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Manuscript Draft

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Article Type: Regular Articles

Keywords: Organ culture; Cerebral arteries; Vascular smooth muscle cells, Lipid-soluble cigarette smoking particles, Endothelin ETB receptor, MAPK.

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Organ culture per se induced transcriptional upregulation of endothelin ETB2 receptors in vascular smooth muscle cells. This upregulation was further increased at the transcriptional level by addition of lipid-soluble cigarette smoking particles, but not by nicotine or water-soluble cigarette smoke particles. The additional enhanced upregulation of endothelin ETB receptors by lipid-soluble cigarette smoking particles was inhibited by U0126, SP600125, actinomycin D, and cycloheximide, suggesting that the underlying molecular mechanisms involved in this process include activation of MAPK MEK and JNK, via transcriptional and translational mechanisms. Understanding how cigarette smoke increases the risk for cerebral vascular disease like stroke may provide new strategies for the treatment.

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Date 2010-06-14

Dear Sirs,

Please find the enclosed manuscript “Upregulation of contractile endothelin type B2 receptors by lipid-soluble cigarette smoking particles in rat cerebral arteries via activation of MAPK” by H. Sandhu, C.B. Xu, and L. Edvinsson, which we hereby submit for publishing to Toxicology and Applied Pharmacology. The paper has not been submitted before or published in whole or in part. The authors have no conflict of interest in the results published. Conflict of interest declaration is attached as a separate document. All the authors have read and approved the submission.

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Upregulation of contractile endothelin type B2 receptors by lipid-soluble cigarette smoking particles in rat cerebral arteries via activation of MAPK

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Abstract

Cigarette smoke exposure is a strong risk factor for cerebral vascular disease such as stroke. However, the underlying molecular mechanisms to explain how cigarette smoke exposure leads to cerebral vascular disease are poorly understood. The present study was designed to examine the hypothesis that upregulation of endothelin ET_{B2} receptors that locate in cerebral vascular smooth muscle cells might be the molecular mechanism that mediates the cigarette smoke-associated increase in risk for cerebral vascular disease. Furthermore, the mechanisms of the mitogen activated protein kinase (MAPK)-mediated upregulation of endothelin receptors involved in this process were explored.

Rat cerebral arteries were incubated in serum free medium for 24 h in the presence of lipid-soluble cigarette smoking particles with or without specific inhibitors: MEK specific U0126, p38 specific

SB202190, JNK specific SP600125, NF-κβ specific antagonists BMS- or IMD-0354, transcription inhibitor actinomycin D, or translation blocker cycloheximide. Contractile responses to the endothelin ET_B receptor agonist sarafotoxin 6c were investigated by a sensitive myograph. The expression of the endothelin ET_{B2} receptors were verified by measurement of mRNA with quantitative real time PCR and protein using immunohistochemistry.

Organ culture *per se* induced transcriptional upregulation of endothelin ET_{B2} receptors in vascular smooth muscle cells. This upregulation was further increased at the transcriptional level by addition of lipid-soluble cigarette smoking particles, but not by nicotine or water-soluble cigarette smoke particles. The additional enhanced upregulation of endothelin ET_B receptors by lipid-soluble cigarette smoking particles was inhibited by U0126, SP600125, actinomycin D, and cycloheximide, suggesting that the underlying molecular mechanisms involved in this process include activation of MAPK MEK and JNK, via transcriptional and translational mechanisms. Understanding how cigarette smoke increases the risk for cerebral vascular disease like stroke may provide new strategies for the treatment.

Keywords: Organ culture; Cerebral arteries; Vascular smooth muscle cells, Lipid-soluble cigarette smoking particles, Endothelin ET_B receptor, MAPK.

1 Introduction

Cerebral vascular disease is one of the leading causes of death (Strong *et al.*, 2007) and disability in the developed countries (Rosamond *et al.*, 2008). Most of the clinical cases are of ischemic origin (Zia *et al.*, 2007). Major risk factors for stroke include hypertension, elevated blood levels of low-density lipoprotein, and exposure to cigarette smoke (Braunwald, 1997). All three risk factors mod-

ify the progression of local vascular inflammation and atherosclerosis, and associate with elevated production of endothelin-1 from the endothelial cells (Zeiher *et al.*, 1995;Ihling *et al.*, 1996) as well as increase in the expression of G-protein coupled receptors of the endothelin ET_A and ET_B receptors (Winkles *et al.*, 1993). These data suggest that endothelin system plays an important role in pathogenesis of cerebral vascular disease.

Endothelin ET_A receptors are located to the smooth muscle cells and mediate vasoconstriction. Vascular endothelin ET_B receptors are divided into two subtypes: endothelin ET_{B1} and ET_{B2} receptors. The endothelin ET_{B1} receptors are expressed in the endothelial cells and mediate vasodilation through the release of nitric oxide and prostacyclin, while endothelin ET_{B2} receptors that are located to the smooth muscle cells mediate vasoconstriction (Arai *et al.*, 1990;Batra *et al.*, 1993;de Nucci *et al.*, 1988;Kedzierski *et al.*, 2001;Sakurai *et al.*, 1990). The two endothelin ET_B receptor subtypes only represents a single molecular genotype, differing only in function and location (Giller *et al.*, 1997).

Studies demonstrate that contractile endothelin ET_{B2} receptors are inducible and expressed via transcriptional mechanisms in the smooth muscle cells after experimental cerebral ischemia (Stenman *et al.*, 2002), in atherosclerotic lesions (Dagassan *et al.*, 1996;Iwasa *et al.*, 1999), in congestive heart failure *(Cannan et al., 1996),* in subarachnoid haemorrhage (Roux *et al.*, 1995;Hansen-Schwartz *et al.*, 2003), or during organ culture of arteries (Adner *et al.*, 1998;Moller *et al.*, 1998). The function of induced endothelin ET_{B2} receptors include abnormal vessel contraction and/or vasospasm, which may contribute to cerebral ischemia (Zuccarello *et al.*, 1994).

In order to explore how endothelin ET_{B2} receptors are induced and expressed, we have developed an organ culture model and demonstrated that organ culture of isolated cerebral arteries is a useful model for mimicking the G-protein coupled receptor alteration seen in cerebrovascular ischemia *in vivo* (Hoel *et al.*, 2001;Rosamond *et al.*, 2008;Hansen-Schwartz *et al.*, 2002;Henriksson *et al.*, 2003). The organ culture model allows in-depth study of the underlying intracellular mechanisms that are responsible for alteration in expression of the G-protein coupled receptors in cerebral arteries.

The present study was designed to test the hypothesis that cigarette smoke particles may activate mitogen-activated protein kinase pathways and subsequently results in upregulation of the endothelin ET_{B2} receptors via transcriptional mechanisms. This was achieved by studying lipid-soluble smoke particle-induced alteration of endothelin ET_{B2} receptors at function, mRNA, and protein levels in presence and absence of specific inhibitors for MEK1/2, p38, JNK, NF-κβ, transcription, and translation. The results show that lipid-soluble cigarette smoking particles, but not water-soluble cigarette smoke particles or nicotine, induce upregulation of ET_{B2} receptors in cerebral arteries. The underlying molecular mechanisms involve activation of MAPK-mediated transcriptional mechanisms.

2 Materials and Methods

2.1 Reagents

Reference research cigarettes were obtained from the University of Kentucky, USA. Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4 mM L-glutamine, 0.11 mg/ml sodium pyruvate), antibiotics mix (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml am-

photericin), and Trizol were from Invitrogen, USA. 1-bromo-3-chloropropane, dimethyl sulfoxide (DMSO), absolute ethanol, nicotine, U0126, SB202190, SP600125, IMD-0354, actinomycin D, cycloheximide, 5-hydroxytryptamine, carbachol, and other high grade chemicals were purchased from Sigma-Aldrich, USA. In addition we used BMS-345541 (Merck, Germany), Sarafotoxin 6c (PolyPeptide Group, France), and RNase free water (Qiagen, USA).

2.2 Extraction of DMSO lipid soluble cigarette Smoking Particles (DSP)

Three cigarettes (0.8 mg nicotine per cigarette, reference research cigarettes, University of Kentucky, USA) were smoked by the aid of vacuum through a sterile cotton filter and dissolved in 1 ml DMSO or distilled water. Detailed description of the extraction and measurement of the nicotine concentration has been described before (Zhang *et al.*, 2006). Preliminary tests have revealed that water-soluble cigarette smoke particles or nicotine (25 ng/ml, dose found in plasma of smokers) have no effect on the G-protein coupled receptor alteration; therefore, we only focus henceforth on the DSP effect. The concentration of DSP used in the present study (0.15 µl DSP pr. ml DMEM) is equivalent to the plasma level of approximately 25 ng/ml nicotine found in smokers (Benowitz *et al.*, 1994;Foulds *et al.*, 2003).

2.3 Isolation of the cerebral arteries and organ culture

Studies were approved by the Danish Animal Experiments Committee guidance (no. 2006/561-1139). Male Sprague-Dawley rats (n = 136; 300-350 g) (Taconic, Denmark) were anaesthetized with CO_2 and decapitated. The brains were removed and immediately chilled in ice-cold bicarbonate buffer solution (for composition see below). The right and left middle cerebral arteries were isolated and dissected free of adhering tissue.

Middle cerebral artery segments (2.0– 3.0 mm long), free from surrounding cerebral tissue with intact endothelium, were studied immediately (fresh, 0 h organ culture) or incubated for 24 h at 37 °C in humidified 5 % CO_2 and 95 % air in 1 ml serum free DMEM supplemented with an antibiotics mix (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin).

The concentrations of the inhibitors were adjusted so that maximum inhibition without toxic effect was achieved. The concentrations tested were: U0126 = 10^{-7} M, 10^{-6} M, and 10^{-5} M (10^{-5} M U0126 chosen); SB202190 = 10^{-7} M, 10^{-6} M, and $10^{-5.5}$ M; SP600125 = 10^{-7} M and 10^{-6} M (10^{-6} M SP600125 chosen); BMS-345541 = 10^{-8} M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, and $10^{-5.5}$ M; IMD-0354 = 10^{-8} M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, and $10^{-5.5}$ M; actinomycin D = 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M (10^{-8} M actinomycin D chosen); cycloheximide = 10^{-8} M, $10^{-7.5}$ M, 10^{-7} M, and 10^{-6} M (10^{-7} M cycloheximide chosen).

DMSO, DSP, inhibitors, or DSP along with inhibitors were added to the DMEM medium at the initiation of the incubation. The final volume of DMSO added to the 1 ml DMEM was always kept at 2.5 µl. This DMSO dose did not modify the upregulation of receptors.

Fresh or organ cultured cerebral artery ring segments were either mounted in myographs for in vitro pharmacology or snap-frozen with dry ice and kept at -80 °C for real time PCR or immunohistochemistry.

2.4 In vitro pharmacology

A sensitive myograph was used for recording of the isometric tension in isolated cerebral vessel segments (Hogestatt *et al.*, 1983;Mulvany *et al.*, 1977). The vessel segments were threaded on two 40 µm-diameter stainless steel wires and mounted on a Mulvany-Halpern myograph (Danish Myo Technology A/S, Denmark). One of the wires was connected to a force displacement transducer attached to an analog-digital converter unit (PowerLab from ADInstruments, New Zealand), while the other wire was attached to a movable displacement device allowing fine adjustments of vascular tension by varying the distance between the two wires. The measurements were recorded on a computer using the software Chart 5.4.2 (ADInstruments, U.K.).

The segments were immersed into a temperature-controlled (37 °C) buffer solution (composition in mM/ml; NaCl = 119, $NaHCO_3$ = 15, KCl = 4.6, $MgCl_2$ = 1.2, NaH_2PO_4 = 1.2, $CaCl_2$ = 1.5, and glucose = 5.5). The buffer was continuously gassed with 5 % CO_2 in O_2 resulting in a physiological pH at 7.4. The vessels were given an initial pre-tension of 2 mN/mm and were adjusted to this tension for 1 hour. Contractile capacity was determined by exposure to potassium rich (60 mM K^+) buffer solution with the same composition as the bicarbonate buffer solution except that NaCl was exchanged for KCl. The threshold for minimum 60 mM K^+-contraction response was set to 0.7 mN (if the K^+-contraction was lower, the data from the vessel was discarded).

The carbachol resulted in dilatation after 5-hydroxytryptamine mediated contraction was used as measurement of endothelium functionality. Carbachol mediates an endothelium dependent dilation of vessels via release of nitric oxide. Middle cerebral artery segments were pre-contracted with 300 nM 5-hydroxytryptamine and subsequently exposed to 10 µM carbachol. A strong dilation by carbachol indicated intact functional endothelium, whereas a weak or absent dilation indicates dam-

aged or unfunctional endothelium (i.e. due to mechanical damage of the endothelium during mounting on wires). Threshold for intact endothelium function was set at to 30 % carbachol induced dilatation, and only vessels showing this response or more were included in the study. Concentration-response curves were subsequently obtained by cumulative application of the agonist: endothelin ET_B receptor specific agonist sarafotoxin 6c (10^{-14} M to 10^{-7} M). Specificity of detailed receptor charactering has been performed before (Henriksson *et al.*, 2003;Sandhu *et al.*, 2010).

2.5 Molecular Biology

The middle cerebral artery vessel segments were after removal snap-frozen with dry ice in green-cap tubes containing Lysing Matrix D provided in the FastRNA Pro Green Kit (Q Biogene, USA). Total cellular RNA was extracted with the use of the FastRNA Pro Green Kit. The vessels were homogenized in 1 ml of Trizol Solution by using a FastPrep FP120 instrument (Q Biogene, USA). The samples were centrifuged for 15 min at 4 °C at 12,500 g, and the liquid phase was transferred to 300 µl of 1-bromo-3-chloropropane. After centrifugation for 15 min at 4 °C at 12,500 g the upper phase was transferred to 500 µl of 100 % isopropanol and stored at -20 °C overnight. Next day the samples were centrifuged for 20 min at 4 °C at 15,000 g. The supernatant was removed. The pellet was washed with 500 µl 75 % ethanol, air dried and redissolved with RNase free water. Total RNA amount and purity was determined using NanoDrop 2000c (Thermo Science, USA).

Reverse transcription of total RNA to cDNA was performed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, USA) in a GeneAmp PCR System 2400 (Perkin-Elmer, USA). First-strand cDNA was synthesized from 250 ng total RNA in a 20 µl reaction volume with random

hexamers used as primers. The PCR reaction was performed with the following setup: 42 °C for 90 min followed by 72 °C for 10 min. The cDNA was diluted with 30 µl of RNase free.

Real time PCR was performed in a 7500 Fast Real Time PCR sequence detection system (Applied Biosystems, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, USA) with 1 µl cDNA synthesized above as template in a 25-µl reaction volume. A none-template control was included in all experiments. Primer (1 µl of 10 mM) was added to the 25 µl real time PCR reaction. Elongation factor-1 (EF-1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as references genes because they are the product of housekeeping genes and are therefore continuously expressed to a constant amount in cells. Specific primers for the rat endothelin ET_B receptor, EF-1, and GAPDH receptors were designed. Table 1 presents the sequences of the specific genes along with the GeneBank accession ID. The real time PCR was performed with the following profile: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles with 95°C for 15 seconds and 60 °C for 1 minute.

The 7500 Fast Real Time PCR sequence detection software SDS version 1.4 (Applied Biosystems, USA) monitors the growth of DNA in real time by optics and imaging system via the binding of a fluorescent dye to double-stranded DNA.

2.6 Immunohistochemistry

The middle cerebral arteries were placed into Tissue TEK (Sakura Finetek Europe B.V., Netherlands) and frozen. Segments were sectioned into 10-µm-thick slices and mounted on SuperFrost Plus slides (Menzel GMBH & COKG, Germany)

The primary antibodies used were sheep anti-rat ET_B (ALX-210-506A, Alexis, USA), diluted 1:250 and mouse anti-rat β-actin (ab11003, Abcam, USA), diluted 1:500. The endothelin ET_B receptor and β-actin dilutions were done in PBS with 2 % donkey serum (017-000-121, Jackson ImmunoResearch laboratories, Inc., USA).

The secondary antibodies used were donkey anti-sheep DL488 conjugated (713-485-003 Jackson ImmunoResearch laboratories, Inc., USA) diluted 1:200 and donkey anti-mouse DL549 conjugated (715-505-150, Jackson ImmunoResearch laboratories, Inc., USA) diluted 1:200. As control, only secondary antibodies were used. The antibodies were detected at the appropriate wavelength on a confocal microscopy (Nikon, C1plus, Nikon Instruments Inc., USA).

The endothelin ET_B receptor binding site could be blocked by high dose of endothelin-1 irreversibly, and thereby blocked the endothelin-1 immunoreaction (De Mey *et al.*, 2009).

2.7 Calculation and statistical analyses

Data are expressed as mean values ± standard error of the mean (S.E.M.), and n refers to the number of rats. Statistical analyses of myograph data were performed on E_{max}- or pEC_{50}-values with one way ANOVA with Bonferroni's test, and selected groups were compared. When analysing real time PCR data mRNA levels relative to the reference gene was used, and fluorescence mean intensities values were analysed for the immunohistochemistry data.

Groups compared in ANOVA tests: fresh middle cerebral vessel vs. 24 h organ culture with DMSO

and 24 h organ culture with DMSO vs. 24 h organ culture with DMSO; 24 h organ culture with DMSO vs. 24 h organ culture with 10 µM U0126, 0.1 µM SB202190, 1 µM SP600125, 0.1 µM BMS-345541, 0.01 µM IMD-0354, 0.01 µM actinomycin D, or 0.1 µM cycloheximide; 24 h organ culture with DSP vs. 24 h organ culture with DSP and 10 µM U0126, 0.1 µM SB202190, 1 µM SP600125, 0.1 µM BMS-345541, 0.01 µM IMD-0354, 0.01 µM actinomycin D, or 0.1 µM cycloheximide. Statistics information given in Table 2. $P< 0.05$ was considered statistically significant ($P< 0.05$=*, $P< 0.01$=**, $P< 0.001$=***).

2.7.1 In vitro Pharmacology

Contractile response of each segment is expressed as percentage of the 60 mM K^+ induced contraction. ET_{max} value represents the maximum contractile response elicited by an agonist and the pEC_{50} the negative logarithm of the drug concentration that elicited half the maximum response.

2.7.2 Quantitative real time PCR

Data were analysed with the comparative cycle threshold (CT) method (Hansen-Schwartz *et al.*, 2002). The CT values of EF-1 or GAPDH mRNA were used as a reference to quantify the relative amount of endothelin ET_B receptor mRNA. The relative amount of mRNA was calculated with the CT values of endothelin ET_B receptor mRNA in relation to the CT values of EF-1 or GAPDH mRNA in the sample by the formula $X_0/R_0=2^{CtR-CtX}$, where X_0 is the original amount of endothelin ET_B receptor mRNA, R_0 is the original amount of EF-1 or GAPDH mRNA, CtR is the C_T value for EF-1 or GAPDH, and C_TX is the C_T value for the endothelin ET_B receptor. Data with EF-1 as the reference gene is shown in results section.

2.7.3 Immunohistochemistry

Images were analysed by Nikon EZ-C1 software version 3.70. Fluorescence intensity was measured on the whole smooth muscle cell area in 3 sections; the mean of this was used. β-actin staining was used as reference of smooth muscle cell localization. Analysis was done by having the red 549 nm channel (β-actin conjugated) overlay; however, only green 488 nm channel (endothelin ET_B receptor conjugated) image is given for visual simplicity.

3 Results

Middle cerebral arteries were incubated for 24 h with DMSO or DSP with and without specific inhibitors for MEK1/2, p38, JNK, NF-κβ, transcription, and translation. The total volume of DMSO in the organ culture was always 2.5 µl/ml of DMEM. Carbachol (10 µM) elicited dilatation of the 5-hydroxytryptamine (300 nM) pre-contracted middle cerebral artery segments by 50 ± 12 % in fresh middle cerebral vessels (n=6). After 24 h of organ culture with DMSO the dilatation by carbachol was 27 ± 7 % (n=8), and after 24 h the organ culture with DSP the dilatation by carbachol was 11 ± 2 % (n=8, $p< 0.05$). Thus, organ culture reduced the endothelial function somewhat; addition of DSP to the organ culture further reduced the endothelial function.

3.1 DSP concentration study

DSP was tested in different doses; 0.05 µl – 0.40 µl DSP/ml DMEM. The K^+-induced contraction, S6c E_{max}, and pEC_{50} values were the same for 0.05 µl and 0.15 µl DSP (data not shown). Addition of 0.25 µl DSP/ml DMEM or a higher dose of DSP reduced the 60 mM K^+-induced contraction, indicating a direct toxic effect on the vessels started to be at this DSP concentration in the or-

gan culture. Doses of 0.30 µl or 0.40 µl DSP completely abolished the 60 mM K^+-induced contraction. Therefore, we chose the DSP dose 0.15 µl henceforth. The 60 mM K^+-induced contraction, E_{max} in % of 60 mM K^+, and pEC_{50} values are given in Table 2.

3.2 Potassium-induced contraction of fresh and incubated vessels

The 60 mM K^+-induced contraction (depolarisation) of the smooth muscle cells and rapid influx of extracellular calcium ions and was used as internal reference (Hogestatt *et al.*, 1983). Agonist mediated contractions are given in % of the 60 mM K^+-induced contraction. This 60 mM K^+-induced contraction (mN) did not differ statistically between the fresh and DMSO groups (Table 2, 60 mM K^+-induced contraction). However the addition of 0.15 µl DSP/ml DMEM reduced the K^+-induced contraction (Table 2, 60 mM K^+-induced contraction: 24 h organ culture with 0.15 µl DSP = 1.23 ± 0.19 mN ($p< 0.05$). The addition of the various blockers with DSP did not further alter the K^+-induced contraction.

3.3 Endothelin ET_B receptor mediated contraction

The specific endothelin ET_B receptor agonist sarafotoxin 6c induced a weak contraction in fresh middle cerebral arteries segments (5 ± 1 %). After 24 h of organ culture the endothelin ET_B receptor mediated contraction was enhanced significantly (103 ± 7 %; $p<0.01$). As shown before the organ culture meditated increased of endothelin ET_B receptor contraction was reduced by U0126, actinomycin D, and cycloheximide; demonstrating transcriptional upregulation via the MEK/ERK pathway (Henriksson *et al.*, 2004;Sandhu *et al.*, 2010). The sarafotoxin 6c induced response was

blocked by the specific endothelin ET_B receptor inhibitor BQ788, showing that the response was modulated by endothelin ET_B receptors. The endothelin ET_B receptor mediated contraction was further increased significantly when DSP was added to the 24 h incubation (from 103 ± 7 % to 191 ± 14 %;$p<0.05$)(Fig. 1a, Table 2). The enhanced sarafotoxin 6c response was attenuated by co-incubation with U0126 (10^{-5} M), SP600125 (10^{-6} M), actinomycin D (10^{-8} M), and cycloheximide (10^{-7} M) (Figures 1b, 1d, 1g, and 1h, Table 2). The sarafotoxin 6c pEC_{50} values were, however, not changed by the addition of DSP. This supports our view of attenuating in the number of the same receptor subtype (Adams *et al.*, 1999;Moller *et al.*, 1998).

3.3.1 Inhibitor effect on endothelin ET_B receptor mediated contraction when DMSO or DSP is added to the organ culture

Once a specific inhibitor showed inhibition effect in DSP organ culture (24 h organ culture with 0.15 µl DSP + 10^{-5} M U0126: E_{max} is reduced from 191 ± 14 % of DSP alone to 91 ± 11 % when U0126 is added), the effect of this specific inhibitor was tested on the organ culture method itself (24 h organ culture with DMSO + 10^{-5} M U0126: E_{max} is reduced from 103 ± 7 % of DMSO alone to 74 ± 3 % when U0126 is added). This was done to estimate on what level the inhibitor had an effect: organ culture mediated upregulation of endothelin ET_B receptor mediated contraction or DSP mediated upregulation of endothelin ET_B receptor mediated contraction.

The DSP mediated upregulation of ET_B mediated contraction (E_{max} = 191 ± 14 %) was reduced by the following inhibitors: 10^{-5} M U0126 (E_{max} = 91 ± 11 %), 10^{-6} M SP600125 (E_{max} = 126 ± 13 %), 10^{-8} M actinomycin D (E_{max} = 22 ± 2 %), and 10^{-7} M cycloheximide (E_{max} = 114 ± 14 %).

SB202190, BMS-345541, and IMD-0354 failed to inhibit the DSP mediated upregulation of ET_B mediated contraction in the concentrations tested. The inhibition effect of U0126, SP600125, actinomycin D, and cycloheximide were tested on vessels incubated with vehicle (DMSO). They all showed a tendency towards lowering the ET_B mediated contraction of organ culture with DMSO (E_{max} = 103 ± 7 %), but these results were without statistical significance (Table 2).

The pEC_{50} values of sarafotoxin 6c induced contraction of the vessels after organ cultured with DMSO or DSP, with or without the specific inhibitors were not altered.

3.4 Immunohistochemistry

Organ culture induced a strong endothelin ET_B receptor mediated contraction, and this contraction was further enhanced by addition of DSP to the organ culture. Immunohistochemistry revealed that this upregulation was seen on the protein level as well. The endothelin ET_B receptor protein level was upregulated from fresh middle cerebral artery sections to 24 h organ cultured ones. This endothelin ET_B receptor protein upregulation was further upregulated by addition of DSP (Figure 3b).

Addition of U0126 (10^{-5} M) and cycloheximide (10^{-7} M) to the 24 h organ culture with DSP significantly reduced the endothelin ET_B receptor protein level, whereas SP600125 (10^{-6} M) and actinomycin D (10^{-8} M) had no effect. When the inhibitors were added to 24 h organ culture with DMSO, only cycloheximide gave a significant drop in endothelin ET_B receptor protein level, whereas U0126 also lowered the endothelin ET_B receptor protein level, but this was without statistical significance. SP600125 and actinomycin D did not change the endothelin ET_B receptor protein level in 24 h organ culture with DMSO.

3.5 Real time PCR

Gene expression of the receptors was investigated in middle cerebral arteries by real time PCR. Standard curves for the respective primer pairs for ET_B, EF-1, and GAPDH had almost the same slopes. The respective genes were therefore amplified with the same efficiency during the real time PCR (data not shown). None template controls were used as negative controls. Both the EF-1 and GAPDH reference genes gave similar results. In this article we therefore only show the mRNA levels of the receptors relative to the EF-1 mRNA expression (X_0/R_0) (Fig. 2).

Endothelin ET_B receptor mRNA level is upregulated significantly from fresh middle cerebral arteries to organ cultured for 24 h with DMSO. Addition of DSP to the 24 h organ culture incubation 24 h did not further upregulate the endothelin ET_B receptor mRNA. Addition of 10^{-5} M U0126, 10^{-6} M SP600125, or 10^{-7} M cycloheximide to 24 h of organ culture with DSP shows tendency towards a reduction of the endothelin ET_B receptor mRNA, but it was only statistical significant for 10^{-8} M actinomycin D. Only actinomycin D showed strong statistical significant downregulation of the endothelin ET_B receptor at the mRNA level.

4 Discussion

Cigarette smoking and second-hand smoke exposure are well-known risk factors for cerebrovascular diseases. However, the underlying molecular mechanisms that are mediating the increased risk of developing cerebral vascular disease associated with smoke exposure are largely unknown. Here, we show that it is the lipid-soluble cigarette smoking particles, but not water-soluble cigarette smoke particles and nicotine *per se*, that induce upregulation of contractile endothelin ET_{B2} receptors in rat cerebral arteries through activation of MAPK-mediated transcriptional mechanisms. The

upregulation of endothelin ET_{B2} receptors with enhanced contraction of cerebral arteries may lead to cerebral ischemia as demonstrated in previous studies *in vivo* (Beg *et al.*, 2006;Stenman *et al.*, 2002;Wackenfors *et al.*, 2004). Thus, targeting the MAPK-mediated molecular mechanisms may provide new strategies for treating cerebral diseases.

Lipid-soluble cigarette smoke particles in the circulation are transported by lipoproteins to the arterial wall (Shu *et al.*, 1983), where they can directly cause damage to the arterial wall (Xu *et al.*, 1993;Xu *et al.*, 2008). Increased expression of endothelin ET_A receptors (Granstrom et al., 2006) and endothelin ET_{B2} receptors (Xu et al., 2008) receptors as well as thromboxane A2 (Zhang et al., 2008) are seen, when the arteries and bronchi are exposed to lipid-soluble smoke particles at concentrations similar to those that occur in smokers (Benowitz *et al.*, 1994;Foulds *et al.*, 2003). While water-soluble smoke particles or nicotine at physiological levels (measured in smokers) do not have such effects on the receptor expression (Zhang *et al.*, 2006). Identifying all of the toxins in cigarette smoke has been a challenge, as cigarette smoke contains more than 4000 different substances (Repace *et al.*, 1980). Nicotine is present as an "essential reward" molecule in cigarette smoke, but does not mediate endothelin ET_B receptor upregulation (Zhang et al., 2006). However, some effects of the nicotine have been observed in high non-physiological concentraction such as 0.48 and 0.96 mg/l (Zhang et al., 2009a). Polycyclic aromatic hydrocarbons (dimethylbenzanthracene and benzpyren) and oxidant-like free radicals are present in the DSP preparation. These ingredients may cause cell proliferation and the release of vasoactive substances (Pessah-Rasmussen *et al.*, 1991;Stavenow *et al.*, 1988). There are reports that suggest that endotoxin lipopolysaccharide (LPS) may be a group one of the toxic substances found in cigarette smoke (Larsson *et al.*, 2004;Sebastian *et al.*, 2006). LPS is a specific agonist for Toll-like receptor 4 (TLR4), and activates innate immu-

nity and inflammation. Activation of TLR4 by LPS activates MAPK pathways (Bachar *et al.*, 2004), which may subsequently lead to upregulation of vascular endothelin receptors and vascular damage. However, this was not observed in a recent study on coronary arteries (Ghorbani *et al.*, 2010). The present study demonstrates for the first time in cerebral arteries that lipid-soluble cigarette smoking particles, but not water-soluble cigarette smoke particle or nicotine, induces upregulation of endothelin ET_{B2} receptors. This agrees well with previous findings obtained from studying the effects of cigarette smoke on rat mesenteric arteries (Xu *et al.*, 2008;Zhang *et al.*, 2008).

The MAPKs are a group of serine/threonine kinases that are evolutionary well-conserved in all eukaryotes. There are three well-characterized MAPK pathways, namely MEK/ERK1/2, p38, and JNK (Lewis *et al.*, 1998). Both MEK/ERK1/2 and p38 play important roles in cerebral ischemia, and treatment with specific inhibitors of these pathways have in experimental models of cerebral ischaemia revealed neuroprotection (Alessandrini *et al.*, 1999;Barone *et al.*, 2001;Lennmyr *et al.*, 2002;Namura *et al.*, 2001;Ansar *et al.*, 2008). In order to understand the role of MAPKs in the upregulation of cerebrovascular endothelin ET_B receptors, we have examined the specific effects of different protein kinase inhibitors (Xu *et al.*, 2008) in the organ culture model and demonstrated that both MEK/ERK1/2 and p38 pathways are involved in the upregulation of endothelin ET_{B2} receptors (Nilsson *et al.*, 2008). In prevous studies, U0126 and SB386023 decreased organ culture-induced endothelin ET_B receptor upregulation in rat cerebral arteries (Henriksson *et al.*, 2004). In addition, immunohistochemistry revealed positive endothelin ET_B receptor immunoreactivity in the media layer (smooth muscle cells), supporting the upregulation of the endothelin ET_B receptors in vascular smooth muscle cells after organ culture. A crucial role of the MEK/ERK1/2 in endothelin ET_B receptor upregulation after organ culture was demonstrated by Western blot in combination

with specific inhibitors (Uddman *et al.*, 2003); this has also been seen after cerebral ischemia (Maddahi *et al.*, 2008;Maddahi *et al.*, 2009). The initiation of the upregulation is still not known but could be related to changes in flow or pressure (shear stress), and the subsequent activation of tyrosine kinase receptors or G-protein coupled receptors. Cardiovascular risk factors may induce vascular smooth muscle cell endothelin ET_B receptor upregulation via activation of MAPK-mediated NF-κB inflammatory signal pathways (Zhang *et al.*, 2009b). We have shown that lipid-soluble smoke particles can induce activation of MEK/ERK1/2, p38, and the downstream transcriptional factor NF-κB within 3 hrs, with a subsequent upregulation of vascular smooth muscle cell endothelin ET_{B2} receptors after 6 hrs (Xu *et al.*, 2008;Zheng *et al.*, 2010). This agrees well with the present findings that inhibition of MEK/ERK1/2, p38, or NF-κB activities by their specific inhibitors significantly attenuates the lipid-soluble smoke particles-induced upregulation of vascular smooth muscle cell endothelin ET_{B2} receptor expression in the cerebral arteries.

In the present study organ culture increased endothelin ET_B receptor mediated contraction, as well as receptor mRNA and protein levels via both transcription and translation through activation of the MEK/ERK pathway. Addition of lipid-soluble smoke particles to the organ culture further increases the endothelin ET_{B2} receptor-mediated contraction and protein, but with no significant effect on the mRNA level. This suggests a translational mechanism is in play, and supported by the fact that the MEK 1/2 specific inhibitor U0126 significantly inhibited this DSP-mediated elevated endothelin ET_B receptor mediated contraction and protein level. A significant attenuation of endothelin ET_B receptor mediated contraction and protein have been observed by the translation specific inhibitor cycloheximide. A decrease in lipid-soluble smoke particle-mediated elevated endothelin ET_B receptor mediated contraction was observed when SP600125 and actinomycin D, but real time PCR

and immunohistochemistry studies could not explain this, suggesting post-translational mechanisms involved. The specific inhibitors used in this study were tested at the organ culture-induced increase in endothelin ET_B receptor mediated contraction. Only U0126 deceased the endothelin ET_B receptor mediated contraction significantly, but this fall in contraction is not linked to transcription or translation. As expected actinomycin D and cycloheximide lowered the organ culture-induced increase in endothelin ET_B receptor mediated contraction. All in all this indicates a strong involvement of MAPK signal pathways in endothelin ET_B receptor expression after organ culture and this is further enhanced by lipid-soluble smoke particles. The role of the endothelin ET_B receptor is not limited to contractions only, but also promote proliferation and migration of endothelial cells (Ziche *et al.*, 1995).

5 Conclusion

Lipid-soluble cigarette smoking particles, but not water-soluble cigarette smoke particle or nicotine, induce upregulation of endothelin ET_{B2} receptors in rat cerebral arteries. The underlying molecular mechanisms that mediate upregulation of endothelin ET_{B2} receptors include activation of MAPK and transcriptional mechanisms. This may advance us understanding how cigarette smoke increases the risk for cerebral vascular disease, such as stroke. Understanding the molecular mechanisms behind upregulation of endothelin ET_{B2} receptors may provide novel strategies for treatments of cerebral vascular diseases.

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Figure 1

Contractile response to the cumulative application of sarafotoxin 6c to fresh or incubated middle cerebral arteries with DMSO (vehicle), or DSP (a). Additional addition of specific inhibitors: 10^{-5} M U0126 (b), 10^{-7} M SB202190 (c), 10^{-6} M SP600125 (d), 10^{-7} M BMS-345541 (e), 10^{-8} M IMD-0354 (f) 10^{-8} M Actinomycin D (g), or 10^{-7} M Cycloheximide (h). Values given represent means ± S.E.M. (n = 6-8). Statistical values for myograph experiments are given in Table 2.

Figure 2

Real time PCR of fresh or incubated middle cerebral arteries with DMSO (vehicle) or DSP ± inhibitors: 10^{-5} M U0126, 10^{-7} M SB202190, 10^{-8} M actinomycin D, or 10^{-7} M cycloheximide. Data are expressed as mean ± s.e.m. values relative to EF-1 mRNA levels (n= 5-7). Comparison of fresh vessels versus organ culture with DMSO (*** $p< 0.001$), organ culture with DMSO versus organ culture with DSP, organ culture with DMSO versus organ culture with specific inhibitors (* $p< 0.05$ for organ culture with DMSO versus organ culture with actinomycin D), or organ culture with DSP versus organ culture with DSP added specific inhibitors.

Figure 3

Immunohistochemistry double staining against endothelin ET_B receptor and β-actin of fresh or incubated middle cerebral arteries with DMSO (vehicle) or DSP ± inhibitors: 10^{-5} M U0126, 10^{-7} M SB202190, 10^{-8} M actinomycin D, or 10^{-7} M cycloheximide. Green channel (488 nm, endothelin ET_B receptor staining) is shown, 60x magnification. Data are expressed as mean ± s.e.m. (n= 4-5). Statistical comparison: Fresh vessels versus organ culture with DMSO (*** $P< 0.001$), and organ culture with DMSO versus organ culture with DSP (*** $P< 0.001$), organ culture

with DMSO versus organ culture with specific inhibitors (*** P< 0.001 for organ culture DMSO versus organ culture with cycloheximide), and organ culture with DSP versus organ culture with DSP added specific inhibitors (*** P< 0.001 for organ culture with DSP versus organ culture with DSP added U0126 or cycloheximide).

Table 1

Gene	GenBank accession ID	Forward primer	Reverse primer
ET_B	NM_017333	5'GATACGACAACTTCCGCTCCA-3'	5'-GTCCACGATGAGGACAATGAG-3'
EF-1	NM_175838	5'-GCAAGCCCATGTGTGTTGAA-3'	5'-TGATGACACCCACAGCAACTG-3'
GAPDH	NM_017008	5′-GGCCTTCCGTGTTCCTACC-3′	5′-CGGCATGTCAGATCCACAAC-3′

Table 1 - Primer sequences of the respective genes analysed by real time PCR

Table 2

	n	60 mM K^+	S6c E_{max}	S6c pEC_{50}
Fresh	6	2.79 ± 0.52	5 ± 1	-
DMSO	8	2.39 ± 0.32	103 ± 7 [b]	10.45 ± 0.25
DMSO + U0126 10^{-5} M	6	2.36 ± 0.47	74 ± 3	9.39 ± 0.07
DMSO + SP600125 10^{-6} M	6	2.51 ± 1.00	93 ± 20	10.65 ± 0.17
DMSO + Actinomycin D 10^{-8} M	6	2.85 ± 0.65	73 ± 19	10.90 ± 0.22
DMSO + Cycloheximide 10^{-7} M	6	2.16 ± 0.46	67 ± 9	11.18 ± 0.34
DSP	8	1.23 ± 0.19	191 ± 14 [a]	10.99 ± 0.26
DSP + U0126 10^{-5} M	7	0.94 ± 0.15	91 ± 11 [c]	10.45 ± 0.47
DSP + SB202190 10^{-7} M	6	1.18 ± 0.28	198 ± 42	10.02 ± 0.54
DSP + SP600125 10^{-6} M	8	1.08 ± 0.18	126 ± 13 [c]	10.06 ± 0.30
DSP + BMS-345541 10^{-7} M	6	0.98 ± 0.22	241 ± 40	10.07 ± 0.43
DSP + IMD-0354 10^{-8} M	6	0.91 ± 0.20	226 ± 27	9.40 ± 0.21
DSP + Actinomycin D 10^{-8} M	8	1.77 ± 0.48	22 ± 2 [c]	10.42 ± 0.51
DSP + Cycloheximide 10^{-7} M	6	1.18 ± 0.37	114 ± 14 [b]	10.98 ± 0.26

Table 2 – E_{max} and pEC_{50} values of fresh or 24 h incubated middle cerebral arteries with DMSO or DSP ± inhibitors: MEK 1/2 inhibitor U0126, p38 inhibitor SB202190, JNK inhibitor SP600125, NF-κβ inhibitor BMS-345541 and IMD- 0354, transcription inhibitor Actinomycin D, and translation inhibitor Cycloheximide. Values given for all agonist experiments and as mean ± S.E.M. Statistical comparison of E_{max} values were performed: Fresh versus organ culture with DMSO, organ culture with DMSO versus organ culture with DSP, organ culture with DMSO versus organ culture with specific inhibitors, and organ culture with DSP versus organ culture with DSP added specific inhibitors ([a] $p< 0.05$, [b] $p< 0.01$, and [c] $p< 0.001$).

Figure 1A

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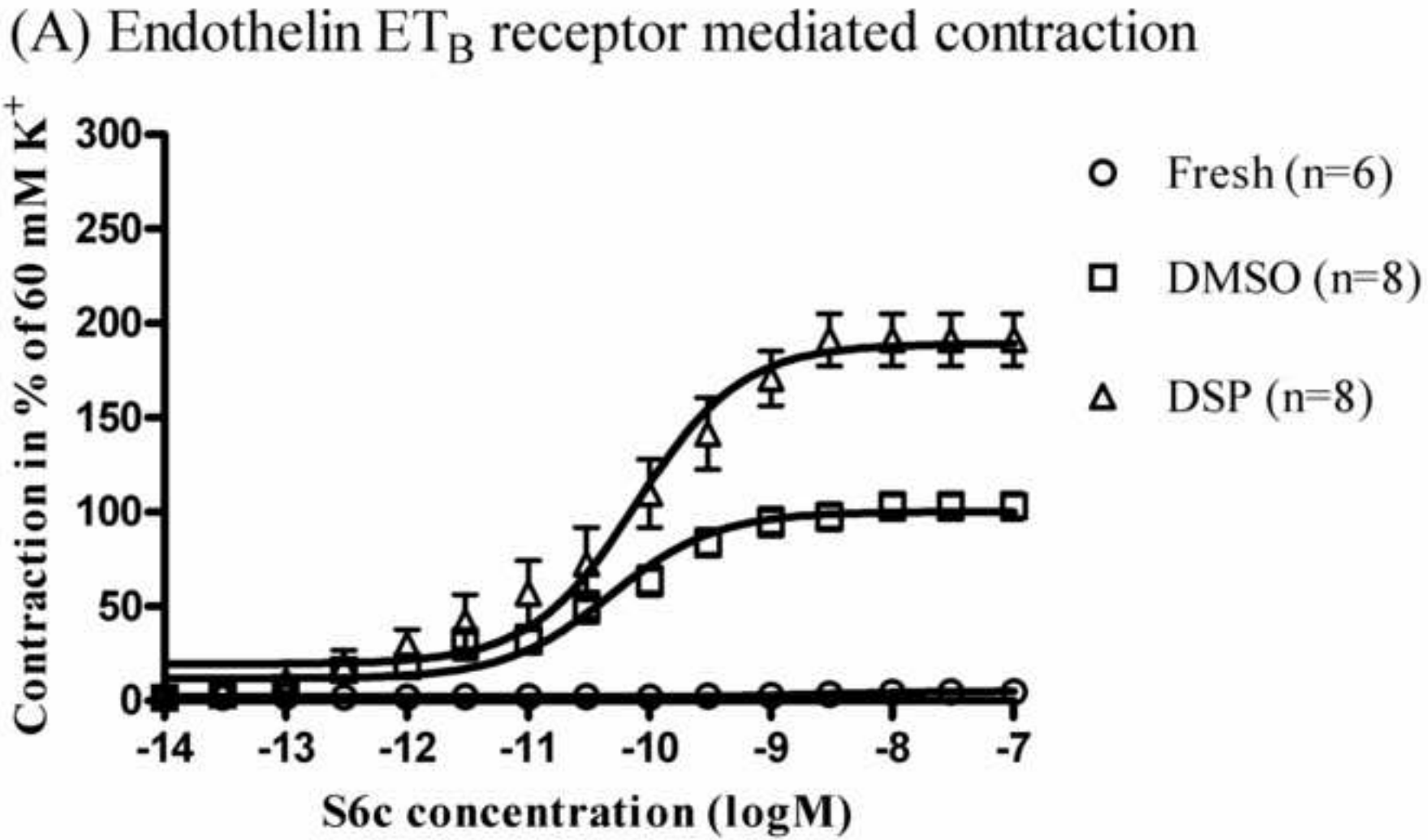

Figure 1B
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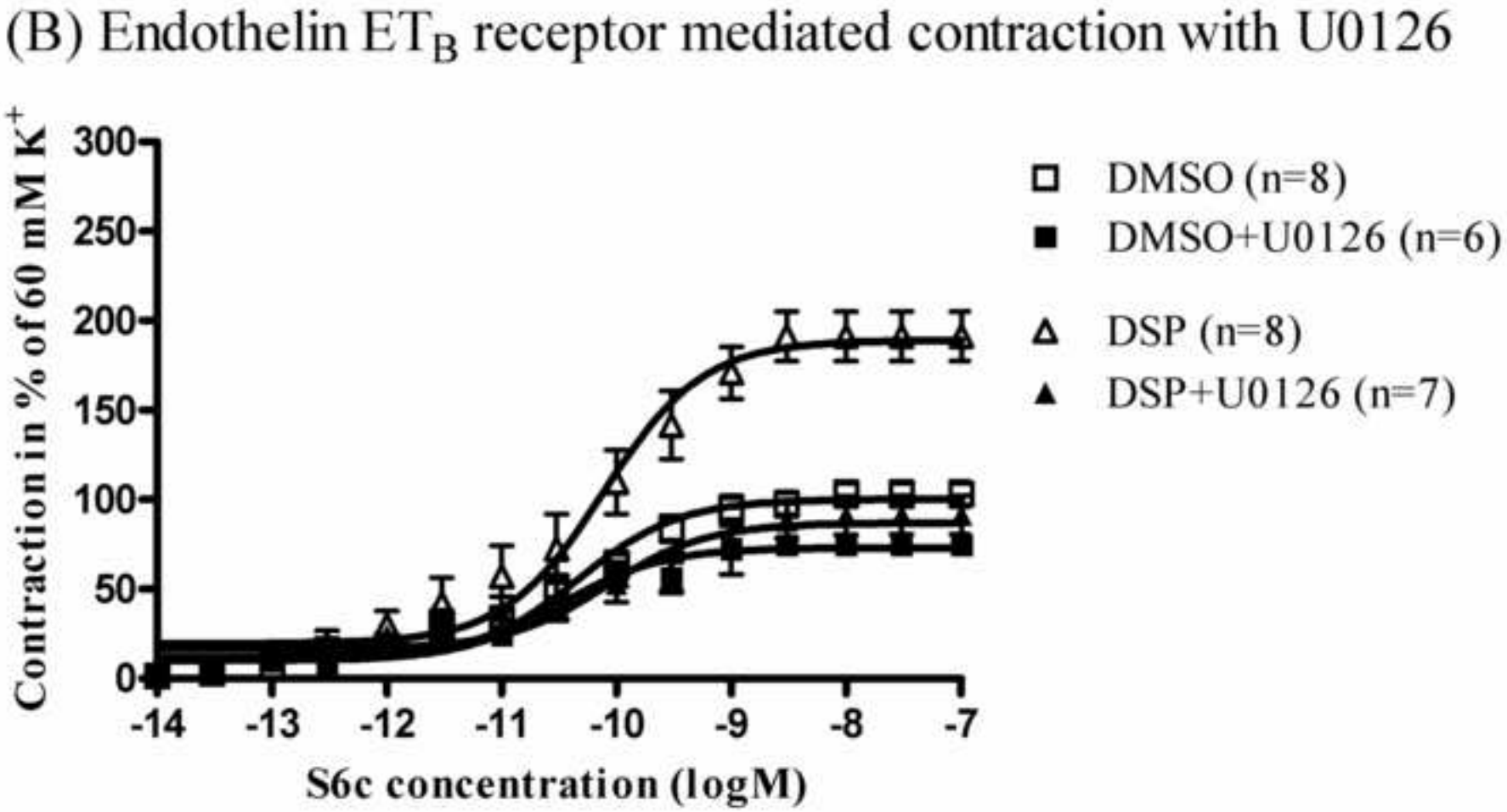

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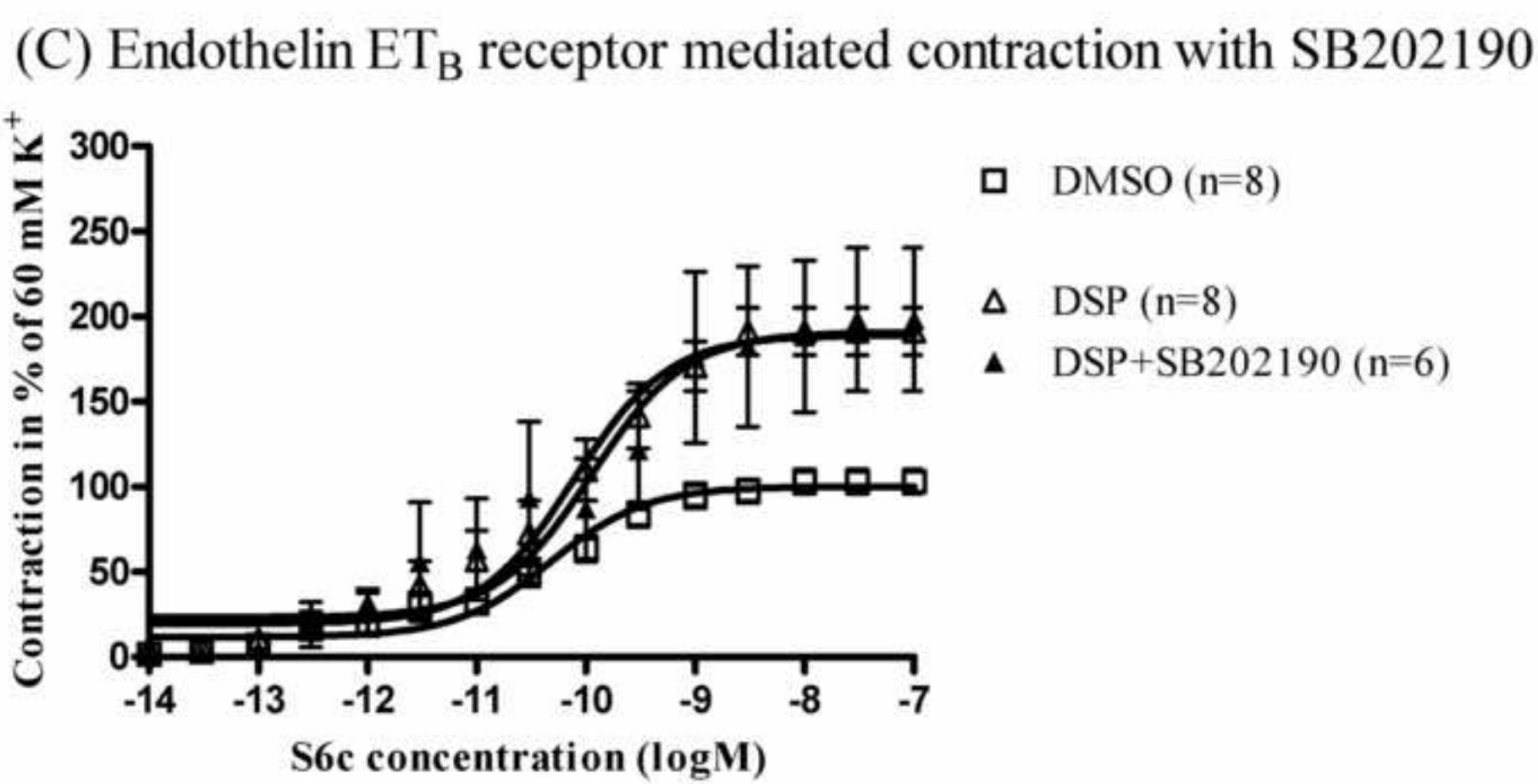

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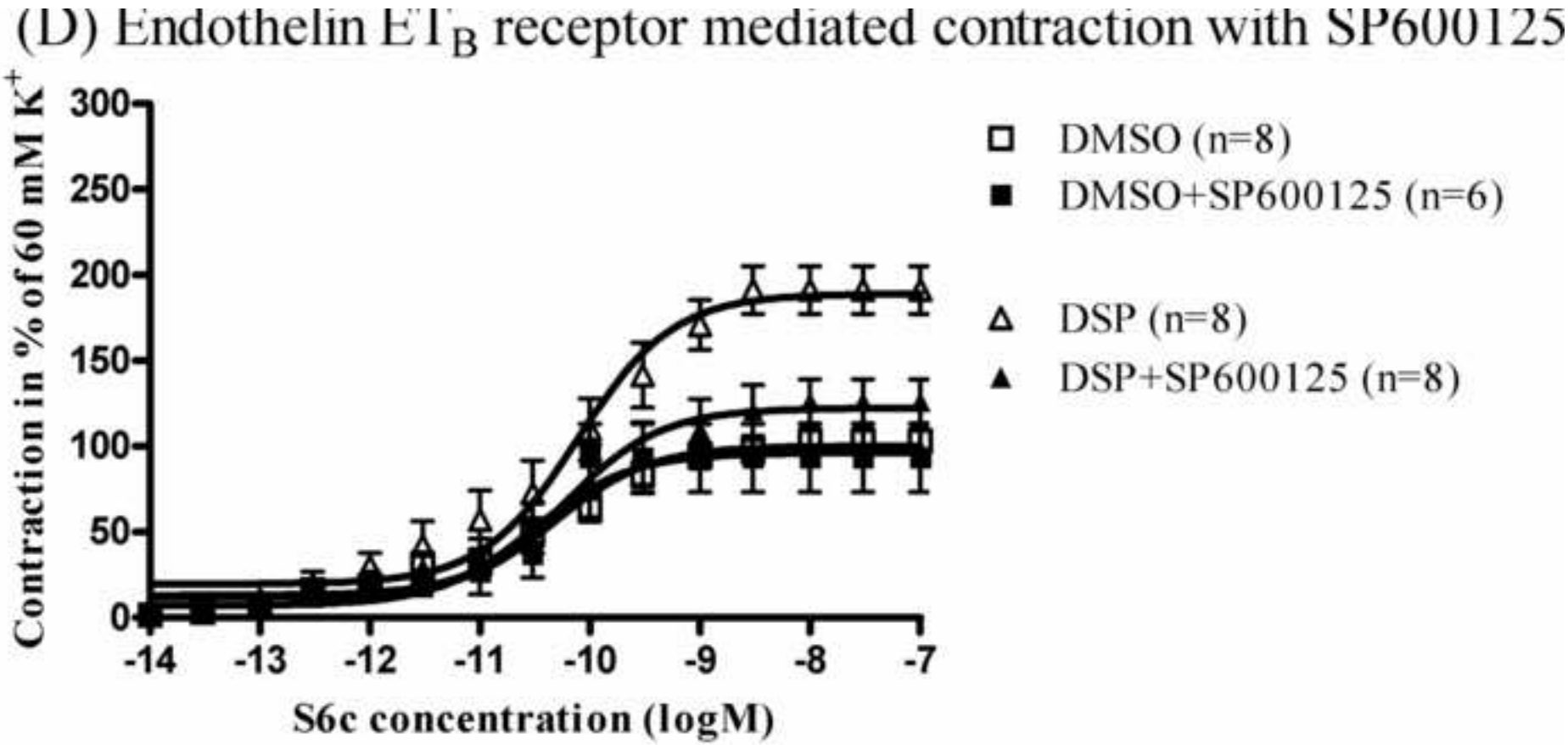

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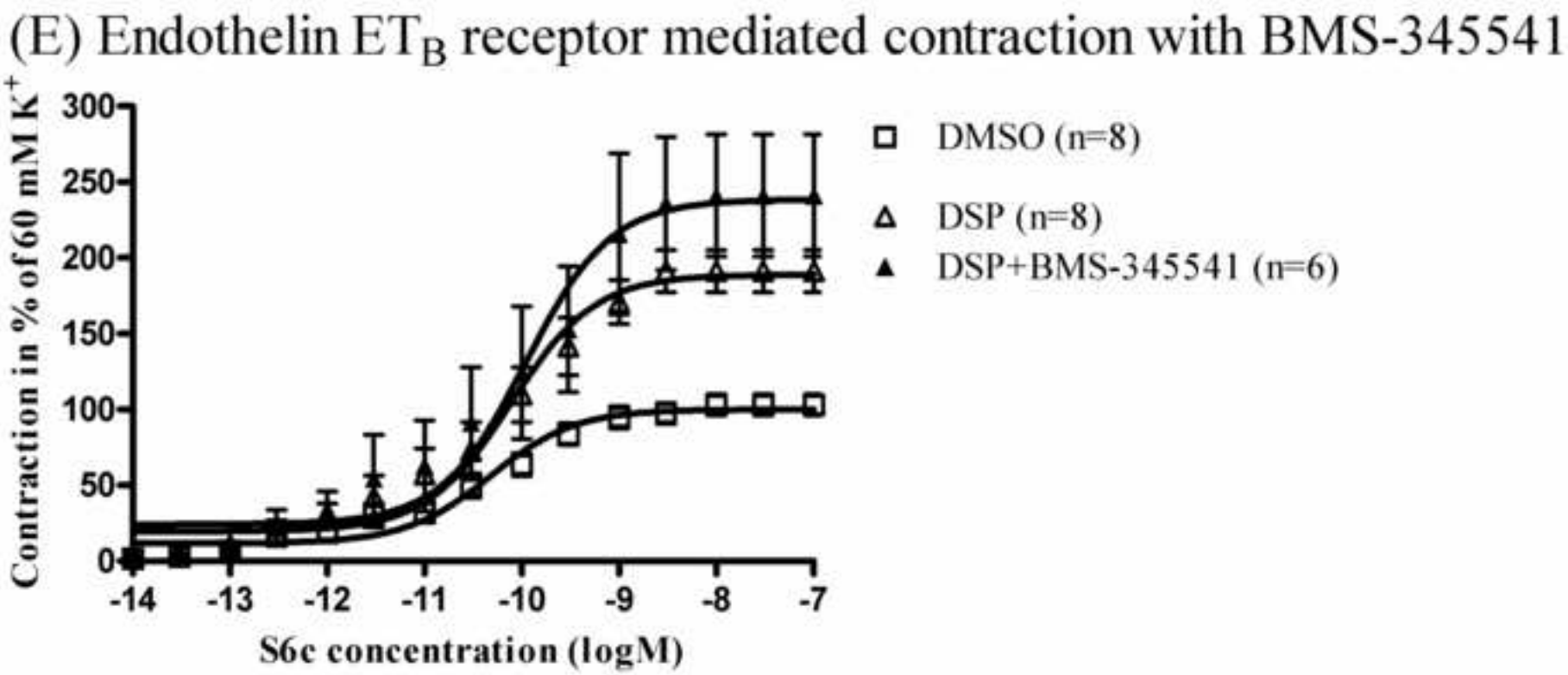

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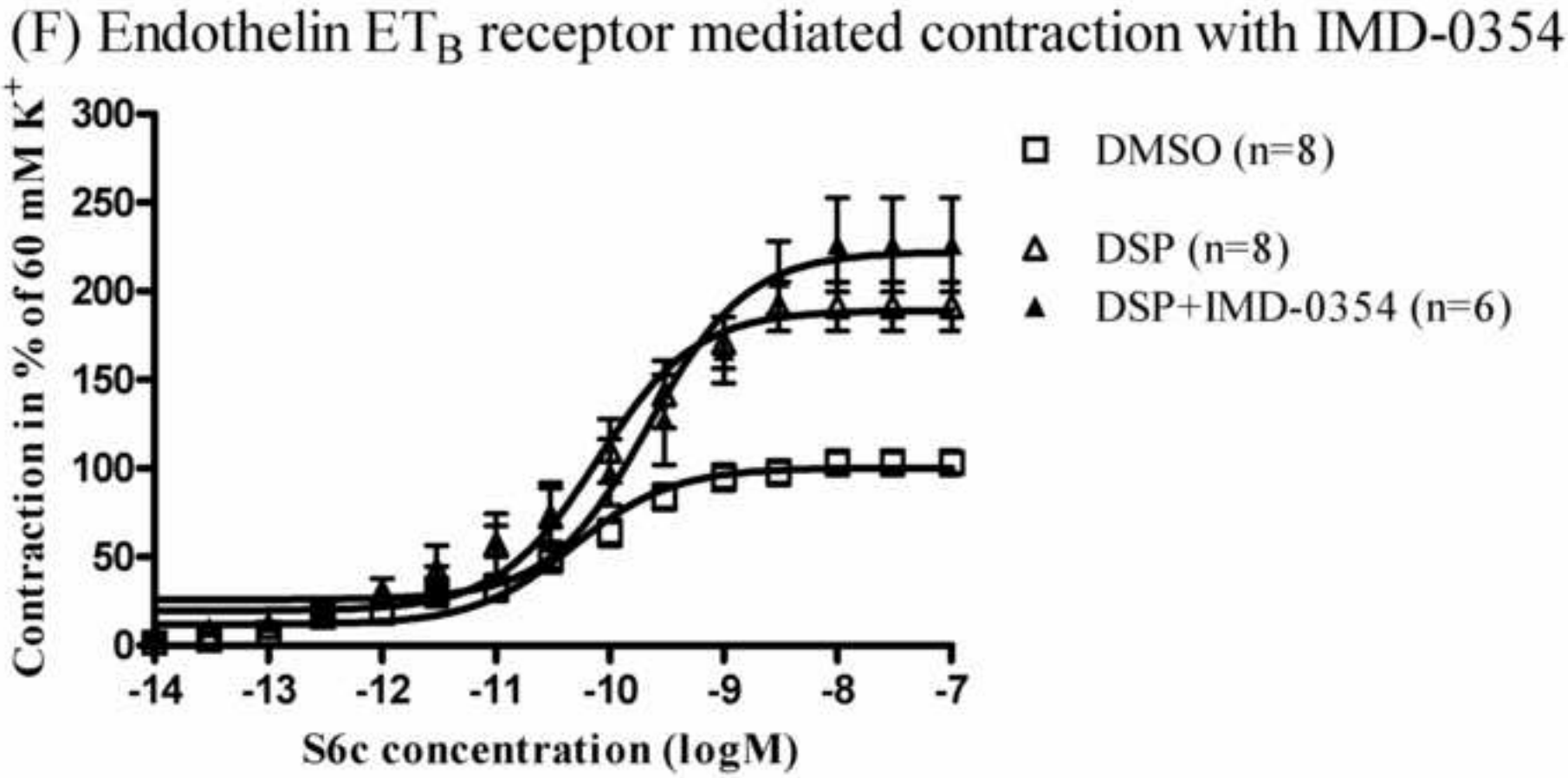

Figure 1G

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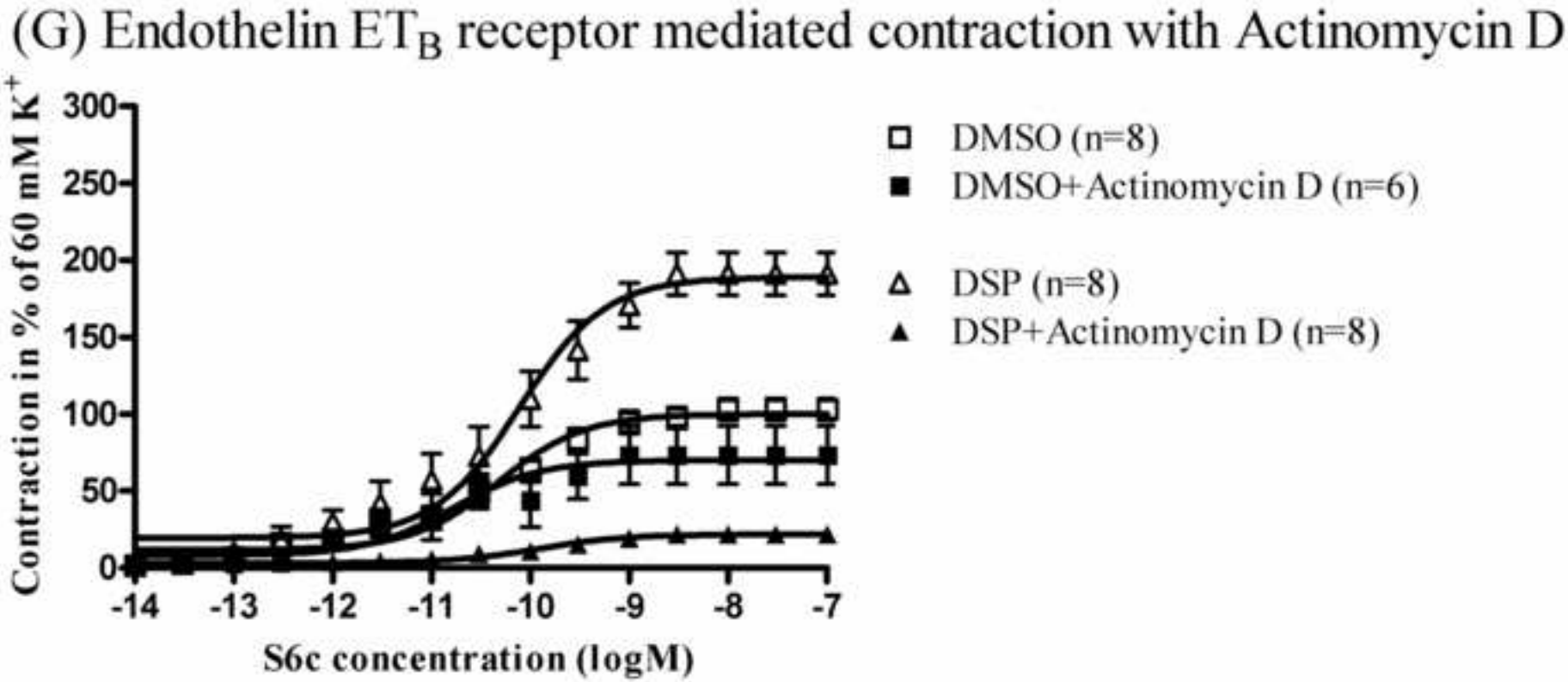

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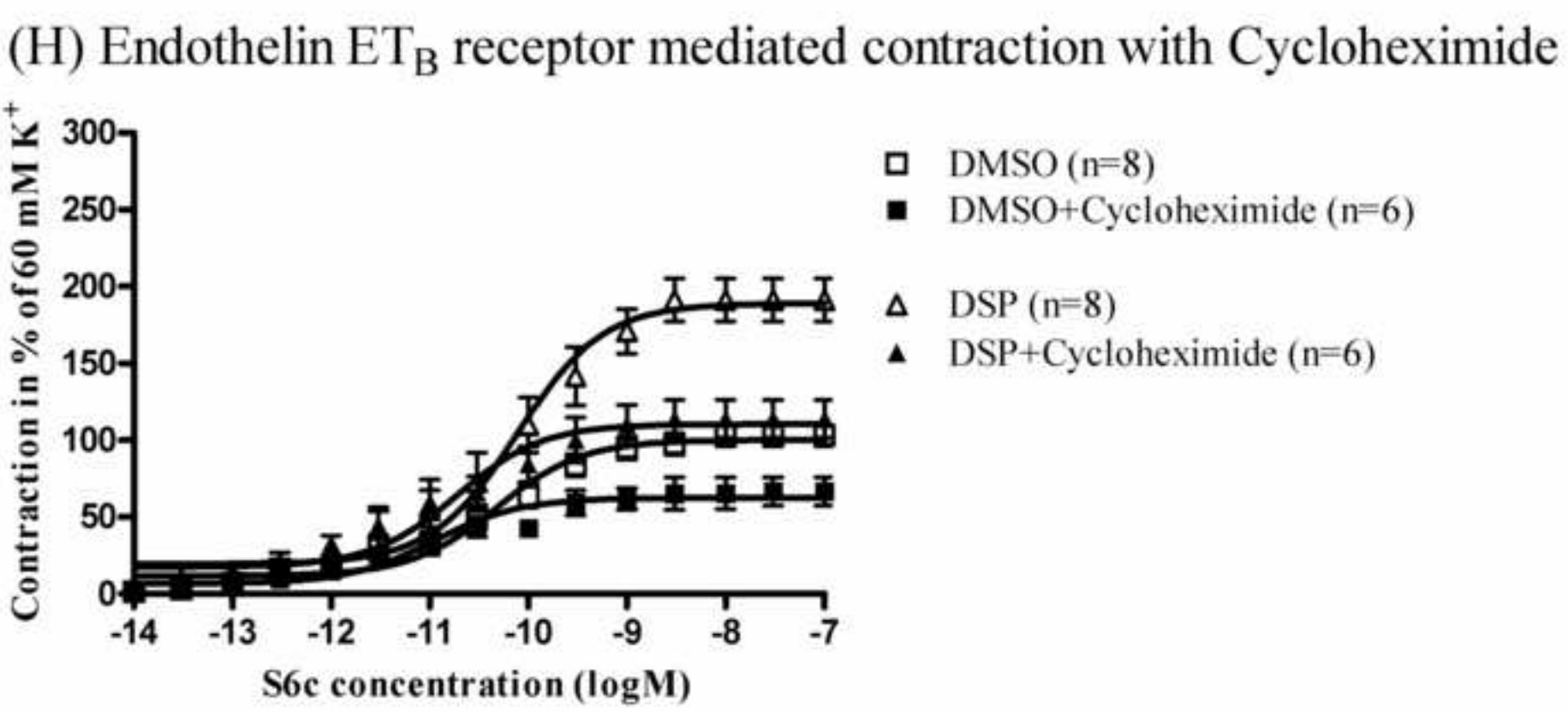

Figure 2

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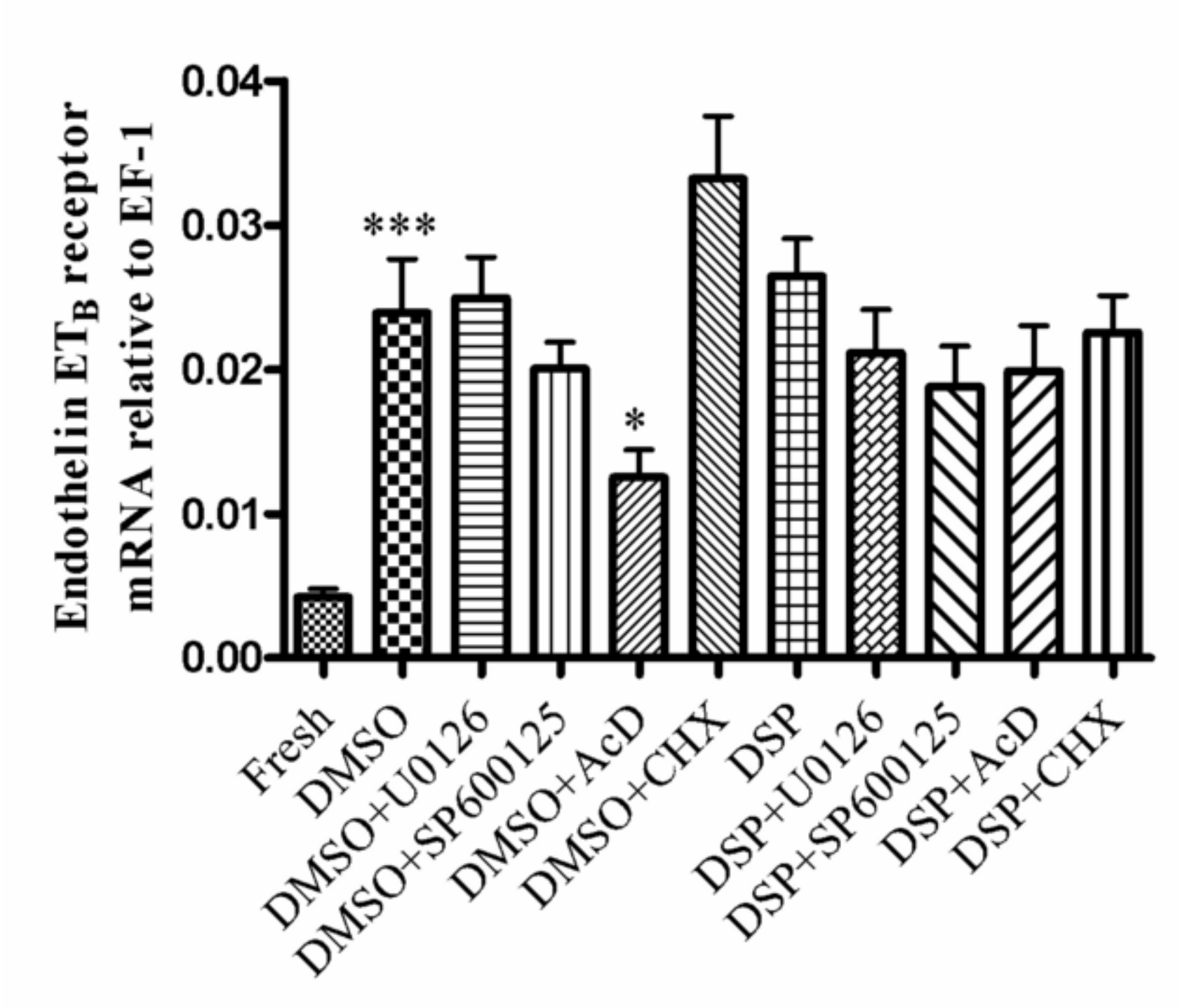

Figure 3

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