Involvement of Protein Kinases on the Upregulation of Endothelin Receptors in Rat Basilar and Mesenteric Arteries

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Endothelin$_B$ (ET_B) receptors are upregulated in experimental stroke or after 24 hrs of organ culture. This upregulation is manifested both as stronger contraction and as an increase in ET_B receptor messenger RNA (mRNA) levels. The present study was designed to evaluate the importance of protein kinases (c-Jun N-terminal kinase [JNK], protein kinase C [PKC], and extracellular signal–regulated kinase [ERK1/2]) in ET_B receptor upregulation after organ culture. Rat basilar and mesenteric arteries were incubated for 24 hrs in Dulbecco's modified Eagle's medium (DMEM) with or without the PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; or the JNK inhibitor, SP600125, added 3, 6, or 12 hrs after initiation of incubation. Subsequently, vessel segments were mounted in myographs and the contractile responses to ET-1 and sarafotoxin 6c were studied. The ET_B and ET_A receptor mRNA levels were determined with a real-time polymerase chain reaction (PCR). The cellular localization and protein level of ET_B receptors were evaluated by immunohistochemistry. The PKC and ERK1/2 inhibitors attenuated the contraction induced by S6c in the basilar arteries more than in the mesenteric arteries. The efficiency of the inhibitors was proportional to the incubation time. Real-time PCR showed a decrease in the ET_B receptor mRNA levels in arteries treated with PKC or ERK inhibitors. The JNK inhibitor had a significant inhibitory effect on ET_B receptor upregulation in the basilar arteries. Immunohistochemistry revealed that the ET_B receptor upregulation occured in the smooth-muscle cells and that it had the same pattern as in the quantitative PCR. Our results show that the PKC, ERK1/2, and JNK are more important for the upregulation of contractile ET_B receptors in cerebral arteries compared with mesenteric arteries. ERK1/2 seems to be more important for the ET_B receptor upregulation, as compared with PKC and JNK. The evaluation of the time dependency suggests that the phenomenon can be reversed even after its initiation. Exp Biol Med 231:403–411, 2006

Key words: endothelin; sarafotoxin 6c; ET_B receptor; protein kinases

Introduction

Endothelin (ET)-1 is a vasoactive peptide produced in the endothelium of blood vessels and is one of the most potent contractile agonists known. Its actions are mediated by two G-protein–coupled receptors, the ET_A and the ET_B receptors (1). The ET_A receptor is a contractile receptor situated on the smooth-muscle cells of the vessels, whereas the ET_B receptor is mainly found on the endothelium, mediating dilation. In addition, there is a small population of contractile ET_B receptors expressed on the smooth-muscle cells in some arteries and veins (2).

It was previously revealed that contractile ET_B receptors on vascular smooth-muscle cells are upregulated *via de novo* transcription after organ culture (3, 4) and experimental focal cerebral ischemia (5). However, the intracellular signaling pathways responsible for this upregulation remain elusive. We have evidence for the involvement of protein kinase C (PKC) in the upregulation both in cerebral and mesenteric arteries after organ culture (6, 7). In addition, mitogen-activated protein kinases (MAPK) represents a group of serine/threonine kinases that play an important role in the intracellular signaling in response to extracellular stimuli (8). The different MAPKs are involved in such important cellular functions as proliferation, differentiation, and survival. There are three major MAPK pathways in mammals: the extracellular signal–regulated kinase (ERK1/2), the p38 and the c-Jun N-terminal kinase (JNK; Ref. 9). Of these, the ERK1/2 pathway is activated by mitogenic stimulation, whereas JNK and p38 are so-called stress-activated protein kinases (SAPK), activated by various stress stimuli, for example, cytokines (10). We have evidence for the involvement of ERK1/2 in the ET_B receptor upregulation after 24 hrs of incubation (11).

The aim of this study was to evaluate whether ET_B receptor upregulation can be attenuated by treatment with PKC or MAPK inhibitors after initiation of the upregulation

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procedure and to evaluate the time dependency. This information will hypothetically be essential in defining the therapeutic situations in which ET_B receptor upregulation can be modified.

Materials and Methods

Removal of Vessels and Organ Culture Procedure. Male Sprague-Dawley rats weighing 250 g (Scanbur, Stockholm, Sweden) housed two to a cage, were used for the study. The animals were anesthetized with CO_2 and killed by decapitation. The basilar and mesenteric arteries were isolated and placed in a cold buffer. One basilar artery (diameter ~0.5 mm) was divided in 4–6 ring segments. The endothelial layer of the basilar arteries was mechanically removed (using a thin steel thread). The successful removal was confirmed by monitoring relaxant responses to 10^{-5} *M* acetylcholine (Chemicon, Malmö, Sweden) after ET-1–induced precontraction. A distal branch of the rat superior mesenteric artery (outer diameter <1 mm) was chosen as the experimental target. The endothelium was denuded *via* perfusion of the vessel for 10 secs with 0.1% Triton X-100 (Sigma-Aldrich, Stockholm, Sweden) followed by a physiologic buffer solution. The arteries were removed, dissected free from adherent tissue, and cut into 1- to 2-mm-long circular segments. Vessels used for *in vitro* pharmacology were incubated for 24 hrs at 37°C in humidified 5% CO_2 and air in Dulbecco's modified Eagle's medium (DMEM; Gibco, Stockholm, Sweden) supplemented with 100 μg/ml penicillin (Sigma-Aldrich) and thereafter mounted in myographs, as described previously (12). Two agonists, ET-1 (an ET_A and ET_B receptor agonist) and sarafotoxin 6c (S6c; an ET_B receptor agonist) were studied. Contractile responses were obtained by cumulative administration of the agonists. By first studying S6c, the ET_B receptors were desensitized, and the subsequent administration of ET-1 resulted in specific activation of ET_A receptors (13). The stimulation with ET-1 in fresh or incubated arteries induced a long-lasting contraction that could not be washed out. The ET_B receptor stimulation, however, was transient and it was possible to wash out the contraction induced by S6c.

By first studying the S6c responses, we obtained a selective activation of ET_B receptors. However, this procedure desensitizes the ET_B receptors, and the subsequent application of ET-1 caused the selective activation of ET_A receptors (3, 12). When using inhibitors, the PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; and the JNK inhibitor, SP600125, were added 3, 6, or 12 hrs after initiation of the incubation. Thus, each segment was incubated with inhibitor for 21, 18, and 12 hrs, respectively, and a 24-hr incubation with dimethylsulfoxide (DMSO; Sigma-Aldrich) was the control. The Animal Ethics Committee, Lund University, Sweden, approved the experiments.

***In Vitro* Pharmacology.** Sensitive myographs were used for recording the isometric tension in isolated basilar artery segments. The basilar artery segments were threaded on two 40-μm-diameter stainless steel wires and mounted in Mulvany-Halpern myographs (Danish Myo Technology A/S, Aarhus, Denmark). One wire was connected to a force displacement transducer and attached to an analog-to-digital converter unit (AD Instruments, Hastings, UK). 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 PC computer using the software program, Chart (AD Instruments). The mesenteric artery segments were mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer (Grass Instruments, Quincy, MA) connected to a Power Lab unit (AD Instruments) for continuous recording of isometric tension. The segments were immersed in temperature-controlled (37°C) tissue baths containing physiologic salt solution buffer for basilar segments and standard buffer solution for mesenteric segments. A tension of 2.5 mN for mesenteric artery or 1.2 mN for basilar artery was applied to each segment, and the segments were allowed to stabilize at this tension for 1 hr before being exposed to a K^+-rich (63.5 m*M*) buffer solution (with the same composition as the standard solution, except that NaCl was replaced by an equimolar concentration of KCl). The choice of basal tone to be used was evaluated in detail in previous studies. The K^+-induced contraction was used as a reference for the contractile capacity. Concentration-response curves for the specific ET_B receptor agonist, S6c, were obtained by cumulative administration of the peptide. The vessels were washed and allowed to recover before ET-1 concentration-response curves were performed. The specific receptor stimulation was analyzed in detail before using selective antagonists (13; for further methodologic details, see Refs. 6 and 12).

Segments to be used for immunohistochemistry were cultured as explained above in "Removal of Vessels and Organ Culture Procedure" for 24 hrs with or without inhibitors added 6 hrs after initiation of incubation. All of the segments were rapidly frozen and subsequently stored at –80°C until use.

Molecular Biology. Segments to be used for polymerase chain reaction (PCR) were cultured for 24 hrs and the PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; or the JNK inhibitor, SP600125, were added after 3, 6, or 12 hrs after initiation of the incubation. Thus, each segment was incubated with inhibitors for 21, 18, or 12 hrs, respectively, and 24 hrs of incubation with DMSO was used as a control. Vessel segments were snap frozen at –80°C, and total cellular RNA was extracted using the FastRNA, Pro Green kit (Qbiogene, Illkersh, France) for 60 secs in the FastPrep FP120 instrument (Qbiogene), according to the suppliers' instructions. The resulting pellet was finally washed with 70% ethanol, air-dried, and redissolved in 50 μL diethyl-pyrocarbonate–treated water. Reverse transcription of total RNA to complementary DNA (cDNA) was carried out using the GeneAmp RNA PCR kit (Perkin-

Elmer Applied Biosystems, Foster City, CA) in a Perkin-Elmer DNA Thermal cycler. First-strand cDNA was synthesized from 200 ng total RNA in a 40-μL reaction volume using random hexamers as primers. The reaction mixture was incubated at 25°C for 10 mins, 48°C for 15 mins, heated to 95°C for 5 mins, and chilled to 5°C for 5 mins. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR-Green kit (Perkin-Elmer Applied Biosystems) with the cDNA synthesized above as the template in a 40-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 were designed as follows: ET_A receptor, forward: 5′-ATTGCCCTCAGC-GAACAC-3′, reverse: 5′-CAACCAAGCAGAAA-GACGGTC-3′; and ET_B receptor, forward: 5′-GATACGACAACTTCCGCTCCA-3′, reverse: 5′-GTCCACGATGAGGACAATGAG-3′.

The product of a housekeeping gene that is continuously expressed to a constant amount in cells, elongation factor-1 (EF-1) messenger RNA (mRNA), forward: 5′-GCAAGCCCATGTGTGTTGAA-3′, and reverse: 5′-TGATGACACCCACAGCAACTG-3′, was used as a reference. The real-time PCR was carried out with the following profile: 50°C for 2 mins, 95°C for 10 mins, followed by 40 cycles at 95°C for 15 secs and at 60°C for 1 min.

Immunohistochemistry. Segments were inserted in a block of Tissue TEK (Sakura Finetek Europe, Zoerterwoude, The Netherlands) and were frozen. The segments were cut into 8-μm-thick sections with a cryostat and stored at −80°C until use. The sections were fixed with 4% paraformaldehyde at room temperature for 15 mins. The segments were washed with phosphate-buffered saline (PBS) and incubated overnight with primary antibodies to ET_B receptor (rabbit anti-human ET_B, 1:400 dilution; IBL 16207; Immuno-Biological Laboratories, Gunma, Japan), ET_A receptor (goat anti-human ET_A, 1:100 dilution; sc-21194; Santa Cruz Biotechnologies, Santa Cruz, CA), and 10% fetal calf serum in PBS. The next day, they were washed and incubated with secondary antibodies, donkey anti-rabbit conjugated to Cy5, and donkey anti-goat conjugated to Cy2 (Jackson ImmunoResearch, Soham, Cambridgeshire, UK) for 2 hrs and observed using a Leica confocal microscope (Solms, Germany). As a control for the efficiency of antibodies, segments were incubated with only secondary antibody. All of the groups (control, PKC, ERK1/2, and JNK) for each vessel were examined at the same time. For each group, three rats were used and studied in four different sections.

Drugs and Solutions. The standard buffer solution used for mesenteric artery consisted of: 119 m*M* NaCl, 15 m*M* $NaHCO_3$, 4.6 m*M* KCl, 1.2 m*M* $MgCl_2$, 1.2 m*M* NaH_2PO_4, 1.5 m*M* $CaCl_2$, and 5.5 m*M* glucose. The physiologic salt solution buffer used for basilar artery had the following composition: 125 m*M* NaCl, 5 m*M* $NaHCO_3$, 5 m*M* KCl, 0.5 m*M* NaH_2PO_4, 2 m*M* $MgCl_2 \cdot 6H_2O$, 1.8 m*M* $CaCl_2$, 0.05 m*M* bovine serum albumin (BSA), and 10 m*M* glucose. The pH was adjusted to 7.4 with NaOH. Analytical grade chemicals and double-distilled water were used for preparing all solutions. ET-1 and S6c were purchased from Sigma and Alexis Biochemicals (Lausen, Switzerland), respectively, and dissolved in 0.9% saline with 10% BSA (Kabi, Stockholm, Sweden). RO-31–7549 (Calbiochem, Nottingham, UK), SB386023, and SP600125 (Sigma) were dissolved in DMSO.

Statistics. The E_{max} values refer to the maximum contraction calculated as percentage of the contractile capacity of potassium. Results are given as mean ± SEM, and *n* refers to the number of vessel segments. There were 4–6 rats in each group and 4–6 vessel segments from each rat. The pEC_{50} value is the negative logarithmic value of the concentration of the stimulator (S6c or ET-1) that is needed to produce half of the maximal contraction in our vessels. When the vessels were incubated with inhibitors, the pEC_{50} decreased, which means that higher concentrations of S6c are required to induce the same maximal contraction. This value was calculated from the line between the concentrations above and below the midpoint of the concentration-response curve.

The Kruskal-Wallis nonparametric test with Dunn's *post hoc* test was used for all statistical analyses. The level of significance was for all tests set to *P* values less than 0.05.

Results

Pharmacology. We have previously shown that PKC, ERK1/2, and JNK pathways are important in the upregulation of the ET receptors, by co-incubation of segments of rat mesenteric and basilar arteries for 24 hrs. Here, we tested whether administration of inhibitors after initiation of the upregulation process can attenuate the receptor expression. The vessel segments were incubated for 21, 18, or 12 hrs with different inhibitors of PKC, ERK1/2, or JNK. The contractile responses to the ET_B receptor activation *via* S6c, and ET_A receptor activation *via* ET-1 (after ET_B receptor desensitization) were investigated.

PKC Inhibition. In the basilar arteries, administration of the PKC inhibitor, 3 hrs after initiation of the organ culture, abolished upregulation of ET_B receptors at mRNA and functional levels.

Basilar arteries incubated with the PKC inhibitor (Fig. 1a) showed attenuated contraction by S6c (Table 1); the response to S6c at 21 hrs of incubation with RO31–7549 was 0% ($P < 0.0001$) and at 18 hrs of incubation was 12 ± 5% of the potassium-induced responses ($P < 0.001$). The pEC_{50} value was 8.87 ± 0.16 in control and 6.86 ± 1.15 at 12 hrs incubation, and there was a significant difference ($P < 0.01$) between control and 21 or 18 hrs, but not between control and 12 hrs.

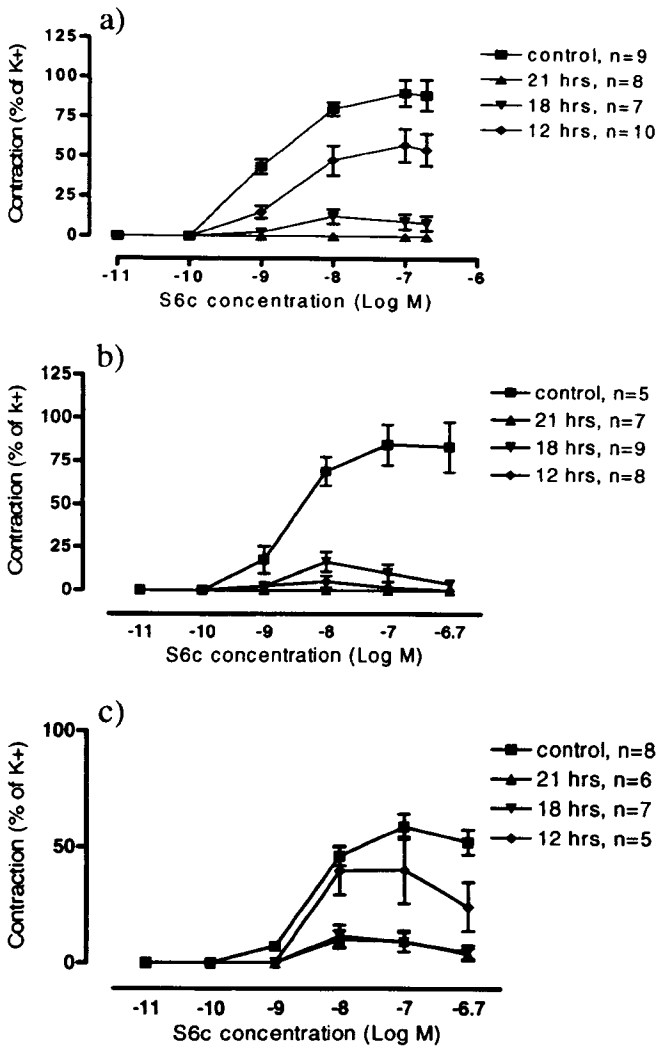

Figure 1. Concentration-response curves of rat basilar arteries for S6c. Rat basilar arteries were incubated for 24 hrs in DMEM with or without the (a) PKC inhibitor, RO-31–7549; (b) the ERK1/2 inhibitor, SB386023; and (c) the JNK inhibitor, SP600125, added 3 (21 hrs), 6 (18 hrs), or 12 (12 hrs) hrs after initiation of incubation. Controls are the arteries that were incubated with DMSO for 24 hrs. Data are shown as mean ± SEM; $n = 5$–11.

In mesenteric arteries, incubation with the PKC inhibitor also attenuated the contraction induced by S6c, but to a somewhat lesser degree than in the basilar artery, from 197 ± 38% to 63 ± 30% at 21 hrs, and to 90 ± 27% at 18 hrs of incubation. The Dunn's *post hoc* test showed a significant difference ($P < 0.05$) between control and 21 hrs but not between control and 18 or 12 hrs. The pEC_{50} value for the control was 8.08 ± 0.16, after 12 hrs of incubation, it was 8.23 ± 0.15, which was not significant (Fig. 2a; Table 2).

ERK1/2 Inhibition. Incubation of basilar arteries with the ERK1/2 inhibitor showed markedly attenuated S6c-induced contractions; 0% of potassium-induced contraction at 21 and 12 hrs of incubation, and, at 18 hrs, there was a slight contraction, 10 ± 5%, with a pEC_{50} value of 2.15 ± 1.14, which was significantly lower ($P < 0.001$) compared with control. A significant difference ($P < 0.0004$) was found for all three groups and the Dunn's *post hoc* test ($P < 0.01$) between control and the 21 hrs, 18 hrs, and 12 hrs groups (Fig. 1b; Table 1).

Incubation of mesenteric artery segments with the ERK1/2 inhibitor, SB386023, attenuated the contraction induced by S6c; to 0% at 21 hrs and 18 hrs incubation but had almost no effect at 12 hrs of incubation. The results show that the effect of SB386023 was significant ($P < 0.003$). Between groups, the effect was significant only for 21 hrs ($P < 0.05$) and 18 hrs ($P < 0.01$), and was not significant for 12 hrs. The pEC_{50} value was significantly lower at 21 hrs and 18 hrs ($P < 0.01$), but not at 12 hrs (Fig. 2b; Table 2). Incubation of basilar and mesenteric arteries with the ERK1/2 inhibitor showed a marked decrease in the pEC_{50} value, indicating that higher concentrations of S6c are required to induce the same maximal contraction in these vessel segments.

JNK Inhibition. In basilar arteries, incubation with the JNK inhibitor, SP600125 (Fig. 1c; Table 1) showed attenuated contraction after 21 and 18 hrs of incubation with S6c, from 66% in control to 6% and 8%, respectively, of the potassium-induced responses. This effect of the JNK inhibitor decreased with time of incubation and was less effective (38 ± 11%) at 12 hrs. The pEC_{50} value for control (8.48 ± 0.07) was significantly ($P < 0.0001$) lower at 21 hrs and 18 hrs, but not at 12 hrs.

In mesenteric arteries, the JNK inhibitor had a weak effect only when added 3 hrs after the start of incubation. The contraction induced by S6c was reduced from 95 ± 13% to 73 ± 15% of the potassium-induced contraction. The P value for all groups was not significant ($P < 0.17$). The pEC_{50} value was 8.18 ± 0.14 in control and was 7.87 ± 0.19 at 12 hrs of incubation, which were not significantly different (Fig. 2c; Table 2).

Effect of Inhibition on ET_A Receptors. After S6c desensitization of the ET_B receptors, the addition of ET-1 induced selective activation of ET_A receptors. As can be seen for the basilar (Table 1) and mesenteric arteries (Table 2), there were no changes in the ET_A receptor–mediated responses after co-incubation with either of the protein kinase inhibitors.

Real-Time PCR. To address the question of whether the alterations in S6c induced responses were caused by changes in receptor expression, we studied changes in ET_B receptor mRNA levels with real-time PCR after organ culture with or without the inhibitors. Organ culture with the inhibitors, RO31–7549, SB386023, and SP600125, for 21 hrs, 18 hrs, and 12 hrs resulted in reduced levels of the ET_B receptor mRNA expression as compared with the control group, which consisted of organ segments cultured for 24 hrs with DMSO (Fig. 3).

In basilar arteries incubated with the PKC inhibitor, RO31–7549, there were reduced ET_B receptor mRNA levels at 21 hrs and 18 hrs, but the results were not significant.

Table 1. Basilar Arteries Co-incubated with Different Inhibitors[a]

	No of segments	S6c			ET-1			K^+
		E_{max} (mN)	E_{max} (%)	pEC_{50}	E_{max} (mN)	E_{max} (%)	pEC_{50}	E_{max} (mN)
RO317549								
Control	6	5.40 ± 0.37	94 ± 7	8.87 ± 0.16	7.40 ± 0.66	141 ± 15	8.79 ± 0.12	5.79 ± 0.56
21 hrs	6	0	0***	0***	8.02 ± 0.91	116 ± 2	8.28 ± 0.18	6.36 ± 0.81
18 hrs	8	0.84 ± 0.34	12 ± 5***	2.87 ± 1.81**	8.17 ± 0.9	124 ± 4	8.34 ± 0.12	6.76 ± 0.88
12 hrs	10	3.32 ± 0.61	58 ± 11	6.86 ± 1.15	7.64 ± 0.53	132 ± 3	8.33 ± 0.07	5.8 ± 0.44
SB386023								
Control	5	4.28 ± 0.54	87 ± 13	8.62 ± 0.16	7.40 ± 0.58	154 ± 21	8.78 ± 0.22	5.25 ± 0.92
21 hrs	7	0	0***	0**	8.41 ± 1.27	146 ± 10	8.60 ± 0.16	5.85 ± 0.9
18 hrs	9	0.92 ± 0.5	10 ± 5*	2.15 ± 1.4*	9.58 ± 0.57	178 ± 27	8.57 ± 0.23	6.14 ± 0.81
12 hrs	7	0	0***	0**	6.94 ± 0.94	144 ± 11	8.72 ± 0.19	5.18 ± 0.89
SP600125								
Control	11	4.23 ± 0.59	66 ± 5	8.48 ± 0.07	7.97 ± 0.75	131 ± 12	8.63 ± 0.15	6.73 ± 1.01
21 hrs	6	0.47 ± 0.33	6 ± 4***	2.81 ± 1.76***	7.6 ± 1.41	133 ± 10	8.24 ± 0.16	5.87 ± 1.22
18 hrs	7	0.53 ± 0.36	8 ± 5***	3.88 ± 1.81**	6.61 ± 1.08	125 ± 4	8.59 ± 0.2	5.23 ± 0.5
12 hrs	7	1.29 ± 0.39	38 ± 11	5.94 ± 1.53	5.16 ± 0.7	124 ± 9	8.6 ± 0.08	4.51 ± 1.17

[a] Data presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$, between control and each group.

Incubation with the ERK1/2 inhibitor showed a significant difference ($P < 0.05$) between control and all three experimental groups (21, 18, and 12 hrs of incubation). Basilar arteries incubated with the JNK inhibitor, SP600125, showed lower ET_B receptor mRNA levels, but the result was not significant between the groups. There was no difference in ET_A receptor expression (data not shown).

In mesenteric arteries, incubation with RO31–7549 or SB386023 showed lower mRNA levels for the ET_B receptor expression, as compared with control (Fig. 4), but it was not changed after incubation with SP600125. Statistical analysis showed a significant difference between the control and the arteries that were incubated with SB386023 ($P < 0.01$), or RO31–7549 ($P < 0.05$) for 21 hrs, but not between the other groups (18 hrs and 12 hrs). None of the inhibitors used in this study affected the ET_A receptor mRNA expression (data not shown). The results from the functional study are more significant than PCR results. It is suggested that the transcription from DNA to mRNA happens earlier than translation from mRNA to protein or functional levels.

Immunohistochemistry. The protein levels of ET_B receptors were evaluated by immunohistochemistry and viewed with a confocal microscope. Expression differences were evaluated using the program ImageJ for quantitative analysis. There were 7–11 pictures in each group, and the ImageJ program measured the intensity of fluorescence in the designated areas of each section. The results are shown as percent change with respect to control (vessels incubated with DMSO for 24 hrs). The incubation *per se* resulted in elevated expression of ET_B receptors in smooth-muscle cells of the vessel wall.

Basilar arteries (Fig. 5A and 6) showed a decrease in intensity for ET_B receptors after incubation with PKC, ERK1/2, and JNK inhibitors. The ERK1/2 inhibitor had a stronger effect than the other inhibitors. Statistical analysis showed a significant difference between control and inhibitors ($P < 0.001$).

The inhibition was less pronounced in upregulation of ET_B receptors in mesenteric arteries, and statistical analysis did not reveal any significant difference between the groups (Figs. 5B and 7).

Furthermore, we observed no difference in the expression of ET_A receptors (data not shown).

Discussion

The present study is the first to demonstrate that interacting with specific protein kinase pathways can modify the process of ET_B receptor upregulation. The upregulation of ET_B receptors involves enhanced transcription because both mRNA and protein expression are elevated along with the functional responses (14).

We found that the ET receptors are upregulated in stroke, both in models in rat (5, 15) and in human (16). An enhanced ET_B receptor–mediated contraction of the rat basilar artery has been reported after incubation with the proinflammatory cytokines, interleukin-1β and tumor necrosis factor-α, thus, supporting the hypothesis that inflammatory components might be involved. The upregulation of ET receptors after stroke might be caused by changes occurring in the perfusion pressure during and after the occlusion (14). Cattaruzza *et al.* presented data revealing that the ET_B receptor mRNA levels in rat aortic smooth muscle cells are increased by up to 10-fold after periodic stretch (17). Furthermore, several studies have shown that there are increased levels of circulating ET-1 in ischemic stroke (18). These studies all suggest an important role of ET and ET receptors in the pathophysiologic mechanisms in cerebral blood vessels after ischemic stroke.

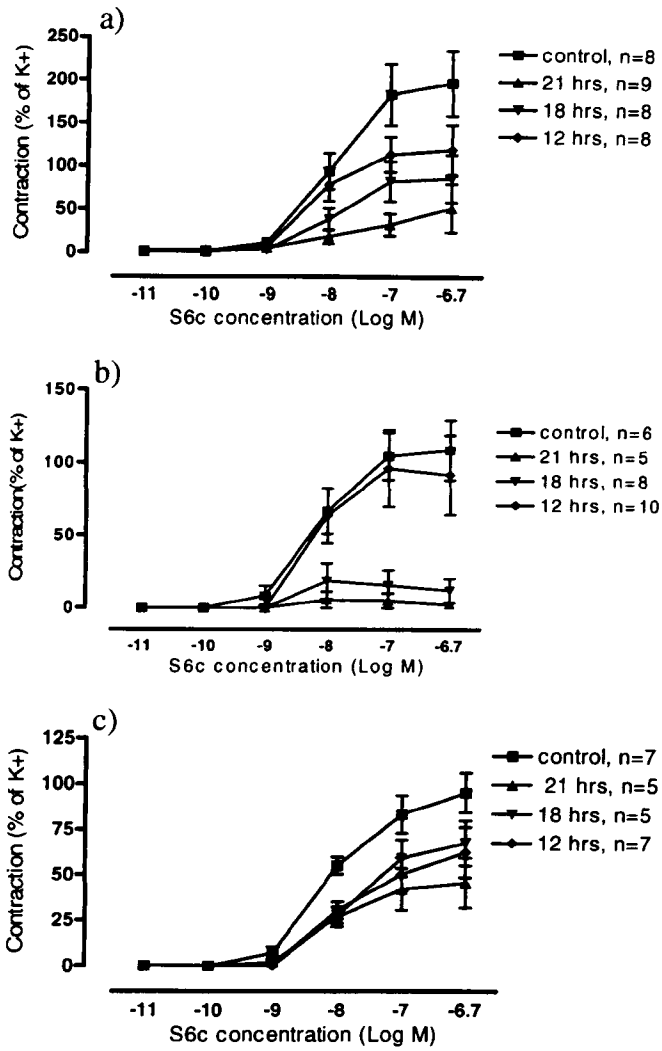

Figure 2. Concentration-response curves of rat mesenteric arteries for S6c. Rat mesenteric arteries were incubated for 24 hrs in DMEM with or without the (a) PKC inhibitor, RO-31–7549; (b) the ERK1/2 inhibitor, SB386023; and (c) the JNK inhibitor, SP600125, added 3 (21 hrs), 6 (18 hrs), or 12 (12 hrs) hrs after initiation of incubation. Controls are the arteries that were incubated with DMSO for 24 hrs. Data are shown as mean ± SEM; $n = 5$–11.

Experimental studies using the organ culture model have provided a method to mimic these changes and to study the intracellular mechanisms involved in more detail, such as the time course of both mRNA and functional receptor changes (19). The receptor upregulation occurs *via de novo* transcription with subsequent translation (3, 19). It was previously shown that co-incubation with an ERK1/2 inhibitor for 24 hrs in organ culture study attenuates the upregulation of ET_B receptors in the mesenteric artery. The present study focused on understanding whether the upregulation can be attenuated by treating the tissue after the event has been initiated. This might provide a basis for consideration in clinical situations in which one hopes to intervene. The reasons for initiation of receptor upregulation are still unclear, but occur in arthrosclerosis, in ischemia, and in situations of reduced blood flow. Hypothetically,

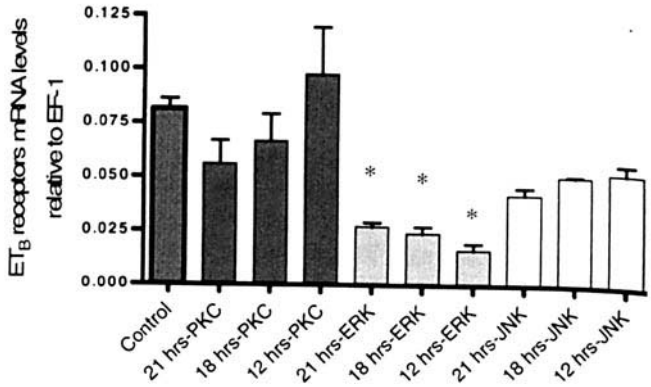

Figure 3. Relative ET_B receptor mRNA levels in the organ-cultured rat basilar artery. Rat basilar arteries were incubated for 24 hrs in DMEM with or without the (a) PKC inhibitor, RO-31–7549; (b) the ERK1/2 inhibitor, SB386023; and (c) the JNK inhibitor, SP600125, added 3 (21 hrs), 6 (18 hrs), or 12 (12 hrs) after initiation of incubation. Controls are the arteries that were incubated with DMSO for 24 hrs. Data are presented as mean ± SEM relative to EF-1; $n =$ 3–6. $^*P < 0.05$.

changes in shear stress is one way to increase the ET_B receptor upregulation, as can be seen by elevation in intramural pressure in a vascular segment.[1] The molecules triggering this on the cell surface have, thus far, not been identified. The further events that occur in the process involves several cellular signaling pathways; such as release of intracellular calcium and activation of MAPK-dependent signaling pathways (20, 21). ERK1/2 and JNK are involved in the signaling pathways from the cytoplasm to the nucleus, where they regulate gene expression. These signaling cascades result in rapid increases in the early response genes, *c-fos*, *c-jun*, and *c-myc* (21), which result in cell proliferation and enhanced contraction (22, 23).

[1] Lindstedt I, Edvinsson L. 2005. Unpublished data.

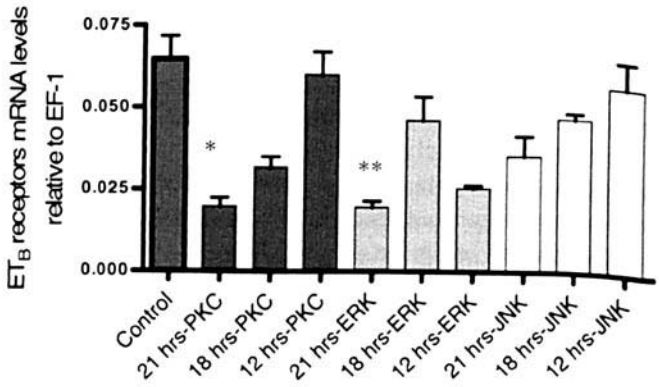

Figure 4. Relative ET_B receptor mRNA levels in the organ-cultured rat mesenteric arteries. Rat mesenteric arteries were incubated for 24 hrs in DMEM with or without the (a) PKC inhibitor, RO-31–7549; (b) the ERK1/2 inhibitor, SB386023; and (c) the JNK inhibitor, SP600125, added 3 (21 hrs), 6 (18 hrs) or 12 (12 hrs) hrs after initiation of incubation. Controls are the arteries that incubated with DMSO for 24 hrs. Data are presented as mean ± SEM relative to EF-1; $n = 3$–6. $^*P < 0.05$.

Table 2. Mesenteric Arteries Co-incubated with Different Inhibitors[a]

	No. of segments	S6c			ET-1			K^+
		E_{max} (mN)	E_{max} (%)	$_pEC_{50}$	E_{max} (mN)	E_{max} (%)	$_pEC_{50}$	E_{max} (mN)
RO317549								
Control	6	7.46 ± 1.14	197 ± 38	8.08 ± 0.16	9.8 ± 1.02	219 ± 20	7.94 ± 0.22	4.45 ± 0.56
21 hrs	7	2.05 ± 0.8	63 ± 30*	7.85 ± 0.8	8.27 ± 0.84	159 ± 16	8.12 ± 0.26	5.04 ± 0.86
18 hrs	6	5.89 ± 1.66	90 ± 27	8.26 ± 0.19	10.25 ± 1.58	152 ± 16	8.07 ± 0.12	7.19 ± 1.35
12 hrs	7	5.34 ± 1.46	129 ± 29	8.23 ± 0.15	9.61 ± 1.31	273 ± 75	7.79 ± 0.17	5.27 ± 1.55
SB386023								
Control	6	5.77 ± 0.97	116 ± 18	8.12 ± 0.17	8.12 ± 0.81	168 ± 18	8.33 ± 0.22	5.10 ± 0.72
21 hrs	5	0.18 ± 0.18	5 ± 5*	1.71 ± 1.71*	6.17 ± 0.78	264 ± 35	7.64 ± 0.11	2.66 ± 0.44
18 hrs	8	0	0**	0**	8.40 ± 0.74	389 ± 65	8.20 ± 0.18	2.84 ± 0.5
12 hrs	10	2.54 ± 0.82	102 ± 28	4.96 ± 1.35	7.88 ± 0.54	368 ± 49	7.94 ± 0.16	2.72 ± 0.51
SP600125								
Control	7	6.39 ± 1.4	95 ± 13	8.18 ± 0.14	10.09 ± 0.73	191 ± 30	7.94 ± 0.16	6.31 ± 1.12
21 hrs	5	4.38 ± 1.27	73 ± 15	8.18 ± 0.09	10.08 ± 0.86	186 ± 36	7.89 ± 0.11	6.24 ± 1.16
18 hrs	5	5.52 ± 1.13	85 ± 12	7.86 ± 0.08	9.76 ± 1.34	183 ± 43	7.73 ± 0.14	6.42 ± 1.43
12 hrs	6	7.3 ± 2.16	108 ± 11	7.87 ± 0.19	10.7 ± 1.52	197 ± 31	7.81 ± 0.12	6.17 ± 1.3

[a] Data presented as mean ± SEM. $^*P < 0.05$; and $^{**}P < 0.01$, between control and each group.

However, ET_B receptors have the ability to activate the ERK1/2 and JNK pathways *via* parallel pathways that are differentially regulated by PKC and calcium ions (23).

It has been shown that, in vascular smooth muscle cells expressing ET_B receptors, ET-1 is more potent in mitogenic activity (24).

In the present study, we examined specific inhibitors of ERK1/2, PKC, and JNK in the signal transduction for upregulation of ET_B receptor, and also to what extent the ET_B receptors are dependent on these signaling cascades. The PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; and the JNK inhibitor, SP600125, had inhibitory effects on ET_B receptor–mediated contraction in the pharmacologic studies. In the molecular experiments, the inhibitors had an effect on ET_B receptor mRNA expression but had no effect on ET_A receptor mRNA (25). Immuno-

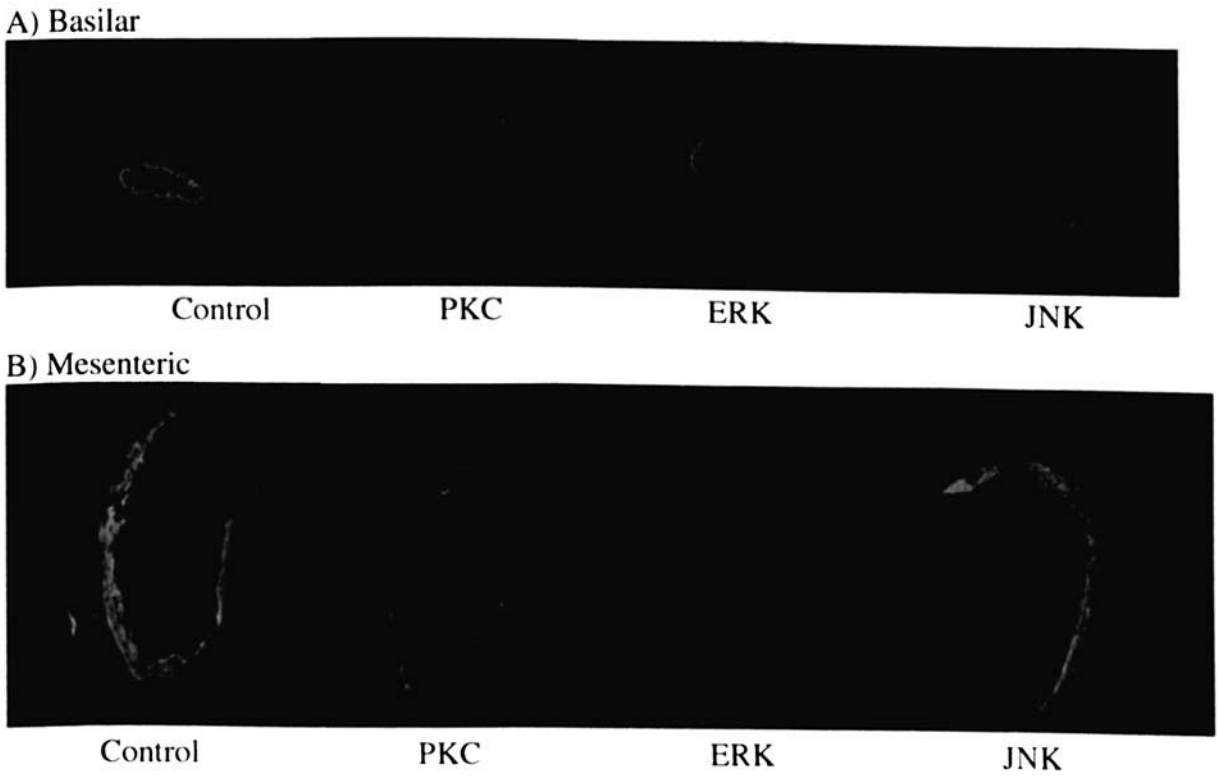

Figure 5. Immunohistochemistry of (A) basilar artery and (B) mesenteric artery. These pictures were taken by Leica microscope with a ×20 objective. All of the factors, such as laser intensity and scanning procedure, were the same in all experiments.

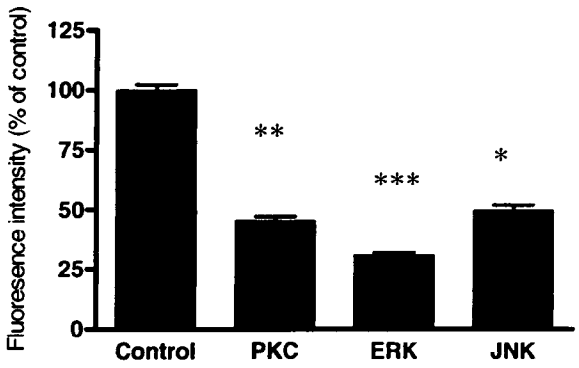

Figure 6. Immunohistochemistry results show the fluorescence intensity of measured areas of ET_B receptors in the vessel walls. Rat basilar arteries were incubated for 24 hrs, and the PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; and the JNK inhibitor, SP600125, were added after 6 hrs (18 hrs), of incubation. Controls are the arteries that were incubated with DMSO for 24 hrs. The results are given as percentage of control; $n = 7–11$. $^*P < 0.05$.

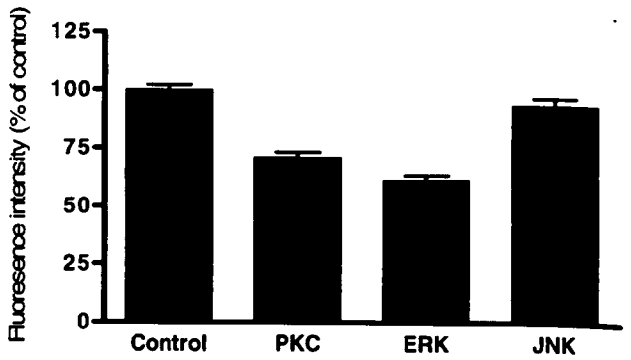

Figure 7. Immunohistochemistry results show the fluorescence intensity of the measured area of ET_B receptors. Rat mesenteric arteries were incubated for 24 hrs, and the PKC inhibitor, RO-31–7549; the ERK1/2 inhibitor, SB386023; and the JNK inhibitor, SP600125, were added after 6 hrs (18 hrs), of incubation. Controls are the arteries that were incubated with DMSO for 24 hrs. The results are given as percentage of control; $n = 7–11$.

histochemical results showing receptor protein expression were parallel to the quantitative PCR results. The effects on mesenteric arteries were less pronounced than those found in basilar arteries, which may depend on vessel size or on being related to the vascular region studied.

In basilar arteries, the most effective inhibitor was the ERK1/2 inhibitor, SB386023, which had an effect even after 12 hrs of organ culture, both on contraction and on the mRNA levels of ET_B receptors. SP600125 and RO317549 were efficient only when the incubation time was 21 and 18 hrs. There was a difference between the pharmacologic studies and mRNA levels of the ET_B receptor with these inhibitors. Even after 18 hrs and 12 hrs of incubation, they decreased the contraction induced by S6c, whereas they did not show a significant decrease in mRNA levels compared with the ERK1/2 inhibitor. It seems that to observe the reaction of mRNA levels by these inhibitors requires more time points of incubation, but they succeed in decreasing the contraction of ET_B receptors in pharmacologic studies. Thus, the ERK1/2 system might have a more central role in the upregulation process.

Our results show that the time point was not as important in the inhibitory effect of the ERK1/2 inhibitor on the contraction induced by S6c. Even the mRNA levels of ET_B receptor did not show a significant difference between 21, 18, and 12 hrs of incubation, and all of them showed a significant difference from control. Therefore, it is reasonable to suggest that the ERK1/2 pathway is the pathway that the cell machinery primarily activates to initiate ET_B receptor upregulation during organ culture. In agreement with this suggestion, it was shown that ERK1/2 phosphorylation after organ culture increased from the first hour and stayed elevated for up to 24 hrs, whereas JNK phosphorylation increased at the first hour and then decreased to a low level after 6 hrs.[2]

[2] Beg S, Edvinsson L. 2005. Unpublished results.

In mesenteric arteries, the ERK1/2 inhibitor also had the strongest inhibitory effect on the upregulation of ET_B receptors and contraction induced by S6c. The mRNA levels of ET_B receptors after incubation for 21 hrs with ERK1/2 inhibitor were significantly different compared with control. In addition, at 18 and 12 hrs of incubation, the inhibitory effect of the ERK1/2 blocker was more pronounced compared with the PKC and JNK inhibitors. The PKC inhibitor had less effect compared with the ERK1/2 inhibitor. The JNK inhibitor had no effect on mesenteric arteries. The immunohistochemical study did not show any significant difference between the groups ($P = 0.14$), possibly because of only a single time point being studied and a lower degree of quantification with that method. However, the upregulated ET_B receptors were located on the smooth muscle cells, because, in our experiment, we removed the endothelial layer and, thus, the receptor upregulation happens in the media and the protein kinase inhibitors were able to reduce this expression.

We studied ET_A and ET_B receptor upregulation at three different levels; at the mRNA, functional receptor, and protein levels. By comparing the results from mesenteric and basilar arteries, it is suggested that the signal transduction involved in ET_B receptor upregulation is more effective in basilar arteries than in mesenteric arteries. In a previous study, we compared a large number of arteries and veins in relation to ET receptor upregulation. The pattern that emerged is that the vessel size and the functional role of the vessel are important. Resistance arteries show better upregulation than conductance arteries and veins (13).

Our results show that ET_B receptor upregulation involves the protein kinase systems (6, 7). The MAPK pathways are thought to act downstream from PKC in the smooth muscle cell regulatory cascade. Thus, it is reasonable to speculate that ET_B receptor upregulation is initiated by a pathway from PKC, *via* raf and ERK1/2.

Clearly, more experiments are required to delineate all of the aspects of the signal transduction involved.

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