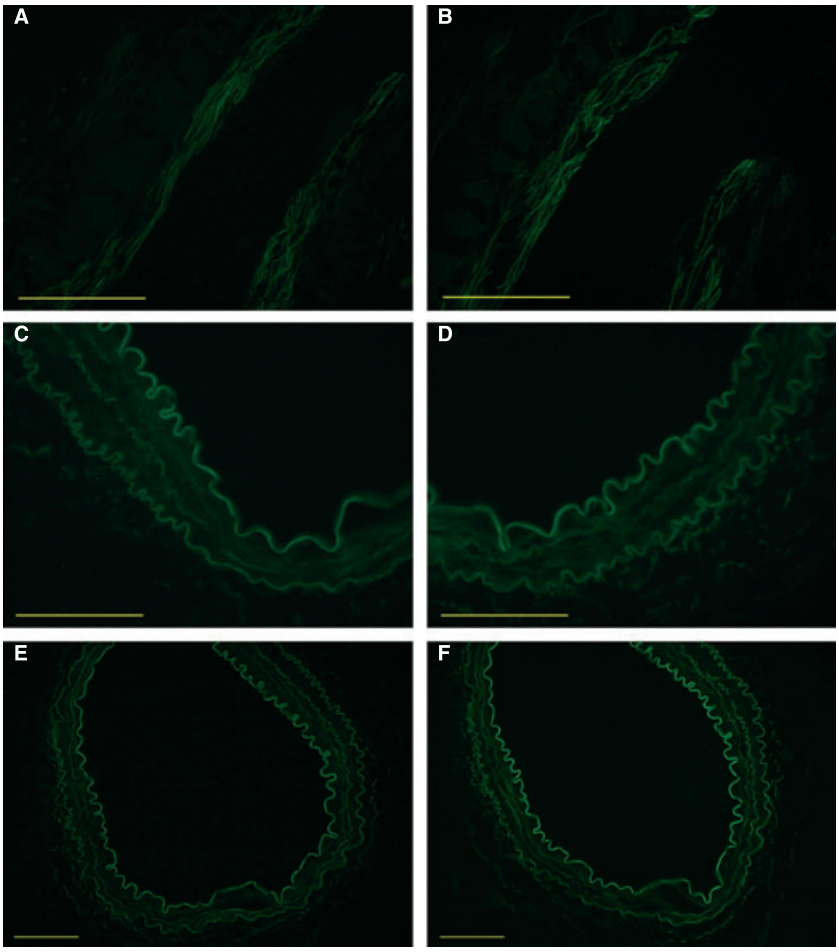


Fig. 5. The primary (A, C, E) and secondary negative controls (B, D, F) of ET_B, ET_A and TP receptors expressions.

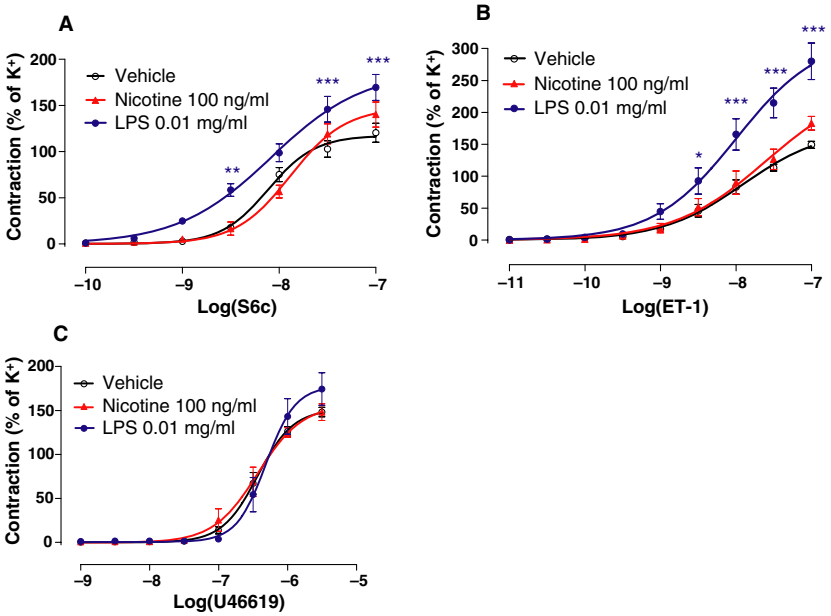


Fig. 6. Effects of LPS and nicotine-induced contraction by S6c (A), ET-1 (B) and U46619 (C). Renal artery segments were organ-cultured for 24 hr in the presence of 0.1 mg/ml LPS or 100 ng/ml nicotine. The contractile responses are presented as the percentage of contractile response induced by 60 mM K^+. Data are shown as means ± S.E.M. Each data point is derived from six to eight experiments.

induce direct vasoconstriction [39,40]. However, in hypertensive rats and patients, ET_{B2} receptors expressed in vascular smooth muscle cells induces stronger vasoconstriction by more than normal blood pressure controls, which is in parallel with up-regulation of ET_A receptors [41]. The present study revealed that both ET_A and ET_B receptors are up-regulated during organ culture in presence of DSP in the renal arteries. Recently, we have shown that DSP induces ET_B up-regulation on the mesenteric vascular smooth muscle cells with enhanced vascular smooth muscle cells contraction via activation of mitogen-activated protein kinase and NF-κB pathways [6]. The present study suggests that in the renal artery, both the ET_A and ET_B receptors were up-regulated caused by DSP and the DSP induced up-regulation of ET_B receptor was mediated by NF-κB signal pathway-mediated transcriptional mechanisms, while the up-regulation of ET_A receptors was independent on the NF-κB signal pathways.

There are other mechanisms for changes in contraction independent of receptor amount. Renal endothelial cell dysfunction is manifested as a decrease in the release of the vasodilatory mediators, such as NO, prostacyclin, and endothelium-derived hyperpolarizing factors (EDHF), and/or an increase in vasoconstrictive mediators, such as endothelin, angiotensin and TP. Also, an increase in the amount/activity of intracellular Ca^{2+} concentration, protein kinase C, Rho kinase and mitogen-activated protein kinase in vascular smooth muscle promotes renal vasoconstriction [10]. In the present study, only endothelium-denudated segments were used, so we could focus on the alteration of vascular smooth muscle cells GPCR expression and the molecular mechanisms involved.

In conclusion, the present study has demonstrated for the first time that lipid-soluble cigarette smoke particles induced up-regulation of ET_B, ET_A and TP receptors with enhanced contractility of rat renal vascular smooth muscle cells. The up-regulation of ET_B receptor, but not ET_A and TP receptors, most likely occurred through activation of NF-κB signal pathway. Both nicotine and LPS were unlikely involved in DSP effects, although LPS at the higher concentrations had such effects. The increased renal vascular ET_B, ET_A and TP receptor expressions may contribute to the development of hypertension, atherosclerosis and other cardiovascular diseases. Pharmacological blockage of the intracellular NF-κB signal mechanisms may provide new options for the treatment.

Acknowledgements

This study was supported by the Heart-Lung Foundation (grant no. 20070273), the Swedish Research Council (5958) and the Flight Medical Research Institute (FAMRI, USA). The authors appreciate Ph.D. student Ying Lei for assistance with the real-time PCR analysis.

References

1 Ezzati M, Henley SJ, Thun MJ, Lopez AD. Role of smoking in global and regional cardiovascular mortality. Circulation 2005;**112**:489–97.

2 Xu CB, Pessah-Rasmussen H, 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.

3 Xu CB, Stavenow L, 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.

4 Xu CB, Stavenow L, 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.

5 Zhang JY, Cao YX, Xu CB, Edvinsson L. Lipid-soluble smoke particles damage endothelial cells and reduce endothelium-dependent dilatation in rat and man. BMC Cardiovasc Disord 2006;**6**:3.

6 Xu CB, Zheng JP, Zhang W, Zhang Y, Edvinsson L. Lipid-soluble smoke particles upregulate vascular smooth muscle ETB receptors via activation of mitogen-activating protein kinases and NF-kappaB pathways. Toxicol Sci 2008;**106**:546–55.

7 Guyton AC. Blood pressure control–special role of the kidneys and body fluids. Science 1991;**252**:1813–6.

8 Hall JE, Brands MW, Shek EW. Central role of the kidney and abnormal fluid volume control in hypertension. J Hum Hypertens 1996;**10**:633–9.

9 Reinhardt HW, Seeliger E. Toward an Integrative Concept of Control of Total Body Sodium. News Physiol Sci 2000;**15**:319–25.

10 Ponnuchamy B, Khalil RA. Cellular mediators of renal vascular dysfunction in hypertension. Am J Physiol Regul Integr Comp Physiol 2009;**296**:R1001–18.

11 Hamel E, Assumel-Lurdin C, Edvinsson L, Fage D, MacKenzie ET. Neuronal versus endothelial origin of vasoactive acetylcholine in pial vessels. Brain Res 1987;**420**:391–6.

12 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**:1159–66.

13 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**:608–16.

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

15 Uddman E, Henriksson M, Eskesen K, 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.

16 Adner M, Shankley N, Edvinsson L. Evidence that ET-1, but not ET-3 and S6b, ET(A)-receptor mediated contractions in isolated rat mesenteric arteries are modulated by co-activation of ET(B) receptors. Br J Pharmacol 2001;**133**:927–35.

17 Burke JR, Pattoli MA, Gregor KR, Brassil PJ, MacMaster JF, McIntyre KW *et al.* BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. J Biol Chem 2003;**278**:1450–6.

18 Rhee MY, Na SH, Kim YK, Lee MM, Kim HY. Acute effects of cigarette smoking on arterial stiffness and blood pressure in male smokers with hypertension. Am J Hypertens 2007;**20**:637–41.

19 Jimenez-Ruiz C, Kunze M, Fagerstrom KO. Nicotine replacement: a new approach to reducing tobacco-related harm. Eur Respir J 1998;**11**:473–9.

20 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.

21 Granstrom BW, Xu CB, Nilsson E, Vikman P, 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.
22 Toivanen J, Ylikorkala O, 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.
23 Danner RL, Elin RJ, Hosseini JM, Wesley RA, Reilly JM, Parillo JE. Endotoxemia in human septic shock. Chest 1991;**99**:169–75.
24 Liu SF, Barnes PJ, Evans TW. Time course and cellular localization of lipopolysaccharide-induced inducible nitric oxide synthase messenger RNA expression in the rat in vivo. Crit Care Med 1997;**25**:512–8.
25 Boyle WA III, Parvathaneni LS, Bourlier V, Sauter C, Laubach VE, Cobb JP. iNOS gene expression modulates microvascular responsiveness in endotoxin-challenged mice. Circ Res 2000;**87**:E18–24.
26 Schildknecht S, Bachschmid M, Baumann A, Ullrich V. COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. FASEB J 2004;**18**:757–9.
27 MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M *et al.* Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 1995;**81**:641–50.
28 Larsson L, Szponar B, Ridha B, Pehrson C, Dutkiewicz J, Krysinska-Traczyk E *et al.* Identification of bacterial and fungal components in tobacco and tobacco smoke. Tob Induc Dis 2008;**4**:4.
29 Bachar O, Adner M, Uddman R, Cardell LO. Toll-like receptor stimulation induces airway hyper-responsiveness to bradykinin, an effect mediated by JNK and NF-kappa B signaling pathways. Eur J Immunol 2004;**34**:1196–207.
30 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**:173–80.
31 Sachinidis A, Flesch M, Ko Y, Schror K, Bohm M, Dusing R *et al.* Thromboxane A2 and vascular smooth muscle cell proliferation. Hypertension 1995;**26**:771–80.
32 Katugampola SD, 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.
33 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**:S272–3.
34 Mehta JL, Lawson D, Mehta P, 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, 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 *et al.* 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 Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 1990;**348**:730–2.
38 Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K *et al.* Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 1990;**348**:732–5.
39 Douglas SA, Meek TD, Ohlstein EH. Novel receptor antagonists welcome a new era in endothelin biology. Trends Pharmacol Sci 1994;**15**:313–6.
40 Sudjarwo SA, Hori M, Tanaka T, Matsuda Y, Okada T, Karaki H. Subtypes of endothelin ETA and ETB receptors mediating venous smooth muscle contraction. Biochem Biophys Res Commun 1994;**200**:627–33.
41 Li J, Cao YX, Liu H, Xu CB. Enhanced G-protein coupled receptors-mediated contraction and reduced endothelium-dependent relaxation in hypertension. Eur J Pharmacol 2007;**557**:186–94.