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Vascular endothelin ET_B receptor-mediated contraction requires phosphorylation of ERK1/2 proteins

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Abstract

In cardiovascular diseases, endothelin type B (ET_B) receptors in arterial smooth muscle cells are upregulated. The present study revealed that organ culture of rat mesenteric artery segments enhanced endothelin ET_B receptor-mediated contraction paralleled with increase in the receptor mRNA and protein expressions. The endothelin ET_B receptor-mediated contraction was associated with increase in phosphorylation of extracellular regulation kinase 1 and 2 (ERK1/2) proteins and elevated levels of intracellular calcium. The elevation curve of intracellular calcium consisted of two phases: one rapid and one sustained. Inhibition of ERK1/2 phosphorylation by SB386023 or blockage of calcium channels by nifedipine significantly reduced the endothelin ET_B receptor-mediated contraction ($P<0.05$) and decreased the sustained phase of intracellular calcium level, but not the rapid phase. Thus, phosphorylation of ERK1/2 proteins and elevation of intracellular calcium level are required for endothelin ET_B receptor-mediated contraction in rat mesenteric artery.
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Keywords: Endothelin; ET_B (endothelin type B); Receptor; ERK1/2 (extracellular regulation kinase 1 and 2); Calcium; Contraction

1. Introduction

Endothelin-1 is a strong vasoconstrictor with important role in cardiovascular regulation and atherosclerotic processes. endothelin-1 acts on two G-protein-coupled receptors; endothelin type A (ET_A) and type B (ET_B) receptor (Masaki, 1994). The endothelin ET_A receptor is located on vascular smooth muscle cells and mediates contraction, whereas the endothelin ET_B receptor usually is found on the endothelium and induces dilatation via release of nitric oxide (NO) and prostacyclin (Schiffrin, 1995; Szok et al., 2001). Contractile endothelin ET_B receptors are upregulated in vascular smooth muscle cells during organ culture (Adner et al., 1998a,b) and in pathological conditions, e.g. in cerebral ischemia (Stenman et al., 2002) and in coronary ischemia (Wackenfors et al., 2004).

Increased endothelin ET_B receptor-mediated contraction has been observed in hypertension, myocardial infarction, congestive heart failure and cerebral ischemia (Abassi et al., 2004; Roux et al., 1995; Siren et al., 2000; Alafaci et al., 1990). We have observed that the upregulation of endothelin ET_B receptors can be mimicked by organ culture of arterial segments (Moller et al., 2002; Uddman et al., 2003; Adner et al., 1998a). It is well documented that in fresh mesenteric arteries of rat, there is no contractile response to sarafotoxin 6c, a selective endothelin ET_B receptor agonist (Adner et al., 1998a). However, after organ culture of mesenteric artery segments, an upregulation of endothelin ET_B, but not ET_A receptor, is induced via a transcriptional mechanism and the de novo expressed endothelin ET_B receptors mediate strong contraction (Adner et al., 1998a,b; Moller et al., 2002). While we have, in detail, studied the transcriptional mechanisms that are involved in the enhanced expression of endothelin ET_B receptors, there is limited knowledge regarding the intracellular events that take place during contraction via these de novo expressed endothelin ET_B receptors.

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Protein kinase C (PKC), extracellular signal-regulated protein kinase 1/2 (ERK 1/2) and intracellular calcium have been revealed to be involved in endothelin-1-induced contraction (Kwon et al., 2003). However, the intracellular events that take place during contraction induced via vascular endothelin ET_B receptors are less known. The present study was designed to examine if phosphorylation of ERK1/2 proteins and increase of intracellular calcium level are required for contraction induced via endothelin ET_B receptors in rat mesenteric artery.

2. Materials and methods

2.1. Tissue preparation and organ culture procedure

Sprague-Dawley rats (body weight 250–300 g) were anaesthetized with dried ice of CO_2 and killed by decapitation. The superior mesenteric artery (0.5–1 mm in diameter) was removed gently, immersed in cold buffer solution (for composition, see below) and dissected free of adhering tissue under a light microscope. The endothelium was denuded by perfusion of the vessel for 10 s with 0.1% Triton X-100 and followed by a physiologic buffer solution. The vessels were then cut into 1-mm-long cylindrical segments, used directly (fresh group) or incubated for 24 h at 37 °C in humidified 5% CO_2 in air (organ culture group). The segments for organ culture were placed in a 96-well plate, one segment in each well, containing 300 μl Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The reagent SB386023 (a generous gift from Dr. A. A. Parsons, GSK, UK), a specific ERK1/2 inhibitor (Uddman et al., 2003), was dissolved into dimethylsulfoxide (DMSO), and SB386023 or DMSO were diluted to needed concentration with the DMEM solution. The protocol was approved by the Ethical Committee of Lund University (M 120-01).

2.2. In vitro pharmacology

Fresh or incubated segments were immersed in temperature-controlled (37 °C) tissue baths containing a bicarbonate buffer solution. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. Each segment was mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer (Grass Instr., Quincy, USA) connected to a PowerLab (AD Instruments, Hastings, UK) unit for continuous recording of isometric tension (Adner et al., 1998b). A tension about 2.5 mN was applied to each segment and the segments were allowed to stabilize at this tension at least one hour before being exposed to a potassium-rich (60 mM) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the segments were used only if potassium elicited reproducible responses (i.e. two times of potassium-induced contractions are not different by more than 10%) over 1.0 mN. Concentration–response curves for the vasoconstrictor sarafotoxin 6c were obtained by cumulative administration of the agents. Specific ERK1/2 inhibitor SB386023 was added into the tissue bath 30 min before administration of sarafotoxin 6c concentration–response curves. To investigate phosphorylation of ERK1/2 proteins in the arterial segments during sarafotoxin-induced contraction, the segments were removed from the prongs just as sarafotoxin 6c (10^{-6} M and 10^{-8} M)-induced contraction reached the maximum. They were then stored at −80 °C for further analysis of ERK1/2 phosphorylation (Henriksson et al., 2004).

2.3. Assay of phosphorylated ERK1/2

Smooth muscle isolated from fresh or cultured segments was homogenized in 0.5 ml of extraction buffer (BioSource International, Inc. CA, USA) supplied with fresh 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) and protease inhibitor cocktail (Sigma) by using the FastPrep® instrument (Q-BIOgene, CA, USA). Measurement of phosphorylated ERK1/2 was performed by using a PhosphoELISAs assay kit (BioSource International, Inc. CA, USA) following the instruction from the supplier. Briefly, the sample was boiled for 5 min to denature proteins. The 100-μl sample was added in each pre-coated well and incubated for 2 h at room temperature. Removal of the sample by aspiration and wash with working wash buffer (supplied in the kit) and then addition of 100 μl of primary antibody for phosphorylated ERK1/2 to each well and incubated at room temperature for 1 h. Thereafter, the primary antibody was removed by aspiration, washed away and 100 μl of anti-Rabbit IgG–HRP conjugated secondary antibody added to each well and incubated for 30 min at room temperature. The second antibody was removed and 100 μl of stabilized chromogen was added to each well. The plate was incubation at room temperature for 30 min in darkness to develop color. Stop solution 100 μl was added to each well to stop the reaction. The plate was read at an absorbance of 450 nm. The outcome was normalized by the analysis of total protein (Lowry's method).

2.4. Fura 2 loading and measurement of force development

Mesenteric artery segments (1 mm) were mounted on two 40-μm stainless steel wires connected to a force transducer in the organ bath of a Confocal Wire Myograph, specially designed for obtaining high resolution image of fluorescence dyes (DMT120CW, Danish Myo Technology A/S, Aarhus, Denmark). After mounting, the arteries were equilibrated in PSS at 37 °C, pH 7.4, for 30 min. Mesentric artery segments were loaded with the fluorescent Ca^{2+} indicator dye (Fura 2) by incubation in PSS containing 20 μM Fura 2-AM (an acetoxymethyl ester form of Fura 2; Sigma), 0.5% (v/v) DMSO (Sigma), and 0.08% (v/v) pluronic F-127 (Sigma). Pluronic F-127 (non-ionic detergents) has been reported to improve the efficiency when loading with Fura 2-AM (Roe et al., 1990).The arteries were loaded for 2 h at 37 °C. After loading with Fura 2, physiological salt solution (PSS) that exchanged NaCl to KCl (KPSS) induced tension development with the same time course as observed before loading. KPSS-induced contraction was followed by rinsing, and the buffer was changed to PSS with nifedipine (Sigma), DMSO, or SB386023. Thereafter, sarafotoxin 6c (5×10^{-7} M) was added to induce contraction.

2.5. Measurement of $[Ca^{2+}]_i$

The myograph was placed on an inverted microscope (Olympus IX70, USA) for measurement of intracellular calcium $[Ca^{2+}]_i$. The arteries were illuminated with 340 and 380 nm light from SpectraMASTER (Life Science Resource, USA). During the experiments, fluorescence signals and force signals were captured by charge coupled devices with a camera head (CCD, Life Science Resource, OLYMPUS EUROPE, UK), and saved by a PC computer. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the equation; $[Ca^{2+}]_i = K_d\beta[(R-R_{min})/(R_{max}-R)]$. The dissociation constant, K_d, of the Fura 2–Ca^{2+} complex, is 225 nM at 37 °C (Grynkiewicz et al., 1985). R_{max} and R_{min} are the highest and the lowest concentration of calcium that each segment had in the experiments. R_{max} was determined in each vessel at the end of the experiment by adding a buffer-solution containing 10 mM Ca^{2+}. R_{min} was determined by incubation with the Ca^{2+}-free buffer plus 2 mM EGTA and 20 μM ionomycin (Sigma) for 10 min. The parameter β is the ratio of emission at 380 nm of R_{min} to emission at 380 nm of R_{max} (corrected for background fluorescence signals). The ratio (R) was calculated by dividing the emission at 340 nm illuminations and emission at 380 nm illuminations. Background fluorescence signals were obtained by quenching the calcium-sensitive Fura 2 fluorescence with 20 mM Mn^{2+} at the end of each experiment (Jensen et al., 1992; Grynkiewicz et al., 1985; Roe et al., 1990).

2.6. Western blot

After organ culture, whole vessels were collected and placed on ice, homogenized in lysis-buffer with protease and phosphatase inhibitors (10 mM Tris pH 7.4, 50 mM β-glycerophosphate, 100 μM Na_3VO_4, 0.5% deoxycholate, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 1 mM DTT, 20 μM pepstatin, 20 μM leupeptin, 0.1 U/ml aprotinin, 1 nM calyculin and 1 mM PMSF). Total protein concentration was determined using a BioRad DC kit (Hercules, CA, USA) and a Genesys 6 spectrophotometer (Thermo, Waltham, MA, USA). Same amount of 45 μg total protein was loaded per lane on a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and blotted onto a Hybond Poly-VinyliDene Fluoride (PVDF) membrane for 30 min at 1.5 mA/cm^2. Subsequently the membrane was blocked in 5% non-fat milk for 1 h at room temperature and incubated with primary antibody overnight at 4 °C and with secondary antibody (Pierce, Rockford, IL, USA) for 1 h at room temperature. The membranes were developed using the Supersignal Dura kit (Pierce) and visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Stamford, CT, USA). The endothelin ET_B antibody (Chemicon, Temecula, CA, USA) was used at 1 : 1000.

2.7. Real-time polymerase chain reaction (real-time PCR)

Smooth muscle isolated from fresh or cultured segments was homogenated in 1 ml of the RNApro™ solution (Q-BIOgene) by using a FastPrep® instrument (Q-BIOgene). The total RNA was extracted following a protocol from the FastRNA® Pro kit supplier. Reverse transcription of total RNA to cDNA was carried out using the Gene Amp RT kit (PE Applied Biosystems) in a Perkin-Elmer 2400 PCR machine at 42 °C for 30 min. The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit (PE Applied Biosystems) in a Perkin-Elmer Real-time PCR machine (PE, GeneAmp 5700 sequence detection system). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Specific primers for rat endothelin ET_B receptor was designed based on gene bank data by using Primer Express 2.0 software (PE Applied Biosystems).

Endothelin ET_B receptor primers
Forward: 5′–GATACGACAACTTCCGCTCCA–3′
Reverse: 5′–GTCCACGATGAGGACAATGAG–3′

The house keeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA continuously expressed to a constant amount in the arterial smooth muscle, was compared with the house keeping gene elongation factor-1 (EF-1) in a pilot study by real-time PCR. GAPDH was used as a reference in this study, but both were equally well constant in the tests.

GAPDH primers
Forward: 5′–GGCCTTCCGTGTTCCTACC–3′
Reverse: 5′–CGGCATGTCAGATCCACAAC–3′

The PCR reaction was performed in a 50 μl volume and started at a temperature of 50 °C for 2 min, 95 °C for 10 min and the following 40 PCR cycles with 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were run after the real-time PCR to identify the specific PCR products. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by TAG Copenhagen A/S (Denmark).

Data were analyzed with the comparative cycle threshold (C_T) method. To evaluate the amount of endothelin ET_B receptor mRNA in a sample, GAPDH mRNA was assessed in the same sample simultaneously. The C_T values of 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 C_T values of endothelin ET_B receptor mRNA in relation to the C_T values of GAPDH mRNA in the sample.

2.8. Buffer solutions and drugs

Standard buffer solution (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, MgCl 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5, and glucose 5.5. Analytical grade chemicals and double distilled water were used for preparing all solutions. Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Gibco BRL (Paisley, Scotland) and HEPES from Sigma. Nifedipine, ionomycin, Fura 2-AM and SB386023 were dissolved in DMSO. Sarafotoxin 6c and endothelin-1 (Auspep, Parkville, Australia) were dissolved in sterile water with bovine serum albumin (0.1% w/v) for in vitro pharmacology, or in

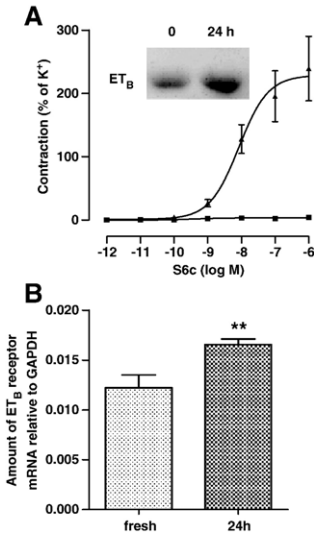

Fig. 1. (A) Effects of sarafotoxin 6c (S6c) on fresh (■) and 24 h organ cultured (▲) rat mesenteric artery segments. Each data point was derived from 9 to 10 experiments and represented as percentage of 60 mM KCl-induced maximal contraction with mean±S.E.M. Western blot by using the specific antibody against endothelin ET_B receptors is shown in both fresh (0 h) and 24 h organ cultured (24 h). The Western blot analysis has been repeated 4–5 times and produced a similar result as shown here. (B) Effects of organ culture on the expression of mRNA for endothelin ET_B receptors. The smooth muscle was isolated from the fresh or organ cultured arteries. The relative amount of mRNA for endothelin ET_B receptors was quantified by the real-time PCR. Each data point was derived from 4 experiments and presented as mean±S.E.M. $^{**}P<0.01$ compared with fresh.

0.9% saline with bovine serum albumin (10% w/v) for intracellular calcium studies. PSS had the following composition (in mM): NaCl 125, $NaHCO_3$ 5, KCl 5, NaH_2PO_4 0.5, $MgCl_2{\cdot}6H_2O$ 2, $CaCl_2$ 1.8, bovine serum albumin (BSA) 0.05, and glucose 10. The pH was adjusted to 7.4 with NaOH. Solutions used for determination of R_{min} and R_{max} contained (in mM): 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) 5, KCl 125, $MgCl_2{\cdot}6H_2O$ 1.17 and glucose 5.5, with addition of 2 mM EGTA or 10 mM $CaCl_2{\cdot}2H_2O$, respectively and pH was adjusted to 7.4 with KOH.

2.9. Calculation and statistics

Data are expressed as mean values±S.E.M. Contractile responses in each segment are expressed as a percentage of the 60 mM potassium-induced contraction. E_{max} represents the maximal contraction induced by an agonist. The pEC_{50} value was calculated from the line between the concentrations above and below the midpoint of the concentration–response curve. Student's *t*-test was applied and $P<0.05$ considered as a significant difference.

3. Results

3.1. Upregulation of endothelin ET_B receptors

The selective endothelin ET_B receptor agonist, sarafotoxin 6c, did not induce any contraction in fresh segments of rat mesenteric artery, while after organ culture of the artery segments for 24 h; it induced strong contraction with an E_{max} of 239±26% and a pEC_{50} of 7.98±0.07 (Fig. 1A). This increase of contraction induced via endothelin ET_B receptors occurred in parallel with a significant increase of the endothelin ET_B receptor mRNA level in the smooth muscle cells ($P<0.01$, Fig. 1B). Western blot using a specific endothelin ET_B receptor antibody confirmed the receptor upregulation at the protein level (Fig. 1A).

3.2. Effect of ERK1/2 inhibitor and calcium channel blocker

SB386023 (10^{-5} M) or nifedipine (10^{-6} M) was added 30 min before administration of sarafotoxin 6c to induce concentration–response curves in organ cultured artery segments. SB386023 significantly reduced the maximum contraction of sarafotoxin 6c (Fig. 2A). The E_{max} decreased from 239±26% ($n=9$) to 89±13% ($n=8$) ($P<0.01$), while the pEC_{50} did not show a significant change (7.98±0.07 vs. 7.89±0.11, $P>0.05$). Nifedipine decreased the maximal contraction induced by sarafotoxin 6c from 205±27% ($n=8$) to 106±18% ($n=11$) ($P<0.01$) with no significant change in the pEC_{50} value (8.12±0.02 vs. 7.92±0.03, $P>0.05$). Incubation of the artery rings in a combination of SB386023 and nifedipine for 30 min further decreased the contraction to an E_{max} of 41±6% ($P<0.05$; Fig. 2B, Table 1).

To further study the inhibitory effect of SB386023, the ERK1/2 pathway inhibitor was added during a steady-state contraction induced by sarafotoxin 6c. Sarafotoxin 6c (10^{-8} M)

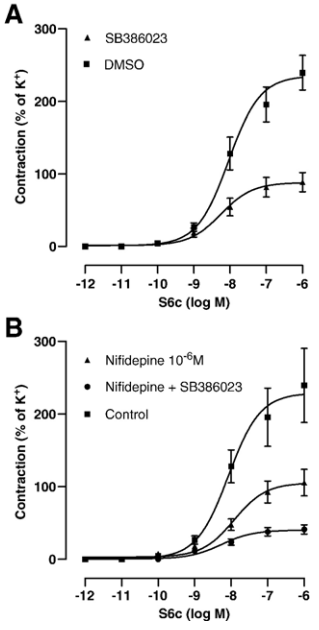

Fig. 2. (A) Sarafotoxin 6c (S6c)-induced contraction curves in the absence (■) or presence (▲) of SB386023 (10^{-5} M). SB386023 was added 30 min before the administration of sarafotoxin 6c to segments cultured for 24 h. DMSO served as control ($n=9$–10). (B) Sarafotoxin 6c (S6c)-induced contraction curves in the absence (■) or presence (▲) of nifedipine (10^{-6} M) or combination SB386023 (10^{-5} M) plus nifedipine (10^{-6} M) (●). The experiments ($n=8$–11) were performed as in (A). Each data point is shown as mean±S.E.M.

Table 1
Effects of SB386023 or nifedipine or the combination on contraction induced by sarafotoxin 6c via endothelin ET_B receptors

	N	K^+ mean	Sarafotoxin 6c		
			E_{max} (mN)	E_{max} (%)	pEC_{50}
Fresh	9	2.15±0.35	0	0	0
Organ culture	9	2.58±0.28	4.64±0.54	239±26	7.98±0.07
with SB386023	10	2.84±0.39	3.14±0.33	89±13 [a]	7.89±0.11
with nifedipine	8	2.43±0.31	3.76±0.42	106±18 [b]	7.92±0.03
with SB386023+ nifedipine	8	2.19±0.25	3.83±0.39	41±6	8.26±0.04
with Control (DMSO)	11	2.38±0.27	4.12±0.47	205±27	8.12±0.02

Data are shown as mean±S.E.M. *N* denotes the number of experiments.
[a] $P<0.01$ vs. Control (DMSO).
[b] $P<0.01$ vs. Organ culture.

elicited an immediate contraction which reached a stable contraction within 1 min and the tone remained stable for more than 10 min. Addition of SB386023 (10^{-5} M or 10^{-4} M) resulted in a time- and concentration-dependent relaxation. The sarafotoxin 6c contraction curves began to decline within 1 min, and it took 9 min for 10^{-5} M SB386023 and 3.5 min for 10^{-4} M SB386023 to reduce contraction by 50% (Fig. 3A). Nifedipine (10^{-6} M) instantly relaxed sarafotoxin 6c-induced contraction to a reduction in the E_{max} of approximately 50% of the maximal contraction within 1 min (Fig. 3B).

3.3. Intracellular calcium

Sarafotoxin 6c induced a contraction that occurred in parallel with a rise in the intracellular calcium level (Fig. 4A). The increase of intracellular calcium consisted of two phases: one phasic, considered due to calcium released from the sarcoplasmic reticulum (SR), and one tonic considered due to calcium influx of extracellular calcium via calcium channels. The ERK1/2 inhibitor, SB386023 (10^{-5} M), significantly decreased the Tonic but not the phasic phase (Fig. 4C). Nifedipine, a specific calcium influx inhibitor, behaved in a similar way; it blocked mainly the sustained tonic phase of the intracellular calcium level and not the initial phasic phase (Fig. 4B). However, during incubation with SB386023 for 30 min, the basic (resting) level of intracellular calcium in the artery segments (492±105 nM; $P<0.05$) was significantly higher than in DMSO (189±70 nM), a phenomenon not seen with nifedipine (108±42 nM) (Fig. 4D).

3.4. ERK1/2 phosphorylation during force development via endothelin ET_B receptors

In a separated series of experiments, the contraction induced by sarafotoxin 6c (10^{-6} M or 10^{-8} M) rapidly reached a stable state of contraction at 2 min ($n=8$) (Fig. 5A). At this time point, the vessel segments were quickly removed from the tissue bath and immediately frozen for examination of the level of phosphorylated ERK1/2 proteins. The results revealed a significant rise in phosphorylated ERK1/2 proteins that occurred in parallel with the contraction (Fig. 5B).

4. Discussion

We have previously demonstrated that in vascular disease, upregulation of contractile endothelin ET_B receptor plays an important role (Wackenfors et al., 2004; Stenman et al., 2002) and this receptor upregulation can be mimicked by organ culture of arteries (Adner et al., 1998a). Since this altered receptor phenotype is seen in vascular disease, it is important to understand its dynamics of action, particularly if we are aiming to alleviate vascular sequelae due to receptor upregulation. The present study has for the first time shown that phosphorylation of ERK1/2 proteins and elevation of intracellular calcium levels both are associated with contraction induced via endothelin ET_B receptors. Activation of the contractile endothelin ET_B receptor by sarafotoxin 6c-induced contraction that occurred in parallel with an increase in intracellular calcium levels and phosphorylation of ERK1/2 proteins. Inhibition of ERK1/2 phosphorylation or blockage of calcium channel significantly reduced the endothelin ET_B receptor-mediated contraction. The increase of intracellular calcium induced via endothelin ET_B consists of two phases: one rapid and one sustained. We have previously shown that isolated arteries respond to high potassium solution with a biphasic contraction that consists of an initial fast partly transient contraction (first phase) and an ensuing more slowly developing contraction (second phase) (Hogestatt et al., 1983; Hogestatt and Andersson, 1984). Studies have subsequently shown that this is due to activation of different types of calcium channels; a rapid type and a second type that allows calcium to pass through the smooth muscle membrane at a slower rate

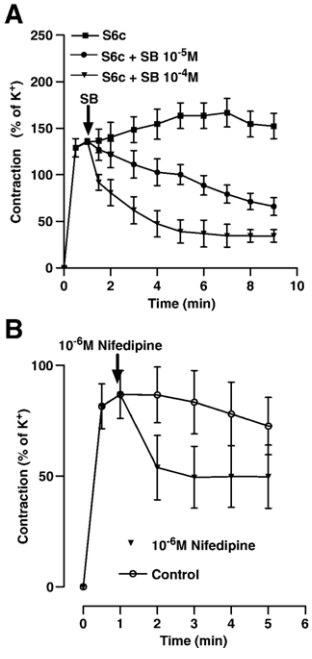

Fig. 3. (A) Time course of the sarafotoxin 6c (S6c)-induced contraction in absence (■) or in the presence of SB386023 (● 10^{-5} M and ▼ 10^{-4} M) were added when the stable contraction has been achieved. Each data point is shown as mean±S.E.M. (B) Time course of the sarafotoxin 6c-induced contraction in absence (●) or in the presence of nifedipine (10^{-6} M) (▼) were added when the stable contraction has been achieved. Each data point is shown as mean±S.E.M.

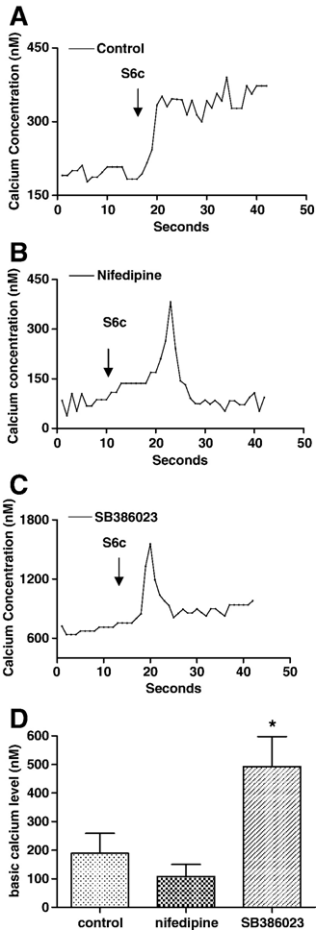

Fig. 4. Typical intracellular calcium concentration curves induced via endothelin ET_B receptors by sarafotoxin 6c in the artery segments in absence (A) or presence of nifedepine (10^{-6} M) (B) or SB386023 (10^{-5} M) (C). Basic (resting) levels of intracellular calcium concentration in the groups are shown in (D). The experiments (n=4–5) were performed as in Fig. 2 and in parallel with assessment of intracellular calcium concentration. Data points in (D) are presented as mean±S.E.M.

(Hogestatt and Andersson, 1984). The later type is typically sensitive to the dihydrophyridine type of calcium entering blocker, e.g. nifedipine. A similar bimodal appearance has been described for the thromboxane A_2 analogue U46619-induced contraction in porcine coronary artery (Nobe and Paul, 2001). The transient phase of contraction to U46619 was associated with Ca^{2+} release from the sarcoplasmic reticulum and a protein kinase C (PKC)-mediated Ca^{2+} release, while in the sustained phase, involves Ca^{2+} influx from the extracellular space and Rho-kinase-mediated Ca^{2+} sensitization (Nobe and Paul, 2001). The present study has revealed that the ERK1/2 inhibitor SB386023 (10^{-5} M) and the calcium influx inhibitor, nifedipine, significantly decreased the sustained contraction. Taken together, we have demonstrated that phosphorylation of ERK1/2 proteins and elevation of intracellular calcium levels are required for contraction induced via endothelin ET_B receptors in rat mesenteric artery and that both calcium-dependent and -independent mechanisms are involved (Ganitkevich et al., 2002; Thorne et al., 2004).

Previously, we have characterized alteration of endothelin receptors during organ culture of rat mesenteric artery (Adner et al., 1998a,b). The fresh arterial smooth muscle cells do not express functional endothelin ET_B receptors, although endothelin ET_B receptor mRNA and protein are present in significant levels (Fig. 1A and B). The lack of contractile ET_B receptors in fresh arterial segments is a general phenomenon that we have seen in mesenteric, cerebral and coronary arteries in rat and in man (Moller et al., 2002; Stenman et al., 2002; Wackenfors et al., 2004). Thus, ET_B receptors expressed in fresh are most likely relaxant ET_B receptors with effects mediated via NO and prostacyclin (Schiffrin, 1995; Szok et al., 2001). However, endothelin ET_B receptor-mediated contraction occurs following organ culture (Moller et al., 2002), in experimental animal models of stroke (Stenman et al., 2002) or subarachnoid hemorrhage (Hansen-Schwartz et al., 2003). This up-regulation of endothelin ET_B receptors has been further documented in vascular disease in humans, including atherosclerotic arteries (Dagassan et al., 1996; Pernow et al., 2000), ischaemic heart disease (Wackenfors et al., 2004) and in cerebrovascular diseases (Hansen-Schwartz et al., 2003). The mechanisms responsible for the upregulation of endothelin ET_B receptors involves gene transcription and include activation of protein kinase (Uddman et al., 2002) and mitogen-activated protein kinase (MAPK) pathways consist of ERK1/2 (Uddman et al., 2003). It has been observed that of MAPK and down stream transcriptional factors regulate the gene expression of G-protein-coupled receptors, such as endothelin ET_B, 5-hydroxytryptamine $(5\text{-HT})_{2A}$ and bradykinin B_1 and B_2 receptors in response to extracellular stimuli (Cao et al., 2005; Zhang et al., 2004, 2005). Typically, these events induce a contractile phenotype (Moller et al., 2002).

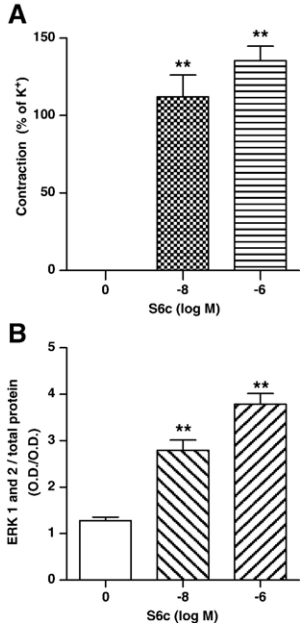

Fig. 5. Force development via endothelin ET_B receptors induced by sarafotoxin 6c (S6c) (10^{-6} M and 10^{-8} M) (A) and phosphorylation of ERK1/2 proteins (B). Each data point is derived from 8 experiments and shown as mean±S.E.M. **P<0.01 compared with control (without sarafotoxin 6c).

On the other hand, MAPK activities have been found to be required for contractions induced by angiotensin II and endothelin-1 (Ishihata et al., 2002; Kwon et al., 2003). In the present study, we focused on the role of MAPK and intracellular calcium to understand the process of contraction induced via endothelin ET_B receptors in rat mesenteric artery and demonstrated that activation of MAPK ERK1/2 is required for the contraction. This is supported by previous findings that contraction induced via G-protein-coupled receptors 5-HT_{2A} receptors in rat mesenteric artery requires ERK1/2 activities (Cao et al., 2005). Nifedipine, a selective calcium channel blocker markedly reduced sarafotoxin 6c-induced contraction. The increase of intracellular calcium induced via endothelin ET_B receptor activation consists of two phases: one rapid and one sustained. The rapid phase of calcium release induced by agonist via endothelin ET_B receptors, is supposed to occur via the inositol 1, 4, 5-triphosphate [Ins(1, 4, 5)P3]-mediated calcium release from the sarcoplasmic reticulum (Nobe and Paul, 2001). This was, however, not inhibited by nifedipine or SB386023. In arteries incubated with nifedipine or SB386023, the contraction and the level of intracellular calcium decreased after the rapid phase, while in control segments there was a step-wise increase of intracellular calcium levels with associated contraction. Surprisingly, when we compared the basic (resting) level in the arteries among control (DMSO), nifedipine and SB386023 treated groups, there was a higher basic level of intracellular calcium in the SB386023 treated group than in control. The mechanism behind this is not known, but it has been suggested that intracellular calcium belongs to two cytosolic compartments; contractile and non-contractile compartments (Abe et al., 1995). The increase in basal level of intracellular calcium induced by the ERK1/2 inhibitor was located in the non-contractile compartment and that is why it was dissociated with the contraction. The same phenomenon has been observed in the guinea pig taenia coli and porcine coronary artery (Thorne et al., 2001, 2004).

In conclusion, the present study has revealed that phosphorylation of ERK1/2 and increments in intracellular calcium levels are required for endothelin ET_B receptor-mediated contraction in rat mesenteric arteries. Both calcium-dependent and -independent mechanisms are involved.

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