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Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries

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1 This study examines the importance of mitogen-activated protein kinases (MAPKs) in upregulation of endothelin type B (ET_B) receptors.

2 Rat middle cerebral arteries (MCAs) were incubated for 24 h with or without kinase inhibitors. Vessel segments were mounted in myographs and the contractile responses to endothelin-1 (ET-1; ET_A and ET_B receptor agonist) and sarafotoxin 6c (S6c; ET_B receptor agonist) were studied. We used real-time PCR to measure the receptor mRNA levels. An ELISA assay showed the activation of ERK1/2 kinases after 3 h. Immunohistochemistry revealed the presence of ET_B receptors on the vessels.

3 After organ culture, S6c induced vasoconstriction. Incubation with the MEK/ERK inhibitors U0126 and SB386023 diminished the contractile response to S6c. The p38 MAPK inhibitor SB239063 did not affect the S6c-induced contraction.

4 The ET-1-induced vasoconstriction was increased after incubation with SB386023 or SB239063, while unaffected by U0126.

5 The ET_B receptor mRNA levels were diminished by SB386023 and U0126. The ET_A receptor mRNA levels were unaffected.

6 The levels of activated ERK1/2 kinases were significantly higher after 3 h of organ culture as compared to fresh vessels.

7 The level of ET_B receptor protein on the smooth muscle cells of the MCA, visualised by immunohistochemistry, was somewhat diminished by SB386023.

8 Our results show that the ERK1/2 MAPK is important in the upregulation of contractile ET_B receptors in MCA after organ culture. Since there is a similar upregulation in models of focal ischaemia and subarachnoid haemorrhage, this may be an important pathophysiological event.
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Keywords: Endothelin; ET_B receptor; upregulation; MAPK; MCA; ERK1/2

Abbreviations: EF-1, elongation factor 1; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; ET_A receptor, endothelin type A receptor; ET_B receptor, endothelin type B receptor; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MCA, middle cerebral artery; MEK, MAP kinase/ERK kinase; PKC, protein kinase C; S6c, sarafotoxin 6c; SAH, subarachnoid haemorrhage

Introduction

Endothelin (ET) peptides are produced in the endothelium of cerebral vessels (Yanagisawa *et al*., 1988). They mediate their vasoactive effects through two different G protein-coupled receptors, the endothelin type A (ET_A) receptor and the endothelin type B (ET_B) receptor. The ET_A receptors are situated on the vascular smooth muscle cells, giving rise to strong contractions, while the ET_B receptors are mainly found on endothelial cells mediating vasodilatation *via* the release of nitric oxide (Masaki *et al*., 1994). However, we have observed a transcriptional and translational upregulation of contractile ET_B receptors in cerebral arteries in organ culture (Leseth *et al*., 1999), in experimental focal cerebral ischaemia (Stenman *et al*., 2002) and in subarachnoid haemorrhage (SAH) (Hansen-Schwartz *et al*., 2003). The intracellular pathways responsible for this upregulation remain unclear, but in the case of organ culture, protein kinase C (PKC) is involved (Henriksson *et al*., 2003).

The general aim is now to elucidate the role of different mitogen-activated protein kinase (MAPK) pathways in the upregulation of ET_B receptors after organ culture. MAPKs are a group of serine/threonine kinases, which are evolutionary well conserved in all eukaryotes. The MAPKs play a pivotal role in intracellular signalling in response to extracellular stimuli and may regulate cellular differentiation, proliferation and survival. There are three well-characterised MAPKs, all responding to various stimuli: the extracellular signal-regulated kinases (ERK) 1/2, the p38 and the c-jun N-terminal kinase (JNK) (Lewis *et al*., 1998). Each of the MAPK pathways includes an MAPK kinase kinase (MAPKKK) that on phosphorylation becomes activated and phosphorylates an MAPK kinase (MAPKK), which in turn phosphorylates the MAPK. The activated MAPK then phosphorylates various gene regulatory proteins, which can give rise to a variety of cellular responses.

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Activation of the ERK1/2 system is often initiated by growth factors binding to tyrosine kinase receptors on the cell surface. The intracellular signalling that follows leads to phosphorylation and activation of ERK1/2 (Boulton *et al*., 1991). p38 and JNK are the so-called stress-activated protein kinases (SAPKs), responding to different types of stress stimuli, for example, cytokines and UV radiation (Raingeaud *et al*., 1995).

Both ERK1/2 and p38 have been shown to play an important role in cerebral ischaemia, and the use of specific inhibitors of these MAPKs in experimental models of cerebral ischaemia have been shown to be neuroprotective (Alessandrini *et al*., 1999; Barone *et al*., 2001; Namura *et al*., 2001; Lennmyr *et al*., 2002).

The aim of the present study was to elucidate the role of ERK1/2 and p38 in the upregulation of contractile ET_B receptors in cerebral vessels following organ culture. Rat middle cerebral arteries (MCAs) were incubated with or without kinase inhibitors for 24 h. The vessels were examined using a myograph method where the contractile responses to endothelin-1 (ET-1; ET_A and ET_B receptor agonist) and sarafotoxin 6c (S6c; ET_B receptor agonist) were measured.

Our results point towards an important role for the ERK1/2 MAPK in the ET_B receptor upregulation following organ culture. The inhibitors selected to target different kinases in the cascade leading to ERK1/2 activation were U0126 and SB386023. U0126 is an inhibitor of the MAP kinase/ERK kinase (MEK) 1/2, the MAPKK of ERK1/2. SB386023 inhibits raf, the MAPKKK of ERK1/2. To inhibit the p38 MAPK, we used the specific inhibitor SB239063. Both inhibitors of the ERK1/2 pathway significantly reduced the ET_B receptor-dependent contractions of the MCA. The ET_B receptor mRNA levels were also decreased, as shown quantitatively by real-time PCR. To confirm the involvement of ERK1/2 MAPK in the receptor upregulation, we used ELISA to measure activated ERK1/2 in the vessels. After 3 h of organ culture, the levels of ERK1/2 were significantly elevated as compared to fresh samples. The p38 MAPK inhibitor SB239063 had no effect on the ET_B receptor-dependent contractions or the ET_B receptor mRNA levels. However, it gave rise to a stronger ET_A receptor response. Immunohistochemistry revealed positive ET_B receptor immunoreactivity in the media layer, thus agreeing with the upregulation of ET_B receptors seen after organ culture. This immunoreactivity had a tendency to be lower in the presence of the raf inhibitor SB386023.

Methods

Removal of cerebral vessels and organ culture

Male Wistar Hannover rats (350–400 g) were anaesthetised with CO_2 and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution (for composition, see below). The right and left MCAs were removed and dissected free from surrounding tissue.

The vessels were incubated for 24 h (or 3 h for ELISA experiments) at 37°C in humidified 5% CO_2 and air in Dulbecco's modified Eagle's medium supplemented with streptomycin ($100\,\mu g\,ml^{-1}$) and penicillin ($100\,U\,ml^{-1}$). Thereafter, vessels were mounted in myographs. For examination by real-time PCR or ELISA, vessels were snap-frozen at −80°C.

When using inhibitors, they were added to the medium before the incubation (U0126, 10 μM; SB386023, 10 μM; and SB239063, 10 μM).

Myograph experiments

A myograph was used for recording the isometric tension in isolated vessel segments (Mulvany & Halpern, 1977; Hogestatt *et al*., 1983). After incubation, the vessels were cut into cylindrical segments, approximately 0.5 mm long. The endothelium was removed mechanically by inserting a thin thread and rubbing the endothelium, and the removal was checked by monitoring responses to 10 μM acetylcholine after 5-HT precontraction.

The vessel segments were threaded on two 40-μm-diameter stainless steel wires and mounted in a Mulvany-Halpern myograph (Danish Myo Technology A/S, Denmark). One wire was connected to a force displacement transducer attached to an analogue–digital converter unit (ADInstruments, Hastings, U.K.). The other wire was attached to a movable displacement device allowing fine adjustments of vascular tension by varying the distance between the wires. The experiments were recorded on a computer by use of the software program Chart™ (ADInstruments, U.K.). The vessel segments were immersed in temperature-controlled (37°C) tissue baths containing a bicarbonate buffer solution of the following composition (mM): 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 solution was continuously gassed with 5% CO_2 in O_2, resulting in a pH of 7.4. The vessel segments were given an initial tension of 1.2 mN, and were adjusted to this level of tension for 1 h. The contractile capacity was determined by exposure to a potassium-rich (63.5 mM) buffer solution with the same composition as the bicarbonate buffer solution except that NaCl was exchanged for KCl. Concentration–response curves for the agonists S6c and ET-1 were obtained by cumulative application of the substances (10^{-12}–$10^{-6.5}$ M). Following S6c administration, the ET_B receptors are desensitised, leaving only ET_A receptors to interact with ET-1 (Lodge *et al*., 1995).

Real-time PCR

Total cellular RNA was extracted using the FastRNA Kit Green (BIO 101, Carlsbad, CA, U.S.A.) following the suppliers' instructions. The resulting pellet was finally washed with ethanol, air-dried and redissolved in 50 μl diethylpyrocarbonate (DEPC)-treated water. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, U.S.A.) in a Perkin-Elmer DNA thermal cycler. First strand cDNA was synthesised from total RNA in a 40 μl reaction volume using random hexamers as primers. The reaction mixture was incubated at 25°C for 10 min, 42°C for 15 min, heated to 99°C for 5 min and chilled to 5°C for 5 min. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, U.S.A.) with the cDNA synthesised above as template in a 50 μl reaction volume. A no template control was included in all

experiments. The GeneAmp 5700 Sequence Detection System monitors the growth of DNA in real time using an optics and imaging system, *via* the binding of a fluorescent dye to double-stranded DNA. Specific primers for the rat ET_A and ET_B receptors were designed as follows:

ET_A receptor	
forward:	5′-ATTGCCCTCAGCGAACAC-3′
reverse:	5′-CAACCAAGCAGAAAGACGGTC-3′
ET_B receptor	
forward:	5′-GATACGACAACTTCCGCTCCA-3′
reverse:	5′-GTCCACGATGAGGACAATGAG-3′

Elongation factor-1 (EF-1) mRNA was used as a reference, since it is the product of a housekeeping gene, continuously expressed to a constant amount in cells. The EF-1 primers were designed as follows:

EF-1 forward:	5′-GCAAGCCCATGTGTGTTGAA-3′
reverse:	5′-TGATGACACCCACAGCAACTG-3′

The real-time PCR was carried out with the following profile: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles with 95°C for 15 s and 60°C for 1 min. To prove that the cDNA of EF-1 and the ET receptors were amplified with the same efficacy during real-time PCR, a standard curve was made where the C_T values were plotted against cDNA concentration based on the equation $C_T = (\lg(1+E))^{-1} \lg(\text{concentration})$, where E is the amplification efficiency with the optimal value of 1 (Stenman *et al*., 2002).

ELISA

The vessels were homogenised in Cell Extraction Buffer (BioSource International Inc., Camarillo, CA, U.S.A.) supplemented with 1 mM phenylmethanesulphonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma) by using the FastPrep FP 120 homogeniser (BIO 101, Carlsbad, CA, U.S.A.). The supernatant was collected after centrifugation at $12{,}000 \times g$ for 20 min.

Total amount of phosphorylated ERK1/2 was measured using the BioSource International Inc. ERK1/2 [pTpY185/187] ELISA kit (BioSource International Inc., Camarillo, CA, U.S.A.) according to the suppliers' instructions. This kit is designed to detect and quantify the level of both dual-phosphorylated ERK2 at threonine 185 and tyrosine 187 and ERK1 at threonine 202 and tyrosine 204. A monoclonal antibody specific for ERK1/2 (both phosphorylated and unphosphorylated) has been coated onto the walls of microtitre wells. Samples are pipetted into these wells, and the ERK1/2 antigens bind to the antibodies. After washing, an antibody specific for phosphorylated ERK1/2 is added and binds to the immobilised ERK1/2 proteins in the wells. Then a horseradish peroxidase-labelled anti-rabbit IgG is added, binding to the second antibody. Finally, a substrate solution is added, which the enzyme uses to produce colour. The intensity of the colour is proportional to the amount of phosphorylated ERK1/2 in the sample. This is measured with a microtitre plate reader at 450 nm.

To quantify the activated ERK1/2 levels, we measured total protein content in each sample and used the (ERK1/2)/protein ratio. The protein was measured by the Folin phenol reagent after alkaline copper treatment (the Lowry method) (Diecke & Beyer-Mears, 1997).

Immunohistochemistry

Fresh artery segments and segments cultured for 24 h with or without the raf inhibitor SB386023 (10 μM) were immersed overnight in a fixative consisting of 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2. After fixation, the specimens were rinsed in sucrose-enriched (10%) Tyrode solution. The MCA segments were serially sectioned in a cryostat at 15 μm thickness and mounted on chrome-alum-coated slides.

The sections were processed for immunohistochemical examination of ET_B receptor localisation using an indirect immunofluorescence method. Briefly, the cryostat sections were rinsed for 15 min in PBS (pH 7.2). They were then incubated with 5% swine serum in PBS for 30 min, followed by incubation with ET_B antibody (1 : 400, anti-human ET_B receptor raised in rabbit, IBL Co Ltd, Japan). The site of the antigen–antibody reaction was revealed by application of a secondary FITC-labelled antibody (1 : 80, swine anti-rabbit IgG, DAKO, Copenhagen, Denmark). In the control experiments, the primary antibody was omitted. Crossreaction with other unknown peptides or proteins sharing amino-acid sequences with the examined receptor cannot be excluded, and hence the product is referred to as immunoreactivity.

Drugs

Myograph experiments ET-1 and S6c (Auspep, Parkville, Australia) were dissolved in 0.9% saline with 0.1% bovine serum albumin. Acetylcholine and 5-HT (Sigma, St Louis, MI, U.S.A.) were dissolved in 0.9% saline. U0126, SB386023 and SB239063 (Sigma, St Louis, MI, U.S.A.) were dissolved in DMSO.

Real-time PCR Oligonucleotides and reagents for the PCR assay were purchased from Perkin-Elmer, Applied Biosystems, Foster City, CA, U.S.A.

ELISA Reagents were purchased from BioSource International Inc., Camarillo, CA, U.S.A., except PMSF and protease inhibitor cocktail (Sigma).

Calculations and statistics

Myograph experiments Contractile experiments were performed on vessels that were incubated for 24 h with or without kinase inhibitors. The E_{max} values refer to maximum contraction calculated as percentage of the contractile capacity of 63.5 mM K^+ and the pEC_{50} values refer to the negative logarithm of the molar concentration that produces half-maximum contraction. Data are expressed as mean values ± s.e.m. and n refers to number of vessel segments. There were 4–5 rats in each group with 1–4 vessel segments from each. Statistical analyses were performed using the nonparametric Kruskal–Wallis test together with Dunn's *post hoc* test, where $P < 0.05$ was considered significant.

Real-time PCR PCR experiments were performed on vessels incubated for 24 h either with or without inhibitors where *n* refers to number of vessels. The amount of ET_A and ET_B receptor mRNA was calculated as relative to the amount of EF-1 mRNA in the same sample by the formula $X_0/R_0 = 2^{CtR-CtX}$, where X_0 is the original amount of ET receptor mRNA, R_0 the original amount of EF-1 mRNA, CtR the C_T value for EF-1 and CtX the C_T value for the ET receptor. Statistical analyses were performed using the nonparametric Kruskal–Wallis test together with Dunn's *post hoc* test, where $P<0.05$ was considered significant.

ELISA The ELISA assay was performed on fresh vessels and vessels incubated for 3 h. The amount of activated ERK1/2 was adjusted to total protein content of the samples. The calculation was made according to (ERK 3 h $\pm\sigma_{ERK}$ 3 h)/(ERK 0 h $\pm\sigma_{ERK}$ 0 h) $= A \pm \sigma_A$, where σ represents s.d. values and A the normalised expression in vessels incubated for 3 h *versus* the fresh control vessels. The formula $\sigma_A = A^*\sqrt{((\sigma_X/X)^2 + (\sigma_y/Y)^2)}$ was used to calculate the change in s.d. The ERK1/2 value for fresh vessels was then defined as 100% and the value for the incubated vessels was adjusted accordingly.

Data are expressed as mean values of the (ERK1/2)/total protein ratio ± s.d. and *n* refers to number of rats. Statistical analyses were performed using Mann–Whitney's nonparametric test, where $P<0.05$ was considered significant.

Results

Myograph experiments

K^+-induced contractions did not differ significantly in the control vessels compared to the ones incubated with MAPK inhibitors (data not shown). In arteries cultured with the MEK1/2 inhibitor U0126 (10 μM) for 24 h, both the S6c-mediated maximum contraction and the EC_{50} value were significantly decreased compared to control ($P<0.05$; Figure 1, Table 1), while the ET-1 response did not differ (Table 1). The raf inhibitor SB386023 (10 μM) also decreased the response to S6c as compared to control ($P<0.001$; Figure 1, Table 1) while the p38 inhibitor SB239063 failed to affect the functional upregulation of ET_B receptors (Table 1). Both SB239063 and SB386023 enhanced the contractile response to ET-1, pointing to a possible functional upregulation of ET_A receptors ($P<0.001$, Table 1).

The same control was used for all experiments.

Previously, we have shown that S6c has no effect in fresh vessels (Henriksson *et al*., 2003).

Real-time PCR

The standard curves of each primer pair had almost similar slopes, indicating that the EF-1, ET_A and ET_B cDNAs were amplified with the same efficiency (data not shown). The values of each slope were close to 3.3, which means that the amplification efficiencies are almost optimal (E is very close to 1). In each PCR experiment, a no template control was included, and there were no signs of contaminating nucleic acids in those samples. The results from the real-time PCR showed diminished, although not significantly, levels of ET_B receptor mRNA relative to the amount of EF-1 mRNA in MCA after organ culture for 24 h with U0126 as compared to control vessels ($ET_B = 0.024 \pm 0.004$ for U0126 and 0.047 ± 0.007 for control, $P>0.05$; Figure 2), while the levels of ET_A remained unchanged ($ET_A = 0.006 \pm 0.001$ for U0126 and 0.005 ± 0.001 for control, $P>0.05$; Figure 2). SB386023 treatment resulted in significantly lower expression of ET_B receptor mRNA in MCA after 24 h of organ culture ($ET_B = 0.006 \pm 0.002$, $P<0.05$; Figure 2). SB239063 did not affect the levels of ET_B receptor mRNA ($ET_B = 0.046 \pm 0.011$; Figure 2). However, incubation with both SB386023 and SB239063 showed a tendency towards an upregulation of ET_A

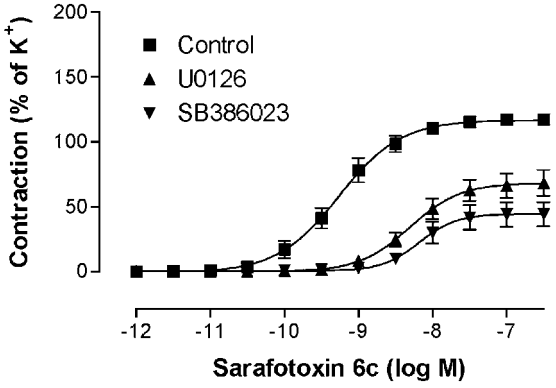

Figure 1 Contractile responses elicited by cumulative application of S6c in rat MCA incubated for 24 h (control) and rat MCA incubated for 24 h with the kinase inhibitors U0126 (10 μM) and SB386023 (10 μM). Each point represents mean values ± s.e.m., $n = 11$–15. For statistical analysis, see Table 1.

Table 1 Contractile effects of S6c and ET-1 in MCA

		S6c		*ET-1*	
	N	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Control	12	117 ± 3	9.30 ± 0.12	110 ± 5	8.45 ± 0.12
U0126	11	68 ± 10*	8.21 ± 0.18**	144 ± 9	7.97 ± 0.15
SB386023	13	44 ± 9***	8.00 ± 0.15***	254 ± 28***	7.58 ± 0.16**
SB239063	15	145 ± 18	8.52 ± 0.22*	227 ± 33***	8.17 ± 0.20

Control represents vessels incubated without inhibitors. Responses to S6c and ET-1 respectively are expressed as E_{max} in per cent of 63.5 mM K^+-induced contraction, and in pEC_{50} values (negative logarithm of the molar concentration that produces half-maximum contraction). *n* represents the number of vessel segments and each value is expressed as mean ± s.e.m. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to control vessels. Statistical analyses were performed using Kruskal–Wallis test together with Dunn's *post hoc* test.

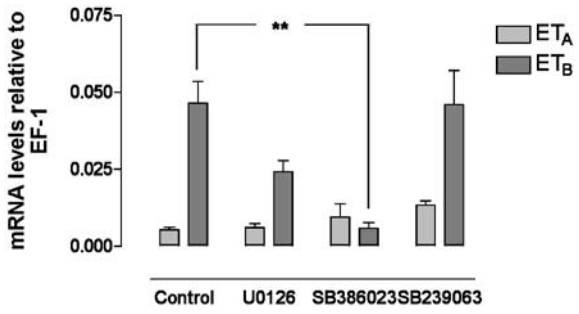

Figure 2 Effect of the kinase inhibitors U0126 (10 μM), SB386023 (10 μM) and SB239063 (10 μM) on the mRNA levels of the ET_A and ET_B receptors after 24 h of organ culture. Data were obtained by real-time PCR and the results are expressed as mean values ± s.e.m. relative to EF-1 mRNA levels, $n = 3$–7. $^{**}P < 0.01$.

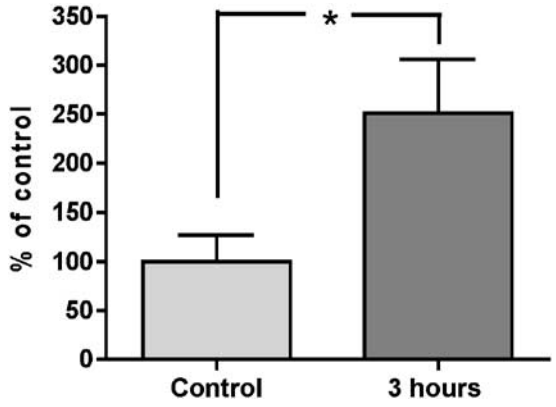

Figure 3 Levels of activated ERK1/2 in fresh vessels and in vessels incubated for 3 h. Data are expressed as per cent of control, $n = 4$. $^{*}P < 0.05$.

receptor mRNA ($ET_A = 0.009 \pm 0.004$ for SB386023 and 0.013 ± 0.001 for SB239063; Figure 2).

ELISA

The protein levels of activated ERK1/2 in the MCAs were significantly increased after 3 h of incubation as compared to fresh vessels (251% of control, $P < 0.05$; Figure 3).

Immunohistochemistry

The fresh MCA segments showed a positive ET_B receptor immunoreactivity localised over the smooth muscle cell medial layer and a faint immunoreactivity over the endothelium (Figure 4a). The immunoreactivity was somewhat more pronounced in artery segments incubated for 24 h and appeared fainter in vessels incubated for 24 h with the raf inhibitor SB386023 (Figure 4b and c).

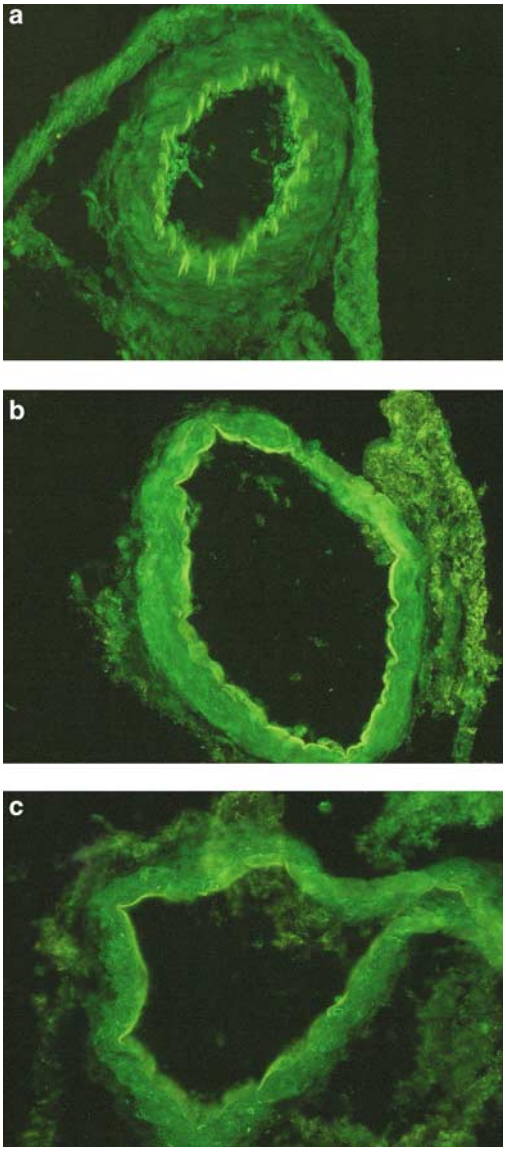

Figure 4 Sections from the rat MCA showing ET_B immunoreactivity in the smooth muscle cell layer in (a) fresh segments, (b) after organ culture and (c) after organ culture in the presence of the raf inhibitor SB386023 (10 μM).

Discussion

Following organ culture of cerebral arteries, there are changes in receptor expression on the vascular smooth muscle cells. Experiments have revealed that there is a time-dependent upregulation of contractile ET_B receptors and that PKC is involved in the intracellular mechanisms leading to this upregulation (Henriksson *et al*., 2003). The present study aimed to further investigate the intracellular pathways involved and more specifically the possible involvement of MAPK in this ET_B receptor upregulation.

We found that the inhibitors U0126 and SB386023 decreased the organ culture-induced ET_B receptor upregulation. U0126, which is a noncompetitive inhibitor with respect to the MEK substrates (Favata *et al*., 1998), blocks the enzymatic activity of MEK1/2 and subsequently inhibits the activation of ERK1/2. SB386023 inhibits the MAPKKK upstream of MEK, that is, the raf family (Yue *et al*., 2000). Raf binds to and activates MEK, and no other MAPKK, which makes it specific for the ERK pathway (Schaeffer & Weber, 1999). We also demonstrated the rapid activation of ERK1/2 in cultured vessels, with a 2.5-fold increase after 3 h.

Immunohistochemistry revealed positive ET_B receptor immunoreactivity over the media layer, thus agreeing with upregulation of the ET_B receptor seen after organ culture. Additionally, there was a tendency to a fainter immunoreactivity after incubation with the raf inhibitor SB386023. The method is not quantitative and hence only points towards the localisation of the upregulated receptors, but it still provides support to the functional results.

Thus, the results of this study point to the crucial role of the ERK1/2 MAPK in the ET_B receptor upregulation after organ culture. The ET_B receptor upregulation is of interest as there is a similar ET_B receptor change in both experimental focal ischaemia and SAH, along with increased levels of ET_B receptor mRNA (Stenman *et al*., 2002; Hansen-Schwartz *et al*., 2003).

In the course of a cerebral ischaemic event of a thromboembolic type, the initial damage results in a central core of ischaemia where the neurons die (Matsumoto *et al*., 2002). Subsequently, the region of damage spreads and a penumbral zone develops, an area that putatively can be rescued by neuroprotective agents. However, clinical studies have so far failed to provide any effective treatment for protection of this area (Davis *et al*., 2000; Lees & Diener, 2002).

The involvement of MAPK in the event of an ischaemic stroke in rat is well known. Both the ERK1/2 and the p38 pathways are important concerning size of the neuronal damage (Alessandrini *et al*., 1999; Barone *et al*., 2001; Namura *et al*., 2001; Lennmyr *et al*., 2002). However, this study shows that in the case of organ culture it is the ERK1/2 of the MAPKs that is of importance in the vascular ET_B receptor upregulation, while the p38 seems to be without effect. This correlates with the previous findings that PKC is involved in the ET_B receptor upregulation induced by organ culture (Henriksson *et al*., 2003), as PKC is known to activate the MEK/ERK pathway at several levels (Schonwasser *et al*., 1998). This involvement of ERK1/2 in ET_B receptor upregulation induced by organ culture has also been observed in mesenteric arteries (Uddman *et al*., 2003).

However, these results do not rule out the possibility that the conditions may be different in the case of ET_B receptor upregulation in cerebral ischaemia, or that the p38 might contribute to the neuronal damage in a different manner.

Interestingly enough, the contractile response of the ET_A receptors towards ET-1 application is enhanced in vessels incubated with SB386023 and SB239063 as compared to control. This phenomenon is not confirmed by the real-time PCR experiments, where the expression of ET_A receptor mRNA is virtually the same in all cases. An explanation to this discrepancy could be that the mRNA levels were elevated at a previous time point and have returned to base level after 24 h. Thus, if SB386023 while decreasing the ET_B responses also increases responses of the ET_A receptors, the overall result of application of this inhibitor may be negative from a therapeutic point of view. This must be taken into account in further experiments.

The initiation of the upregulation is still not known but could be related to changes in flow/pressure, for example, shear stress, activating tyrosine kinase receptors or seven transmembrane receptors. They will in turn activate PKC (Yang & Kazanietz, 2003) and subsequently the ERK1/2 MAPK pathway. The present study and a recent study by Uddman *et al*. (2003) support this theory.

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