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Abstract: Organ culture is an in vitro method for investigating cellular mechanisms involved in upregulation vasoconstrictive G-protein coupled receptors (GPCR). We hypothesize that mitogen activated protein kinase kinase (MEK) and/or extracellular signal-regulated kinase (ERK) specific inhibitors will attenuate the GPCR expression following organ culture.

Rat cerebral arteries were incubated for 48 h in the presence of MEK/ERK specific inhibitors U0126, PD98059, SL327, or AG126 for different time periods. Contractile responses by activation of endothelin receptor type A and type B, serotonin receptor 5-HT_{1B}, thromboxane A₂ receptor, and angiotensin II receptor type 1 and type 2 were investigated. Results were verified by measurement of mRNA with real time PCR and by protein immunohistochemistry.

Organ culture induced transcriptional upregulation of endothelin ET_B receptor and of serotonin 5-HT_{1B} receptor on translational level and increased respective contractions. The thromboxane A₂ receptor mediated contraction curves were left-wards shifted by organ culture. Organ culture was associated with elevated pERK 1/2 in the vascular smooth muscle cells: the MEK 1/2 inhibitor U0126 attenuated the endothelin ET_B receptor mediated contraction at post-translational level or by changing the receptor affinities. The serotonin 5-HT_{1B} receptor and thromboxane A₂ receptor mediated contractions were abolished by U0126. Administration of U0126 6 h after start of incubation blocked the receptor upregulation.

In conclusion, MEK specific inhibitor U0126 is a potent inhibitor of GPCR alteration seen during organ culture. Given the ability to inhibit GPCR alteration at the clinically relevant time-point 6 h post incubation makes it an attractive therapeutic agent for in vivo studies.

Date 2010-01-15

Dear Sirs,

Please find the enclosed manuscript “Comparison of MEK/ERK pathway inhibitors on the upregulation of vascular G-protein coupled receptors in rat cerebral arteries” by H. Sandhu, S. Ansar, and L. Edvinsson, which we hereby submit for publishing to The European Journal of Pharmacology for consideration. The paper has not been submitted before or published in whole or in part. All the authors have read and approved the submission.

The **manuscript** text is written in Microsoft word and the fonts are Times New Roman. It has been saved as PDF. The contents are as follows:

- Title
- Authors
- Affiliations
- Abstract
- Keywords
- Main text
 - o Introduction
 - o Materials and Methods
 - o Results
 - o Discussion
 - o Conclusion
- Acknowledgement
- References

Figures (Figures have been made in PowerPoint, fonts in illustrations are Times New Roman, saved as PDF):

- Figure 1
- Figure 2A
- Figure 2B
- Figure 2C
- Figure 3A
- Figure 3B
- Figure 4A
- Figure 4B
- Figure 5A
- Figure 5B
- Figure 6

Figure legends (The text is written in Microsoft word and the fonts are Times New Roman. It has been saved as PDF)

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We are looking forward to hear the views of you and your reviewers.

Sincerely

Hardip Sandhu

Comparison of MEK/ERK pathway inhibitors on the upregulation of vascular G protein coupled receptors in rat cerebral arteries

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Abstract

Organ culture is an *in vitro* method for investigating cellular mechanisms involved in upregulation vasocontractile G-protein coupled receptors (GPCR). We hypothesize that mitogen activated protein kinase kinase (MEK) and/or extracellular signal-regulated kinase (ERK) specific inhibitors will attenuate the GPCR expression following organ culture.

Rat cerebral arteries were incubated for 48 h in the presence of MEK/ERK specific inhibitors U0126, PD98059, SL327, or AG126 for different time periods. Contractile responses by activation of endothelin receptor type A and type B, serotonin receptor 5-HT_{1B}, thromboxane A₂ receptor, and angiotensin II receptor type 1 and type 2 were investigated. Results were verified by measurement of mRNA with real time PCR and by protein immunohistochemistry.

Organ culture induced transcriptional upregulation of endothelin ET_B receptor and of serotonin 5-HT_{1B} receptor on translational level and increased respective contractions. The thromboxane A₂ receptor mediated contraction curves was left-wards shifted by organ culture. Organ culture was associated with elevated pERK1/2 in the vascular smooth muscle cells: the MEK1/2 inhibitor U0126 attenuated the endothelin ET_B receptor mediated contraction at post-translational level or by changing the receptor affinities. The serotonin 5-HT_{1B} receptor and thromboxane A₂ receptor me-

diated contractions were abolished by U0126. Administration of U0126 6 h after start of incubation blocked the receptor upregulation.

In conclusion, MEK specific inhibitor U0126 is a potent inhibitor of GPCR alteration seen during organ culture. Given the ability to inhibit GPCR alteration at the clinically relevant time-point 6 h post incubation makes it an attractive therapeutic agent *for in vivo* studies.

Keywords: Organ culture; cerebral arteries; vascular smooth muscle cells; GPCR upregulation; MEK/ERK pathway; U0126

1 Introduction

Cerebral ischemia and stroke are the third leading causes of death worldwide and leading causes of long-term disability (Rosamond et al., 2008a). The most common type is thromboembolic stroke which accounts for 87 % of all stroke incidents; the remaining incidents are caused by intracerebral and subarachnoid haemorrhages (SAH) (Rosamond et al., 2007). Substantial efforts have been made to understand the intracellular mechanisms involved in ischemia induced cerebral damage, with focus primarily on cerebral neurons (neuroprotection), and to develop drugs that protect the brain from damage once a stroke has occurred. Despite intense investigation for over three decades, few therapies have proven effective in the clinic (Elting et al., 2002; O'Collins et al., 2006). Experimental and clinical studies have shown increased levels of the locally formed vasoconstrictor endothelin-1, angiotensin II, 5-hydroxytryptamine, and thromboxane in cerebral ischemia (Lincoln et al., 1990; Loesch and Burnstock, 1988; Loesch and Burnstock, 2002); all the substances mediate vasoconstriction via specific vascular GPCR (Boess and Martin, 1994; Dudley et al., 1991; Murphy et al., 1991; Pucell et al., 1991; Sakamoto et al., 1993; Sasaki et al., 1991; Shenker et al., 1991; Tsutsumi et al., 1991).

We have demonstrated that cerebral ischemia is associated with enhanced expression of vasoconstrictor GPCRs in the smooth muscle cells of brain arteries, both in the major arteries belonging to the circle of Willis and in intracerebral microvessels, but not in brain neurons or glial cells (Ansar et al., 2007; Beg et al., 2006; Vikman and Edvinsson, 2006). There is a rapid transcription/translation upregulation of contractile endothelin ET_A and ET_B receptors, angiotensin AT₁ and AT₂ receptors, and serotonin 5-HT_{1B} receptors in the vascular smooth muscle cells after SAH or focal ischemia and reperfusion (Hansen-Schwartz et al., 2003a; Hansen-Schwartz et al., 2003b; Stenman et al., 2002; Stenman and Edvinsson, 2004). Furthermore, the mitogen-activated protein kinase (MAPK) signalling pathways are involved in the process of cerebrovascular receptor upregulation (Ansar and Edvinsson, 2008; Beg et al., 2006; Henriksson et al., 2007; Maddahi and Edvinsson, 2008). In an initial study, the MEK1/2 specific inhibitor U0126 reduced the activity of ERK1/2 and blunted the increase in vasoconstrictive receptor upregulation, reduced the cerebral infarct volume and improved neurological score after experimental focal ischemic stroke in rodents (Henriksson et al., 2007; Namura et al., 2001; Maddahi and Edvinsson, 2008).

We have found that organ culture of cerebral arteries is an *in vitro* method to induce changes in GPCR expression in a pattern which resembles that seen in cerebrovascular disease *in vivo* (Hoel et al., 2001a; Rosamond et al., 2008b). The organ culture model allows for detailed studies of the underlying intracellular mechanisms responsible for the upregulation of GPCR in cerebral arteries. We hypothesise that MEK/ERK pathway specific inhibitors will prevent the upregulation of contractile cerebrovascular GPCRs in smooth muscle cells. The aim of this study was to evaluate the effect of available MEK1/2 specific inhibitors U0126, PD98059, and SL327, and the ERK1/2 specific inhibi-

tor AG126 on vascular GPCR upregulation, to compare their efficacy, and to unravel their treatment time window during which each inhibitor effectively prevents GPCR upregulation.

2 Materials and Methods

2.1 Removal of cerebral vessels and organ culture

Male Sprague-Dawley rats (n=98; 350-400 g) (Taconic, Denmark) were anaesthetized with CO₂ 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 artery were removed and dissected free.

Middle cerebral artery segments (1 mm long), rings with intact endothelium, were studied immediately (fresh, 0 h organ culture) or incubated for 48 h at 37 °C in humidified 5 % CO₂ and 95 % air in serum free Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4mM L-glutamine, 0.11 mg/ml sodium pyruvate) supplemented with an antibiotics mix (10.000 units/ml of penicillin, 10 mg/ml of streptomycin, and 25 ng/ml of amphotericin B). Thereafter vessels 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 protein immunohistochemistry. When using MEK/ERK1/2 inhibitors, they were added to the DMEM medium at 0 h, 6 h, 12 h or 24 h after the initiation of the incubation (Figure 1) (U0126: 0.1 µM, 1 µM or 10 µM; PD98059: 0.1 µM, 1 µM, 10 µM, or 30 µM; SL327: 0.1 µM, 1 µM, 10 µM, 100 µM, or 300 µM; AG126: 0.1 µM, 1 µM or 10 µM). The inhibitors were dissolved in dimethyl sulfoxide. Dimethyl sulfoxide added alone was used as vehicle (placebo, control).

2.2 Drugs

DMEM and the antibiotics mix were from Invitrogen (USA). 1-Bromo-3-chloropropane, dimethyl sulfoxide, AG126, U0126, PD98059, carbachol, 5-hydroxytryptamin, 5-carboxamidotryptamine, PD123319, and U46619 were obtained from Sigma-Aldrich (USA). SL327 was from Biaffin GmbH & Co KG (Germany). Endothelin-1, Sarafotoxin 6c, and Angiotensin II were from NeoMPS S.A. (France).

2.3 *In vitro* pharmacology

A sensitive myograph was used for recording the isometric tension in isolated vessel segments (Hogestatt et al., 1983; Mulvany and Halpern, 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 were 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 6 (ADInstruments, New Zealand).

The segments were immersed in a temperature-controlled (37 °C) buffer solution (composition in mM/ml; NaCl = 119, NaHCO₃ = 15, KCl = 4.6, MgCl₂ = 1.2, NaH₂PO₄ = 1.2, CaCl₂ = 1.5, and glucose = 5.5). The buffer was continuously gassed with 5 % CO₂ in O₂ resulting in a physiological pH at 7.4. The vessels were given an initial pre-tension of 2 mN/mm and adjusted to this tension for 1 hour. The contractile capacity was determined by exposure to a 30 mM potassium buffer solu-

tion with the same composition as the bicarbonate buffer solution except that NaCl is exchanged for KCl. The threshold for minimum 30 mM potassium contraction response was set to 0.8 mN.

Carbachol mediated dilation after 5-hydroxytryptamine mediated contraction is used as measurement of endothelium functionality. Carbachol mediates an endothelium dependent dilation of vessels. 5-hydroxytryptamine mediates a strong smooth muscle cell dependent contraction. This contraction is used as a reference for maximum contraction of the vessel when endothelium functionality is measured. Middle cerebral artery segments were pre-contracted with 3 μ M 5-hydroxytryptamine and subsequently exposed to 10 μ M carbachol. A strong dilation by carbachol indicates intact and/or functional endothelium, whereas a weak or absent dilation indicates damaged or unfunctional endothelium (i.e. due to mechanical damage of the endothelium during mounting on wires). Threshold for maximal endothelium presence/function was set to 30 % carbachol induced dilatation, and only vessels showing this response or less were included, as we are interested in observing the GPCR response in smooth muscle cells only. Concentration-response curves were then obtained by cumulative application of the agonists endothelin-1 (10^{-14} M to 10^{-7} M), sarafotoxin 6c (10^{-14} M to 10^{-7} M), 5-carboxamidotryptamine (10^{-12} M to 3×10^{-5} M), angiotensin II (10^{-12} M to 3×10^{-6} M), and U46619 (10^{-12} M to 3×10^{-6} M). The angiotensin AT₂ receptor antagonist PD123319 was added at a concentration of 30 μ M 30 min prior to the angiotensin II concentration-response curve when the angiotensin AT₁ receptor response was studied.

2.4 Molecular Biology

Isolated middle cerebral artery was snap-frozen with dry ice in green-cap tubes containing Lysing Matrix D provided in the FastRNA Pro Green Kit (Q Biogene, USA) after removal. Total cellular

RNA was extracted with use of the FastRNA Pro Green Kit following the supplier's instructions. The vessels were homogenized in 1 ml of RNAPro 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 % ethanol and stored at minus 20 °C over-night. 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 re-dissolved with RNase free water. Total RNA quantity and purity was determined using a GeneQuant II spectrophotometer (Pharmacia Biotech, UK) measuring the absorbance at 260 nm and 280 nm. Demonstration of a high RNA quality, the A_{260}/A_{280} ratio value was around 1.8 – 2.0.

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 30 µl reaction volume where random hexamers were 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 20 µl of RNase free water when used in the real time PCR reaction.

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

Elongation factor-1 (EF-1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as reference housekeeping genes (Stenman et al., 2002; Stenman and Edvinsson, 2004). Specific primers for the rat endothelin ET_A and ET_B receptors, serotonin 5-HT_{1B} receptor, thromboxane A₂ receptor, EF-1, and GAPDH were designed (Table 1). One µl 10 mM primer was added to the 25 µl real time PCR reaction. 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.

2.5 Immunohistochemistry

The middle cerebral artery was dissected out and then 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 & CO KG, Germany).

The primary antibodies used were sheep anti-rat endothelin ET_B receptor (ab50658, Abcam, UK), diluted 1:200; rabbit anti-rat serotonin 5-HT_{1B} receptor (ab13896, Abcam, UK), diluted 1:500; rabbit anti-rat thromboxane A₂ receptor (ab65150, Abcam, UK), diluted 1:200; rabbit anti-rat pERK1/2 (4370, Cell Signalling, USA), diluted 1:1000; and mouse anti-rat β-actin (ab11003, Abcam, UK), diluted 1:500. The endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, and pERK1/2 dilutions were done in phosphate buffered saline with 2 % donkey serum (017-000-121, Jackson ImmunoResearch laboratories, Inc., USA), while the thromboxane A₂ receptor dilution was done in phosphate buffered saline with 2 % goat and donkey serum (005-000-121 and 017-000-121 Jackson ImmunoResearch laboratories, Inc., USA).

The secondary antibodies used were donkey anti-sheep DL488 conjugated (STAR88D488, AbD Serotec, USA), diluted 1:200; donkey anti-rabbit DL488 conjugated (711-485-152, Jackson ImmunoResearch, USA), diluted 1:200; goat anti-rabbit FITC conjugated (ab6717, Abcam, UK), diluted 1:200; donkey anti-rabbit DL459 conjugated (711-505-152, Jackson ImmunoResearch, USA), diluted 1:200; and donkey anti-mouse DL549 conjugated (715-505-150, Jackson ImmunoResearch, 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).

2.6 Calculation and statistical analyses

Data are expressed as mean values \pm standard error of the mean (s.e.m.), and n refers to the number of rats. Statistical analyses were performed with one way ANOVA (non-parametric Kruskal-Wallis test) with Bonferroni's test (groups compared: organ culture of vehicle vs. fresh or organ culture with 10 μ M U0126 at specific time points), where $P < 0.05$ was considered statistically significant ($P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, and not significant = n.s.).

2.6.1 In vitro Pharmacology

Contractile responses in each segment are expressed as percentage of the 30 mM K^+ induced contraction. E_{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. For biphasic responses, $E_{max(1)}$ and $pEC_{50(1)}$ describes the high affinity phase and $E_{max(2)}$ and $pEC_{50(2)}$ describes the low affinity phase.

2.6.2 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_A and ET_B receptor, serotonin 5-HT_{1B} receptor, and thromboxane A2 receptor mRNA. The relative amount of mRNA was calculated with the CT values of endothelin ET_A and ET_B receptor, serotonin 5-HT_{1B} receptor, and thromboxane A2 receptor and 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 target 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 CtX is the C_T value for the target. Data with EF-1 as the reference gene is shown in results section.

2.6.3 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 488 nm channel (β -actin conjugated) overlay; however, only green 549 nm channel (receptor conjugated) image is given for visual simplicity. Mean fluorescence intensity of the green channel image was compared in the different groups, where fresh group was used as a reference.

3 Results

3.1 Organ culture and receptor upregulation

Middle cerebral arteries were incubated for 48 h with the MEK/ERK inhibitor or their solvent dimethyl sulfoxide (in the dose of 2.5 μ l/ml of DMEM dimethyl sulfoxide had no effect; data not

shown). There was no significant difference of K^+ -induced contractions between fresh and vehicle incubated vessels (30 mM K^+ values as mean \pm s.e.m.: fresh = $2.58 \text{ mN} \pm 0.28$; 48 h organ culture dimethyl sulfoxide vehicle = $2.83 \text{ mN} \pm 0.26$).

3.1.1 Endothelin receptors

Sarafotoxin 6c did not induce any contraction of fresh cerebral artery segments, while endothelin-1 produced a strong concentration dependent contraction (E_{\max} in % of K^+ = $139 \pm 14 \text{ mN}$). After 48 h of organ culture, sarafotoxin 6c resulted in a strong concentration-dependent contraction (Figure 2C, Table 2). The response to endothelin-1 showed no change in maximum contraction or pEC_{50} (Figure 2B, Table 2). As demonstrated previously the sarafotoxin 6c effect was mediated by upregulated endothelin ET_B receptors (Henriksson et al., 2003). Administration of endothelin-1 – following a sarafotoxin 6c mediated contraction curve - resulted in activation of the endothelin ET_A receptors only due to desensitisation of endothelin ET_B receptors (Adner et al., 1994). The upregulation of the endothelin ET_B receptors was confirmed by real time PCR of receptor mRNA levels (Figure 3A) and receptor protein immunohistochemistry (Figure 3B). Endothelin ET_A receptor mRNA or protein was not changed after 48 h of vehicle compared to fresh vessels (data not shown).

3.1.2 5-HT receptors

5-carboxamidotryptamine was used as an agonist because it activates both serotonin $5-HT_{1B}$ receptor and serotonin $5-HT_{2A}$ receptors (Hoel et al., 2001b). Careful study of rat cerebral arteries have shown that they mainly contain the $5-HT_{2A}$ subtype of receptors, however, organ culture resulted in upregulation of the serotonin $5-HT_{1B}$ receptor subtype (Hansen-Schwartz et al., 2002; Hoel et al., 2001b). The rat middle cerebral artery 5-carboxamidotryptamine contraction E_{\max} almost doubled

from fresh to after 48 h of organ culture (Figure 4A, Table 2). The 5-carboxamidotryptamine mediated contraction of fresh middle cerebral artery results in a sigmoid curve, while the curve of incubated vessels gives a biphasic curve. There was no significant difference in the serotonin 5-HT_{1B} receptor mRNA levels (data not shown), but protein immunohistochemistry showed a significant rise in the serotonin 5-HT_{1B} receptor level between fresh vessels and vessels with vehicle after 48 h of organ culture (Figure 4B).

3.1.3 Thromboxane A₂ receptors

The thromboxane A₂ receptor agonist U46619 induced strong contraction of fresh middle cerebral artery segments as well as vessels incubated for 48 h (Figure 5A, Table 2). There is a slight leftward shift from fresh to 48 h incubated middle cerebral artery, but without significance ($p>0.05$). There was a no difference in thromboxane A₂ receptor mRNA levels between fresh and 48 h incubated middle cerebral artery (data not shown). Protein immunohistochemistry did not show any difference in thromboxane A₂ receptor protein level from fresh to 48 h incubated middle cerebral artery (Figure 5B).

3.1.4 Angiotensin AT receptors

We have before observed a weak contraction by angiotensin II of rat middle cerebral artery in fresh vessels and a stronger contraction after cerebral ischemia (Stenman and Edvinsson, 2004). However, organ culture did not reveal any enhanced contraction to angiotensin II whether or not the angiotensin AT₂ receptor antagonist PD123319 (30 μ M) was present (data not shown).

3.2 MEK / ERK inhibitors

Available MEK/ERK1/2 inhibitors were tested primarily on the endothelin ET_B receptor upregulation because this response to organ culture was strong and reproducible. K⁺-induced contractions did not differ significantly in vehicle vessels compared to vessels incubated with MEK/ERK inhibitors (30 mM K⁺ values in mN as mean \pm s.e.m.: 48 h organ culture vehicle = 2.83 ± 0.26 ; 48 h organ culture 10 μ M U0126 = 2.20 ± 0.23 ; 48 h organ culture 10 μ M PD98059 = 3.60 ± 0.38 ; 48 h organ culture 10 μ M SL327 = 2.35 ± 0.32), except for AG126 (30 mM K⁺ values in mN as mean \pm s.e.m.: 48 h organ culture 1 μ M AG126 = 1.72 ± 0.42).

3.2.1 U0126

The direct effect of U0126 was tested on fresh middle cerebral artery and on segments organ cultured for 48 h. U0126 in the concentration interval 10^{-14} M to 10^{-5} M did not alter the basal vascular contractile tone. In addition, 10 μ M U0126 had no dilatory effect on pre-contracted vessels, and did not alter the 30 mM K⁺ max response (data not shown; n=6).

The second set of experiments was performed to evaluate the effects of U0126 on endothelin ET_B receptor activation and expression. Middle cerebral artery co-incubated with 10 μ M U0126 for 48 h showed significantly reduced endothelin ET_B receptor mediated maximum contraction compared to control (Figure 2C, Table 2). There was a time-dependent rightward shift of the concentration-contraction curve to endothelin-1 with prior exposure to sarafotoxin 6c after incubation with U0126 (Figure 2A, Table 2). The concentration of U0126 was kept at 10 μ M because previous study in our group has shown that this concentration is optimal (Henriksson et al., 2004); lower doses (0.1 – 1 μ M) were without significant effect (tested in the present study; data not shown).

3.2.2 PD98059

The MEK1/2 specific inhibitor PD98059 (Alessi et al., 1995;Dudley et al., 1995) was tested at different concentrations; 0.1 μ M, 1 μ M, 10 μ M, and 30 μ M. The result was the same for all these concentrations. PD98059 did not inhibit the upregulation of the endothelin ET_B receptors after 48 h of organ culture of rat middle cerebral artery. PD98059 in concentrations above 30 μ M altered the contractile tone of the vessels, and hence the effect on endothelin ET_B receptor upregulation of doses above 30 μ M was not tested (data not shown).

3.2.3 SL327

The MEK1/2 specific inhibitor SL327 (Favata et al., 1998b) was tested at different concentrations 0.1 μ M, 1 μ M, 10 μ M, 100 μ M, and 300 μ M. SL327 did not inhibit the endothelin ET_B receptor dependent contractions after 48 h of middle cerebral artery organ culture in the doses studied.

3.2.4 AG126

The ERK1/2 specific inhibitor AG126 (Novogrodsky et al., 1994) significantly reduced both the endothelin ET_A and ET_B receptor mediated contractions in middle cerebral artery after 48 h of organ culture. The 30 mM K⁺ contraction was however also reduced by 1 μ M AG126 ($p < 0.05$) when compared to vehicle incubation (30 mM K⁺ values in mN as mean \pm s.e.m.: 48 h organ culture vehicle = 2.83 ± 0.26 and 48 h organ culture 1 μ M AG126 = 1.72 ± 0.42). E_{\max} values of both the endothelin ET_A and ET_B receptor were reduced and pEC_{50} significantly shifted to the right after administration of AG126 at the start of organ culture (endothelin ET_A receptor mediated contraction: 48 h organ culture with vehicle $E_{\max} = 132 \pm 4$ % and $pEC_{50} = 12.1 \pm 0.2$ and 48 h organ culture with 1 μ M AG126 $E_{\max} = 82 \pm 9.1$ % and $pEC_{50} = 10.6 \pm 0.3$; endothelin ET_B receptor

mediated contraction: 48 h organ culture with vehicle $E_{\max} = 121 \pm 6 \%$ and $pEC_{50} = 11.7 \pm 1.0$, and 48 h organ culture with 1 μM AG126 $E_{\max} = 79 \pm 11 \%$ and $pEC_{50} = 10.5 \pm 0.2$). However, AG126 did not inhibit the receptor alteration when it was added to the organ culture at the other time points (6, 12, or 24 h after start of organ culture), or in lower or higher doses (data not shown).

3.3 Expression of pERK1/2

Organ culture resulted in a strong expression in the activated (phosphorylated) form of ERK1/2 (pERK1/2) in the middle cerebral artery smooth muscle cells; pERK1/2 co-localized with actin. Total ERK1/2 was not altered (data not shown). To verify whether pERK1/2 was reduced by the specific MEK/ERK inhibitors we performed immunohistochemistry of vessels incubated for 48 h with vehicle, 10 μM U0126, 10 μM PD98059, 10 μM SL327, and 1 μM AG126. These experiments revealed that the increased pERK1/2 activity by organ culture was attenuated by U0126 and AG126, whereas PD98059 and SL327 failed to do so (Figure 6). In addition, U0126 added 6 h after the start of the organ culture also showed significant reduction in pERK1/2.

3.4 Time study of MEK inhibition

In order to examine in more detail the inhibitory potential of the two MEK/ERK1/2 inhibitors U0126 and AG126 studies were performed adding the blockers to the medium at different time points after initiation of the organ culture. AG126 failed to inhibit the endothelin ET_B receptor upregulation when it was given 6 h after initiation of the organ culture; thus further study of this blocker was discontinued. Vessel segments were incubated with 10 μM U0126 (start of U0126 administration 6 h, 12 h, or 24 h after initiation of organ culture, see Figure 1) and receptor upregulation was studied of the by using myographs, real time PCR and immunohistochemistry.

3.4.1 Endothelin ET_A receptor and endothelin ET_B receptor receptors

Organ culture for 48 h resulted in a strong sarafotoxin 6c induced contraction, associated with elevated expression of endothelin ET_B receptor mRNA and protein, localised to the cytoplasm of the smooth muscle cell (Figures 3A+B). The upregulated endothelin ET_B receptor mediated contraction is time dependently reduced by 10 μ M U0126. The E_{\max} of sarafotoxin 6c is 121 ± 6 % for vehicle vessels relative to contraction elicited by 30 mM potassium. The E_{\max} for the 0 h, 6 h, 12 h, and 24 h incubation with 10 μ M U0126 is reduced to 78 ± 8 %, 10 ± 4 %, 5 ± 1 %, and 70 ± 9 %, respectively (Figure 2C, Table 2). Addition of 10 μ M U0126 after 6 h or 12 h after initiation of organ culture gives the strongest inhibition by U0126.

There was no significant change in the maximum contractile responses to endothelin-1 when 10 μ M U0126 was added to the organ culture in the presence of sarafotoxin 6c desensitising, resulting in only endothelin ET_A receptor activation (Figure 2A). However, there was a shift of the endothelin-1 responses in the lower half of the endothelin-1 concentration-response curve when both ET receptors are activated at the same time (Figure 2A). Organ culture for 48 h shifts the endothelin-1 pEC₅₀ value for endothelin ET_A receptor and endothelin ET_B receptor mediated contraction to the left from fresh vessels (pEC₅₀ = 9.3 ± 0.1) to incubated vehicle vessels (pEC₅₀ = 10.8 ± 0.2). Addition of 10 μ M U0126 after 6 h or 12 h of organ culture initiation gives the most dominant rightward shift by U0126. These are the same time periods for which the endothelin ET_B receptor mediated contraction is inhibited strongest by 10 μ M U0126. The endothelin ET_A receptor mediated contraction was not altered by organ culture (Figure 2A, Table 2). U0126 was therefore not admin-

istered to this curve. There was no significant change in the mRNA levels of endothelin ET_A receptors between organ culture for 48 h with vehicle or 10 μ M U0126 (data not shown).

3.4.2 Serotonin 5-HT_{1B} receptor

5-carboxamidotryptamine induced a biphasic concentration-dependent contraction of rat middle cerebral artery after 48 h organ culture. U0126 (10 μ M) strongly inhibits the serotonin 5-HT_{1B} receptor mediated contraction by almost 100 % at all three time points (Figure 4A, Table 2). There was no change in serotonin 5-HT_{1B} receptor mRNA at the different time points or treatments (data not shown). Protein immunohistochemistry revealed a significant reduction of in the protein level when 10 μ M U0126 was added to the 48 h organ culture (Figure 4B).

3.4.3 Thromboxane A₂ receptor

The thromboxane A₂ receptor agonist U46619 mediated contraction was inhibited strongly by 10 μ M U0126 when added 6 h after initiation of organ culture. All time points of incubation with 10 μ M U0126 (6 h, 12 h and 24 h), also resulted in a right-ward shift of the thromboxane A₂ receptor mediated contraction (Figure 5A, Table 2). Incubation with 10 μ M U0126 for 42 h did not change the thromboxane A₂ receptor mRNA significantly compared to fresh or 48 h organ culture with vehicle (data not shown). Immunohistochemistry showed no difference in the thromboxane A₂ receptor protein level from fresh to organ culture for 48 h. When U0126 was added to the organ culture there was a significant down-regulation of thromboxane A₂ receptor protein level (Figure 5B).

4 Discussion

The present study has demonstrated by functional and molecular studies, that organ culture results in upregulation of endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, and thromboxane A₂ receptor in rat middle cerebral artery, via enhanced transcription and/or translation in the smooth muscle cells. The main aim of this work was to evaluate the effect of available MEK/ERK1/2 inhibitors on the upregulation of cerebrovascular smooth muscle cell receptors and the time-dependency of this effect. We observed that U0126 had a strong antagonistic effect even if it was given up to 12 h after initiation of the organ culture procedure. Thus, the upregulation of GPCR's involves the MEK/ERK1/2 pathway, and U0126 attenuates the upregulation of endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, and thromboxane A₂ receptor, and the pERK1/2 activity (Favata et al., 1998b). AG126 also showed an inhibitory effect but it was effective only when administered at the initiation of the organ culture. The other MEK/ERK inhibitors tested were ineffective in non-toxic doses and this was confirmed by the lack of modification of pERK1/2 in the vessel walls. All the MEK/ERK inhibitors studied have earlier been verified to inhibit this pathway in cell culture studies (Chen et al., 2009; Moon et al., 2002). The difference between the effects of the inhibition in cell culture studies versus organ culture in the present study is probably due to differences in their concentration-effect profile in isolated cells versus a whole vessel segment.

There is a clear association between cerebrovascular receptor upregulation and activation of the MAPK pathway after organ culture; the phenotypic change of GPCR's occurs in the smooth muscle cell and does not involve the endothelium (Favata et al., 1998a; Beg et al., 2006; Maddahi et al., 2009). The organ culture model provides a method which to some extent mimics the receptor changes that are seen *in vivo* (Hansen-Schwartz et al., 2003b; Stenman et al., 2002) and thereby facilitates the study of intracellular mechanisms involved in more detail. *In vivo* studies using West-

ern blot revealed that the MEK/ERK pathway was activated early (within the first hour) while the JNK and p38 MAPK's were only activated at 48 h after cerebral ischemia (Ansar and Edvinsson, 2008). The present study focused on investigating which of the MAPK inhibitors that most effectively prevented the upregulation of contractile GPCRs and if they would be effective even when given after the initiation of the upregulation process. This is particularly important when considering MEK/ERK inhibition as a potential therapeutic target for prevention of the organ culture induced upregulation of vasoconstrictor receptors and the reduced cerebral blood flow in experimental cerebral ischemia.

The study revealed that organ culture caused enhanced contractile responses to sarafotoxin 6c and in part to endothelin-1, due to upregulation of smooth muscle cell endothelin ET_B receptors, as verified by real time PCR of mRNA expression, and protein immunohistochemistry and function. The contractile response to 5-carboxamidotryptamine was increased, and in parallel an increased protein level demonstrated by immunohistochemistry; however there was no change in the serotonin 5-HT_{1B} receptor mRNA. This suggests an upregulation in serotonin 5-HT_{1B} receptors via increased translation. Thromboxane A₂ receptor mediated contraction was shifted left-ward by organ culture, suggesting upregulation of thromboxane A₂ receptor contraction; however, there was no change in mRNA or protein immunohistochemistry. There was no change in the angiotensin receptor function or expression.

Four inhibitors were examined in this study, three of them are MEK1/2 specific (U0126, PD98059, and SL327), while AG126 is an ERK1/2 specific inhibitor. They have been shown to be neuroprotective *in vivo* or *in vitro* (Namura et al., 2001; Alessandrini et al., 1999; Wang et al., 2003; Kann et al., 2004). Most of the potential pharmacologic agents that have been identified recently do not

cross the blood-brain barrier and therefore require intracisternal administration; U0126 and PD98059 fall into this category (Atkins et al., 1998; Garrington and Johnson, 1999), however, if the dose is increased it may have effects *in vivo* (Maddahi and Edvinsson, 2008). SL327 and AG126 on the other hand are able to cross the blood-brain barrier and are effective in the CNS when administered intraperitoneally (Angstwurm et al., 2004; Atkins et al., 1998), this makes them attractive as potential therapeutic agents. U0126 is highly selective towards both MEK 1 and MEK 2, suppressing both inactive and active forms of MEK 1 and / or MEK 2 ($IC_{50} = 72$ nM for MEK 1 and $IC_{50} = 58$ nM for MEK 2) (Davies et al., 2000; Favata et al., 1998b). PD98059 specifically binds to the inactive form of both MEK 1 and MEK 2, thereby preventing MEK 1 and MEK 2 to be phosphorylated by cRAF or MEK kinase ($IC_{50} = 2-7$ μ M for MEK1 and MEK2) (Favata et al., 1998b). SL327 selectively inhibits MEK 1 and MEK 2 ($IC_{50} = 0.18$ μ M for MEK 1 and $IC_{50} = 0.22$ μ M for MEK 2), and thereby inhibits the phosphorylation of ERK 1/2 (Scherle et al., 2000). Furthermore, SL327 also inhibits and delays MEK activation by interfering with MEK activation by Raf (Rauh-Adelmann et al., 2008). Tyrphostin AG126 selectively inhibits the phosphorylation of ERK 1 and ERK 2 ($IC_{50} = 25-50$ μ M) (Hanisch et al., 2001; Novogrodsky et al., 1994).

We observed that under our experimental conditions only U0126 and AG126 could inhibit the activated form of ERK1/2 and the receptor upregulation in concentrations that did not have any effect on the basal vascular tone. AG126 had only effect when given at the beginning of the organ culture. Importantly, U0126 inhibited receptor upregulation also when given 6 h after initiation of organ culture procedure; this is a more interesting profile if we consider administration of a drug after initiation of cerebral ischemia *in vivo*. This result correlates with *in vivo* observations: Maddahi and Edvinsson observed that U0126, given 6 h after middle cerebral artery occlusion for 2 h and then reperfusion for 48 h depressed of the enhanced expression of pERK1/2 in cerebral vessels and the

upregulation of endothelin ET_B receptors (Maddahi and Edvinsson, 2008). We found that there is a clear time-dependency of the effect of U0126 depending on the time of application after organ culture initiation, illustrating a potential window for therapy. Treatment with U0126 up to 6 hrs after middle cerebral artery occlusion attenuated the elevated endothelin ET_B receptor and mediated contraction, reduced the protein levels of endothelin ET_B, angiotensin AT₁, and serotonin 5-HT_{1B} receptors, and phosphorylation of ERK 1/2 and Elk-1 (Maddahi and Edvinsson, 2008). Furthermore, the infarct size was reduced and the neurological score improved by treatment with U0126 (Henriksson et al., 2007;Maddahi and Edvinsson, 2008;Maddahi et al., 2009). Organ culture of rat middle cerebral artery gives a strong endothelin ET_B receptor mediated contraction while U0126 diminished this endothelin ET_B receptor mediated contraction and the elevated endothelin ET_B receptor mRNA level. The serotonin 5-HT_{1B} receptor mediated contraction is upregulated by organ culture and depressed by U0126 at the protein level.

5 Conclusion

This present study has shown that the MEK/ERK1/2 pathway is involved in GPCRs alteration seen after 48 h of organ culture. Since similar receptor alterations are seen in cerebrovascular diseases *in vivo*, organ culture is a convenient way to studying mechanisms involved in GPCR alterations. The MEK1/2 specific inhibitor U0126 provides the most promising results; it has the ability to inhibit upregulation even when added 6 h after the initiation of organ culture. U0126 attenuated the upregulation of endothelin ET_B, serotonin 5-HT_{1B}, and thromboxane A₂ receptors in a time dependent manner, which may open a window of opportunity to modify *in vivo* situations where the MEK/ERK pathway has a central role.

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7 References

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Figure 1

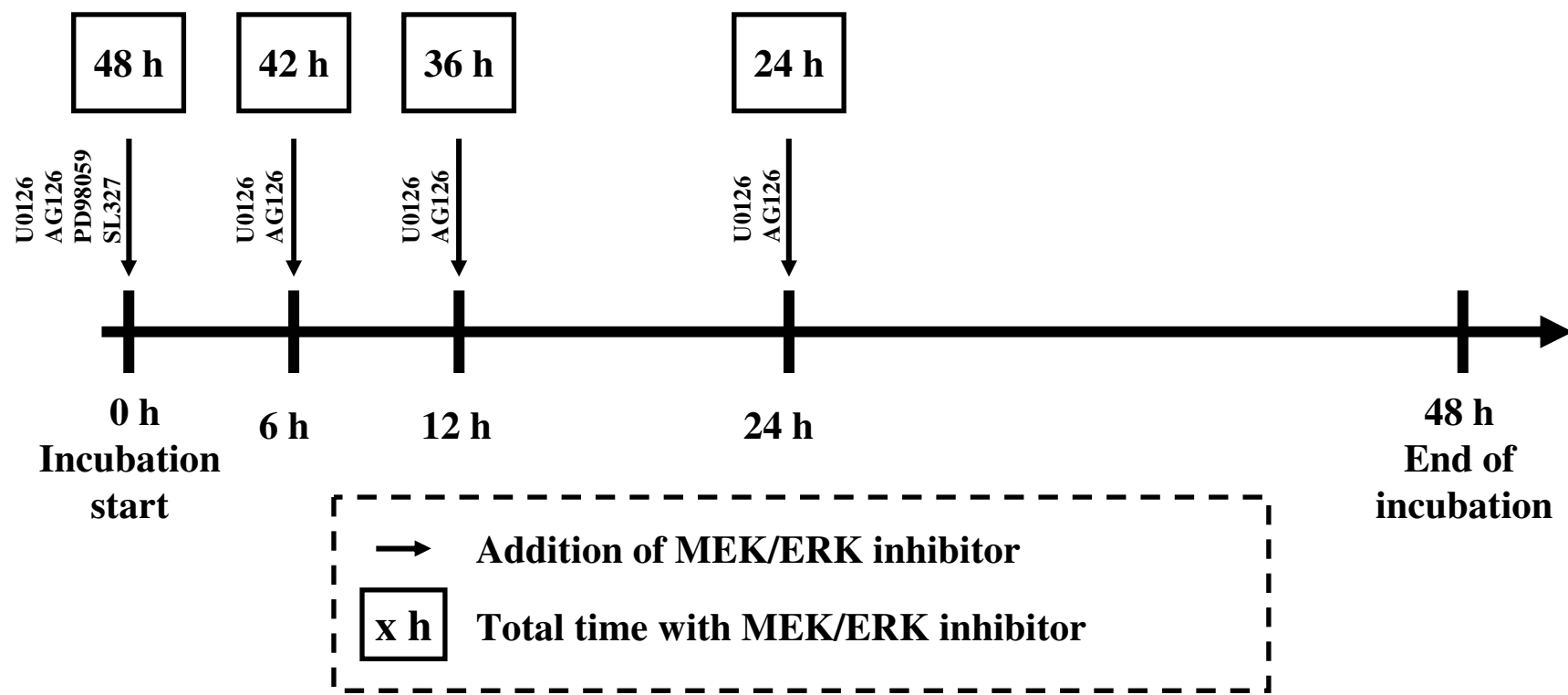


Figure 2A

(A) Endothelin ET_A and ET_B receptor mediated contraction

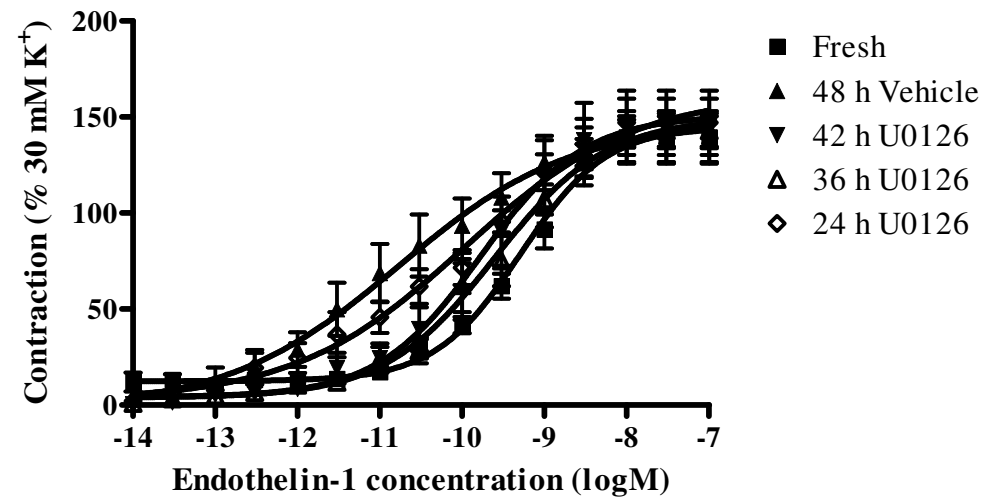


Figure 2B

(B) Endothelin ET_A receptor mediated contraction

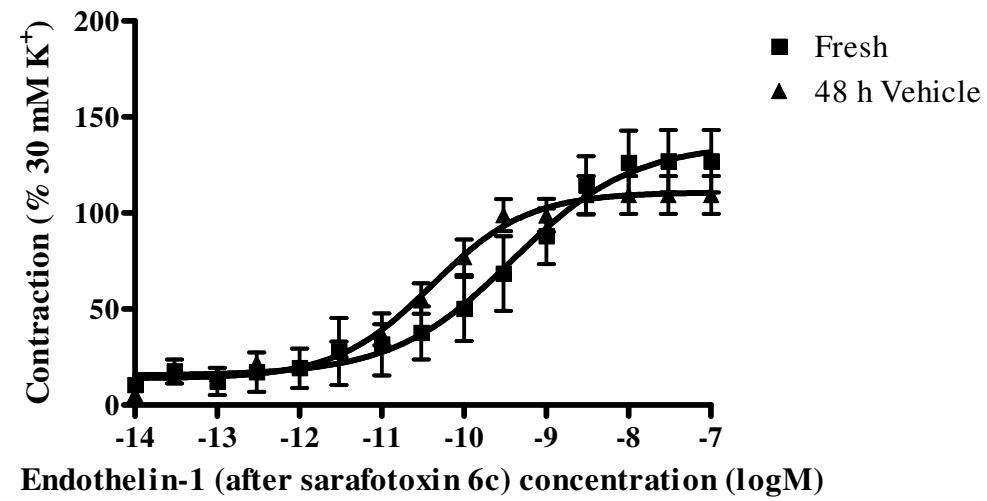


Figure 2C

(C) Endothelin ET_B receptor mediated contraction

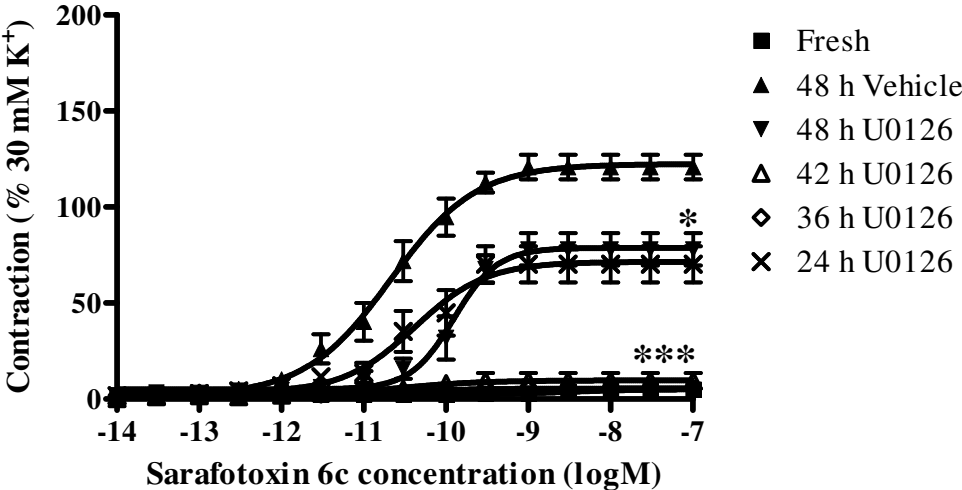


Figure 3A

(A) Endothelin ET_B receptor mRNA

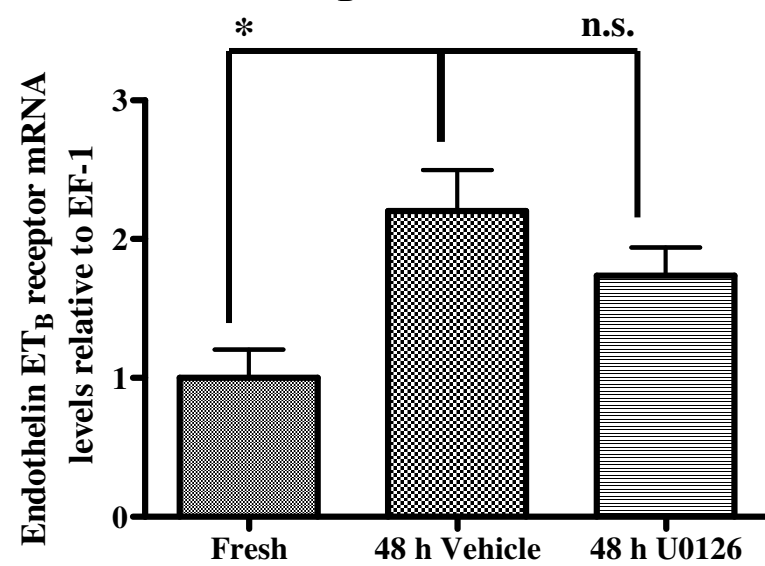


Figure 3B

(B) Endothelin ET_B receptor immunohistochemistry

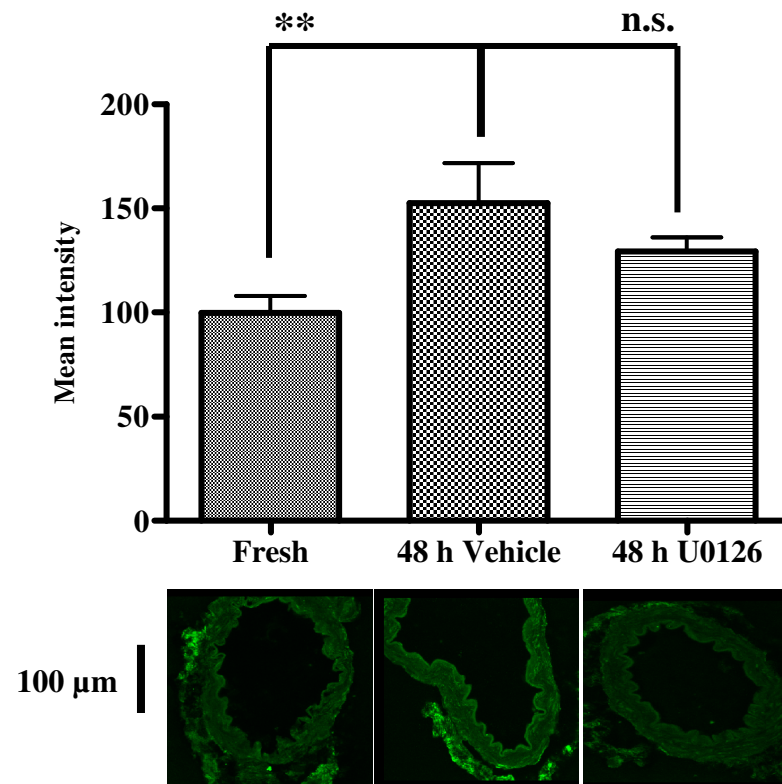


Figure 4A

(A) Serotonin 5-HT_{1B} receptor mediated contraction

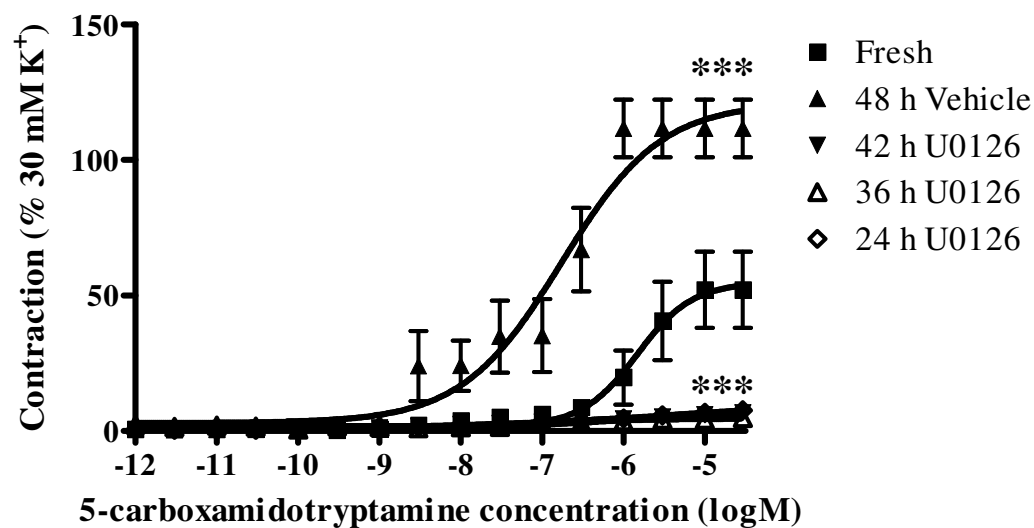


Figure 4B

(A) Serotonin 5-HT_{1B} receptor immunohistochemistry

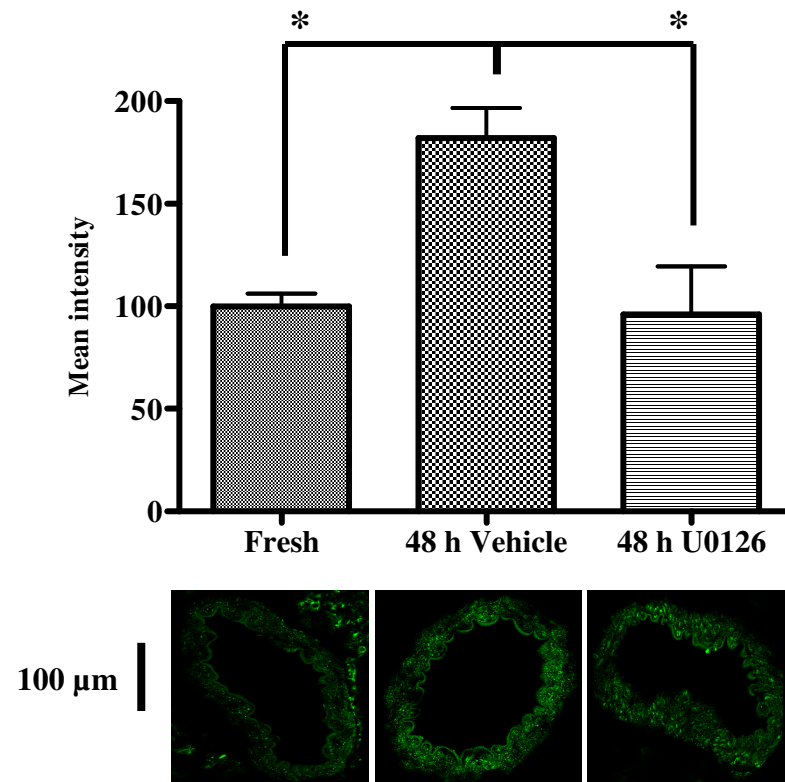


Figure 5A

(A) Thromboxane A2 receptor mediated contraction

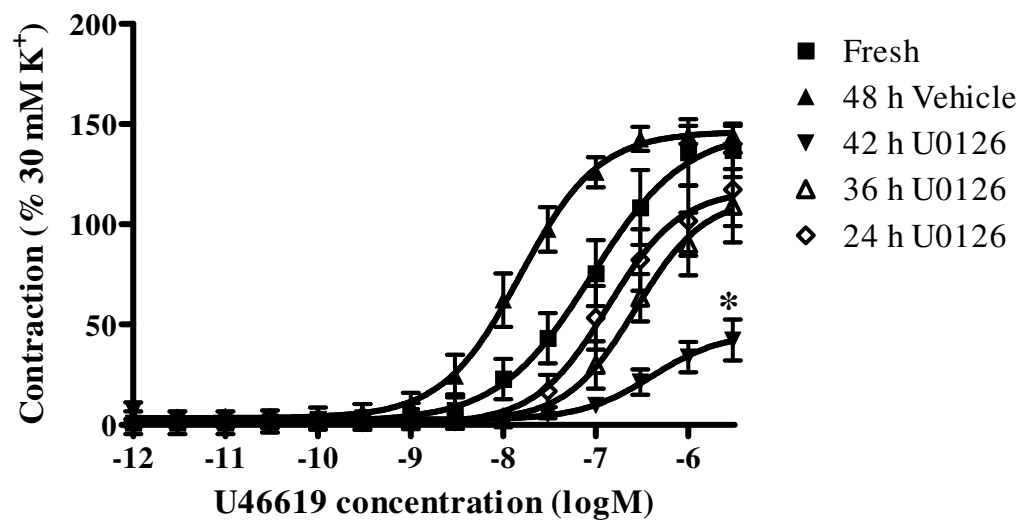


Figure 5B

(B) Thromboxane A2 receptor immunohistochemistry

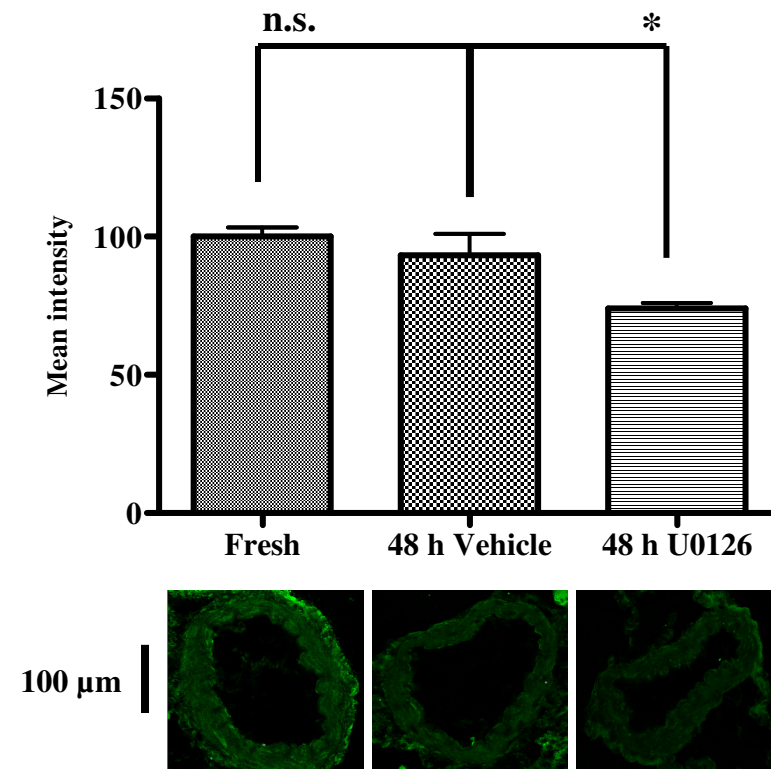


Figure 6

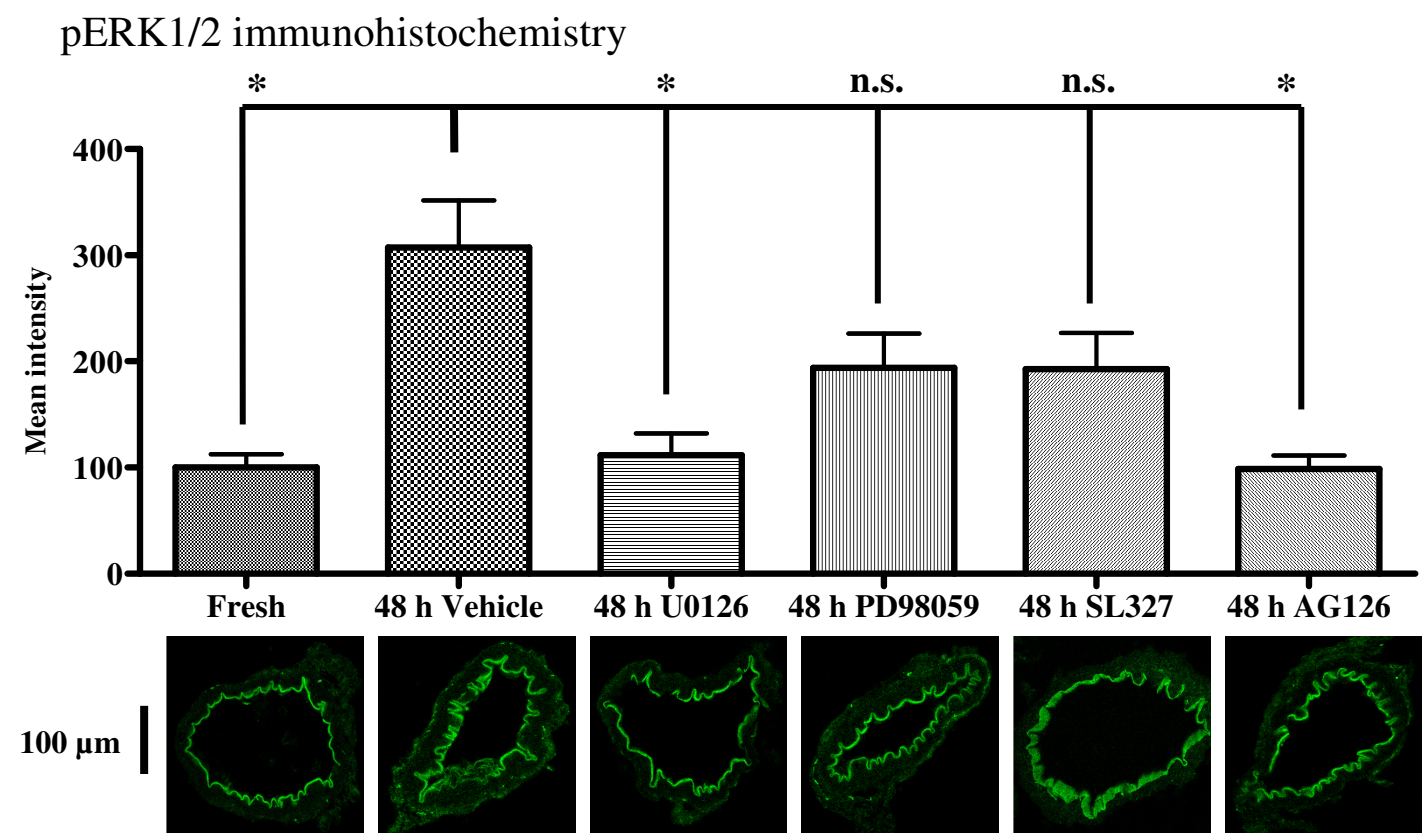


Figure legends

Figure 1 – Organ culture experiment time line

Figure 2 - Contractile responses to the cumulative application of endothelin-1 (A), endothelin-1 after preincubation with sarafotoxin 6c (B), or sarafotoxin 6c (C) to fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126. Values given represent means \pm s.e.m. n=6-16. $P < 0.05 = *$ and $P = 0.001 = ***$.

Figure 3 – Endothelin ET_B receptor mRNA level relative to elongation factor (EF-1) in fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126 (A). Immunohistochemistry double staining against endothelin ET_B receptor and β -actin of fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126, only 488 nm green channel (endothelin ET_B receptor staining) is shown, 60x magnification (B). Values given represent means \pm s.e.m. n=7-8 for real time PCR and n=6 for immunohistochemistry. $P < 0.05 = *$ and $P = 0.01 = **$. n.s. = not significant.

Figure 4 - Contractile responses to the cumulative application of 5-carboxamidotryptamine to fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126 (A). Immunohistochemistry double staining against serotonin 5-HT_{1B} receptor and β -actin of fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126, only 488 nm green channel (serotonin 5-HT_{1B} receptor staining) is shown, 60x magnification (B). Values given represent means \pm s.e.m. n=6-7 for myograph and n=4 for immunohistochemistry. $P < 0.05 = *$ and $P = 0.001 = ***$.

Figure 5 - Contractile responses to the cumulative application of U46619 to fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126 (A). Immunohistochemistry double staining against thromboxane A₂ receptor and β -actin of fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126, only 488 nm green channel (thromboxane A₂ receptor staining) is shown, 60x magnification (B). Values given represent means \pm s.e.m. n=7-11 for myograph experiments, n=7-8 for real time PCR, and n=6 for immunohistochemistry. $P < 0.05 = *$. n.s. = not significant.

Figure 6 - Immunohistochemistry double staining against pERK1/2 and β -actin of fresh or incubated middle cerebral arteries with vehicle or 10 μ M U0126, 10 μ M PD98059, 10 μ l SL327, or 1 μ M AG126, only 488 nm green channel (pERK 1/2 staining) is shown, 60x magnification. Values given represent means \pm s.e.m. n=4 for immunohistochemistry. $P < 0.05 = *$. n.s. = not significant.

Table 1

Gene	GenBank ID	Forward primer	Reverse primer
ET _A	NM_012550	5'-ATTGCCCTCAGCGAACAC-3'	5'-CAACCAAGCAGAAGACGGTC-3'
ET _B	NM_017333	5'-GATACGACAACTTCCGCTCCA-3'	5'-GTCCACGATGAGGACAATGAG-3'
5-HT _{1B}	NM_022225	5'-TCCGGGTCTCCTGTGTACGT-3'	5'-GGCGTCTGAGACTCGCACTT-3'
TA2	NM_017054	5'-ATCTCCCATCTTGCCATAGTCC-3'	5'-CGATGATCCTTGAGCCTAAAG-3'
EF-1	NM_175838	5'-GCAAGCCCATGTGTGTTGAA-3'	5'-TGATGACACCCACAGCAACTG-3'
GAPDH	NM_017008	5'-GGCCTTCCGTGTTCTACC-3'	5'-CGGCATGTCAGATCCACAAC-3'

Table 1 - Primer sequences of the respective genes analysed by real time PCR. ET_A: endothelin ET_A receptor; ET_B: endothelin ET_B receptor; 5-HT_{1B}: serotonin 5-HT_{1B} receptor; TP: thromboxane A2 receptor; EF-1: enlongation factor-1; and GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Table 2

		ET-1	ET-1 (S6c)	S6c	5-CT		U46619
Fresh	E _{max} in % of K+	139 ± 14	127 ± 16	5 ± 1	52 ± 14		137 ± 13
	pEC ₅₀	9.3 ± 0.1	9.4 ± 0.2	-	5.8 ± 0.1		7.0 ± 0.2
48 h Vehicle	E _{max} in % of K+	140 ± 10	109 ± 10	121 ± 6	24 ± 11 E _{max} (1)	112 ± 11 E _{max} (2)	145 ± 4
	pEC ₅₀	10.8 ± 0.2	10.4 ± 0.2	10.7 ± 0.1	8.8 ± 1.3 pEC ₅₀ (1)	6.5 ± 0.3 pEC ₅₀ (2)	7.8 ± 0.1
48 h 10 μM U0126	E _{max} in % of K+	n.d.	124 ± 8	78 ± 8	n.d.		n.d.
	pEC ₅₀	n.d.	11.3 ± 0.2	9.9 ± 0.1	n.d.		n.d.
42 h 10 μM U0126	E _{max} in % of K+	145 ± 19	172 ± 26	10 ± 4	7 +/- 2		42 ± 10
	pEC ₅₀	9.8 ± 0.1	9.9 ± 0.1	-	-		6.4 ± 0.2
36 h 10 μM U0126	E _{max} in % of K+	142 ± 9	196 ± 17	5 ± 1	5 ± 1		109 ± 18
	pEC ₅₀	9.6 ± 0.1	9.5 ± 0.1	-	-		6.6 ± 0.1
24 h 10 μM U0126	E _{max} in % of K+	147 ± 12	154 ± 17	70 ± 9	8 ± 2		117 ± 18
	pEC ₅₀	10.0 ± 0.3	10.1 ± 0.2	10.4 ± 0.2	-		6.9 ± 0.1

Table 1 – E_{max} and pEC₅₀ values of fresh or 48 h incubated MCA with vehicle or 10 μM U0126. Values given for all agonist experiments and as mean ± s.e.m. E_{max}(1), E_{max}(2), pEC₅₀(1), and pEC₅₀(2) values given for 48 h organ culture of MCA 5-CT biphasic sigmoid curve. n.d. = not determined. ET-1: endothelin-1; S6c: sarafotoxin 6c; 5-CT: 5-carboxamidotryptamine.