Cerebral Ischemia Upregulates Vascular Endothelin ET<sub>B</sub> Receptors in Rat

Emelie Stenman, MSc; Malin Malmsjö, MD, PhD; Erik Uddman, MD; Gunilla Gidö, PhD; Tadeusz Wieloch, MD, PhD; Lars Edvinsson, MD, PhD

**Background and Purpose**—Elevated levels of endothelin-1 (ET-1) have been reported in cerebral ischemia. A role for ET may prove more important if the vascular receptors were changed. We addressed whether there is any change in ET receptor expression in cerebral ischemia.

**Methods**—The right middle cerebral artery (MCA) was occluded in male Wistar rats for 2 hours with the intraluminal filament method. The basilar artery and both MCAs were removed after 46 hours of recirculation. The contractile responses to ET-1, a combined ET<sub>A</sub> and ET<sub>B</sub> receptor agonist, and sarafotoxin 6c (S6c), a selective ET<sub>B</sub> receptor agonist, were examined in vitro, and ET receptor mRNA was quantified by real-time polymerase chain reaction.

**Results**—S6c, which had no contractile effect per se on fresh or sham-operated rat cerebral arteries, induced a marked contraction in the occluded MCA (E<sub>max</sub> = 68 ± 68%; P < 0.0001), while there was no difference in the responses to ET-1 after cerebral ischemia. Real-time polymerase chain reaction revealed a significant upregulation of both the ET<sub>A</sub> and ET<sub>B</sub> receptors (both P < 0.05) in the occluded MCA compared with the nonoccluded MCA from the same rats.

**Conclusions**—Focal cerebral ischemia in rat induces increased transcription of both ET<sub>A</sub> and ET<sub>B</sub> receptors, which results in the appearance of a contractile response to the ET<sub>B</sub> receptor agonist S6c. These results suggest a role for ET receptors in the pathogenesis of a vascular component after cerebral ischemia. (*Stroke*. 2002;33:2311-2316.)

**Key Words:** cerebral ischemia ■ endothelins ■ middle cerebral artery ■ receptors, endothelin ■ rats

Since the discovery of an increased concentration of endothelin (ET) in both plasma and cerebrospinal fluid after cerebral ischemia, there has been much discussion regarding its possible involvement in the pathophysiology of cerebral ischemia. As yet the mechanisms of its role remain elusive. In mammals, 2 different subtypes of ET receptors have been found thus far, the ET<sub>A</sub> and ET<sub>B</sub> receptors. The ET<sub>A</sub> receptors are located on the smooth muscle cells and mediate strong contractile effect in cerebral arteries, while the ET<sub>B</sub> receptors are localized mainly to endothelial cells mediating vasodilation. However, ET<sub>B</sub> receptors capable of inducing vasoconstriction have been found in smooth muscle cells in mammals. Upregulation of contractile ET<sub>B</sub> receptors after organ culture has been reported in human omentum and temporal arteries, while organ culture resulted in an upregulation of ET<sub>A</sub> receptors in cortical arteries of the human brain. Studies have been made on the impact of ET receptor antagonists on the outcome of cerebral focal ischemia. Administration of selective ET<sub>A</sub> receptor antagonists has proved to increase the perfusion after cerebral ischemia and decrease the ischemic brain damage. On the other hand, the combined ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist bosen-tan failed to improve the perfusion or decrease the brain damage after cerebral ischemia.

The objective of the present study was to further evaluate the role for ET receptors in cerebral ischemia and specifically to address the question of whether there are phenotypic changes in cerebral ischemia. The contractile responses to the combined ET<sub>A</sub> and ET<sub>B</sub> receptor agonist ET-1, after ET<sub>B</sub> receptor desensitization, and the selective ET<sub>B</sub> receptor agonist sarafotoxin 6c (S6c) were examined in vitro, and the ET receptor mRNA expression after experimental focal cerebral ischemia was quantified with the use of real-time polymerase chain reaction (PCR).

**Materials and Methods**

**Middle Cerebral Artery Occlusion**

Focal cerebral ischemia was induced according to the intraluminal occlusion technique originally described by Koizumi et al (1986). Male Wistar Hannover rats (Møllegaard Breeding Center, Copenhagen, Denmark), weighing 350 to 450 g, were used for the study. The experimental procedures were approved by the Ethical Committee for Laboratory Animal Experiments at the University of Lund (M217-00). The animals were fasted overnight with free access to water. Anesthesia was induced with 4.5% halothane in N<sub>2</sub>O/O<sub>2</sub>.

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were intubated and artificially ventilated with inhalation of 1% to 1.5% halothane in N2/O2 (70:30) during the surgical procedure. A polyethylene catheter was inserted into a tail artery for recording of blood pressure and blood gases and for heparin injection. An electric temperature probe was inserted into the rectum to record the temperature, which was regularly maintained at 37°C. A skin incision of 2 cm was made in the midline of the neck to expose the right common, internal, and external carotid arteries. The external carotid artery was ligated, the common carotid artery was closed by a ligature, and the internal carotid artery was temporarily closed by a microvascular clip. A small incision was made in the common carotid artery, and the occluding filament was inserted into the internal carotid artery. After removal of the microvascular clip, the filament was further advanced 19 mm from the bifurcation between external carotid artery and internal carotid artery to close the origin of the middle cerebral artery (MCA). The filament was fixed in the right common carotid artery by a suture. When the surgical procedures were finished, anesthesia was discontinued, and the animals were allowed to awaken. Two hours after MCA occlusion, the animals were briefly reanesthetized to allow withdrawal of the filament to achieve reperfusion. To avoid hyperthermia after the operation, animals with a body temperature >39°C were placed in a cage with a temperature of approximately 10°C for a maximum of 4 hours. They were then kept for 2 days with free access to food and water. The sham-operated rats went through the same operative procedure as the MCA-occluded rats, except that the filament was immediately withdrawn after insertion. These animals were reanesthetized for 15 minutes after 2 hours.

Removal of Cerebral Vessels and Evaluation of Ischemic Damage

After 48 hours of recovery, the animals were reanesthetized and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution (for composition, see below). The basilar artery (BA) and the right and left MCA were removed and vessel segments were immediately mounted in micrographs for in vitro pharmacology or snap-frozen in −80°C and examined by real-time PCR. The ischemic damage was in all animals confirmed by staining coronal slices of the brains with 1% 2.3.5-triphenyltetrazolium chloride (TTC) dissolved in a saline solution at 37°C for 20 minutes.16

In Vitro Pharmacology, Myograph Tissue Bath

A sensitive myograph was used for recording the isometric tension in isolated vessels segments, as described below.17,18 The vessels were cut into 0.5-mm-long cylindrical segments. The endothelium was removed mechanically by inserting a thin thread and rubbing the vessel wall, and the right and left MCA were mounted in a Mulvany-Halpern myograph (Danish Myotech A/S). One wire was connected to a force displacement transducer attached to an analog-digital converter unit (ADInstruments). One wire was connected to a force displacement transducer attached to an analog-digital converter unit (ADInstruments). 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). The segments were immersed in temperature-controlled (37°C) tissue baths containing a bicarbonate buffer solution of the following composition (mmol/L): NaCl 119, NaHCO3 15, KCl 4.6, MgCl2 1.2, NaH2PO4 1.2, CaCl2 1.5, and glucose 5.5. The solution was continuously gassed with 5% CO2 in O2, resulting in a pH of 7.4. The vessels were given an initial tension of 1.2 mN and were adjusted to this level of tension for 1 hour. The contractile capacity was determined by exposure to a potassium-rich (63.5 mmol/L) buffer solution with the same composition as the bicarbonate buffer solution except that NaCl was exchanged for KCl. The vessels were exposed to 63.5 mmol/L potassium twice, and the second contraction was used as a reference for the contractile capacity. Only vessels responding by at least 0.3 mN to potassium were included in the study. Concentration-response curves for the agonists ET-1 and S6c were obtained by cumulative application (10−12 to 10−6 mol/L). To evaluate the selective activation of the ETA receptors, the ET-1 responses were preceded by application of S6c (10−6 mol/L), which desensitizes (and abolishes) the ETA receptor responses.19

Molecular Biology

After removal of the endothelium, the vessels were snap-frozen in −80°C and total cellular RNA was extracted with the use of the Trizol reagent (Gibco BRL) following the suppliers’ instructions. The resulting pellet was finally washed with 75% ethanol, air-dried, and redissolved in 10 μL diethyl-pyrocarbonate–treated water. Reverse transcription of total RNA to cDNA was performed with the use of the GeneAmp RNA PCR kit (PE Applied Biosystems) in a Perkin-Elmer DNA Thermal cycler. First-strand cDNA was synthesized from 1 μg total RNA in a 20-μL reaction volume with the use of random hexamers as primers. The reaction mixture was incubated at 25°C for 10 minutes and 42°C for 15 minutes, heated to 99°C for 5 minutes, and chilled to 5°C for 5 minutes. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System (Perkin-Elmer, Applied Biosystems) with the use of the GeneAmp SYBR Green kit (Perkin-Elmer, Applied Biosystems) with the cDNA synthesized 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 with an optic and imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers for the rat ETA and ETB receptors were designed as follows: ETA receptor forward: 5′-ATTGCCTCTAGCAGAAAC-3′; ETA receptor reverse: 5′-CAACGACGAGGAAAGGTC-3′; ETB receptor forward: 5′-GATAAAGCCTTCGCTCACA-3′; ETB reverse: 5′-GGTCCAGTGGACCAATGAG-3′. Elongation factor-1 (EF-1) mRNA was used as a reference because 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′-GCAAAGCCTGATGGTTGAA-3′; EF-1 reverse: 5′-TGATGACCCCACAGCAACT-3′. The real-time PCR was performed with the following profile: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds times 40, and 60°C for 1 minute. 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 were made in which the Ct values were plotted against cDNA concentration on the basis of the following equation: Ct = −log(1 + E) / log(concentration), where Ct is the number of PCR cycles performed in 1 sample at a specific point of time, and E is the amplification efficiency with an optimal value of 1. To prove that each primer pair generates only 1 PCR product, an agarose gel electrophoresis with the PCR products was run. The respective lengths of the products were 64 bp for ETα, 86 bp for ETα, and 96 bp for EF-1. This corresponds to the expected size of the ETα, ETα, and EF-1 mRNA from the gene bank as examined with the program Primer Express 3.0.

Drugs

Pharmacology

ET-1 and S6c (Auspep) were dissolved in 0.9% saline with 0.1% bovine serum albumin. Acetylcholine (Sigma) was dissolved in 0.9% saline.

Polymerase Chain Reaction

Oligonucleotides and reagents for the PCR assay were purchased from Perkin-Elmer, Applied Biosystems.

Calculations

Pharmacology

Contractile experiments were performed on 6 MCA-occluded and 6 sham-operated rats. The Emax values refer to maximum contraction, calculated as percentage of the contractile capacity of 63.5 mmol/L K+. Data are expressed as mean±SD. Statistical analyses were
Partial PCO₂ and PO₂, Mean Arterial Blood Pressure, and Rectal Temperature
Measured During Surgical Procedure, Just Before Insertion of Occluding Filament

<table>
<thead>
<tr>
<th>Animals</th>
<th>PCO₂, kPa</th>
<th>PO₂, kPa</th>
<th>Mean Arterial Blood Pressure, mm Hg</th>
<th>Rectal Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA occlusion</td>
<td>5.1±0.6</td>
<td>15.1±2.1</td>
<td>104.7±5.7</td>
<td>37.1±0.3</td>
</tr>
<tr>
<td>Sham operation</td>
<td>4.9±0.4</td>
<td>15.5±2.0</td>
<td>102.6±5.4</td>
<td>37.1±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SD.

Results

MCA Occlusion

In MCA-occluded and sham-operated rats, arterial blood gases, mean arterial blood pressure, and temperature were within acceptable limits during operation (PCO₂ 4.4 to 6 kPa, PO₂ ≥12 kPa, blood pressure approximately 90 to 110 mm Hg with transient fluctuations, and rectal temperature 37°C with transient fluctuations; Table). When coronal slices of the brains were stained with TTC, ischemic damage could be observed in the right cerebral hemisphere from MCA-occluded rats. The extent of damage was compared with the results of a previous study by Memezawa et al (1992), in which the same occlusion technique was used. The damage was uniform, with no major difference between the animals.

There was no damage in the brains from the sham-operated rats.

In Vitro Pharmacology

K⁺-induced contractions did not differ in the vessel segments from MCA-occluded compared with sham-operated rats. The difference in K⁺-induced contractions between right and left MCA were also not significant (E max = 1.5±1.0 and 1.4±1.1 mN for right MCAs; E max = 1.0±0.4 and 0.8±0.3 mN for left MCAs; E max = 3.3±1.7 and 2.3±0.8 mN for BAs). Each vessel segment was exposed to K⁺ twice, and the subsequent contractions did not differ in strength between the first and second exposures. The relaxant responses to acetylcholine were totally abolished, which indicated a properly removed endothelium (data not shown). S6c induced an efficacious contraction in the occluded right MCA, while no contractile response to S6c was detected in the left, nonoccluded MCA from the same rats or in the right MCA from sham-operated rats (E max = 68±68% in the occluded MCA, 0% in the left nonoccluded MCA, and 0% in MCA from sham-operated rats; P<0.0001; Figure 1). There was no difference in the contractile responses to ET-1, after ETB receptor desensitization, in the occluded right MCA compared with the left MCA or the right MCA from sham-operated rats (Figure 2). In the BA there were no differences in the contractile responses to either ET-1, after ETB receptor desensitization, or S6c between MCA-occluded and sham-operated rats (Figure 3). The selectivity of S6c for ETB receptors and ET-1 for ETA/ETB receptors has been evaluated in brain vessels of the rat by Hansen-Schwartz and Edvinsson (2000).
Molecular Biology

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 efficacy (Figure 4). 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). Electrophoresis of the PCR products demonstrated that each primer pair generated only the expected product (Figure 5). 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 real-time PCR showed significantly elevated levels of both ET_A and ET_B receptor mRNA in the occluded right MCA after cerebral ischemia as compared with the contralateral MCA from the same rats (ET_A = 180 ± 63%, P < 0.05; ET_B = 233 ± 102%, P < 0.05; Figure 6). There were no differences in the ET_A or ET_B receptor mRNA levels in the BA from MCA-occluded rats compared with sham-operated rats (data not shown).

Discussion

This is the first study to show that there is a phenotypic change with a local upregulation of contractile ET_B receptors in cerebral vessels after focal ischemia in the rat. The response to S6c was significantly more efficacious in the occluded MCA 48 hours after induction of cerebral ischemia, while the response to ET-1, after ET_B receptor desensitization, in the same vessel was not altered. In the nonoccluded left MCA and the BA there were no differences in the responses to ET-1, after ET_B receptor desensitization, and S6c between MCA-occluded and sham-operated rats, thus revealing the precise and focal nature of the upregulation of the contractile phenotype. The results from real-time PCR are in agreement with the findings of upregulated ET_B receptors from in vitro pharmacology experiments, with upregulated
ET$_A$ receptor mRNA in the occluded MCA compared with the nonoccluded MCA from the same rats. These results suggest that the enhanced responses to S6c are due to de novo synthesis of the ET$_A$ receptors in the smooth muscle cell since the endothelium had been removed before the experiment. This is supported by studies in which perfused MCA was used, which revealed that abluminal S6c had no effect on the vessel tone, while luminal S6c caused an endothelium-mediated relaxation.$^6$ In addition, we observed a significant upregulation of the ET$_A$ receptor mRNA, which did not result in a change in the contractile phenotype.

Accordingly, the event of MCA occlusion seems to have an impact on intracellular pathways coupled to the transcription of both ET receptors in vascular smooth muscle cells in the affected hemisphere. In a study by Möller et al (1997),$^{22}$ it was mediated via increased transcription and subsequent translation of ET$_A$ receptor mRNA. The mRNA level reaches its maximum at 24 hours, while the contractile response has its maximum at 48 hours (S. Möller, PhD, et al, unpublished data, 2001). As in the human genome, the rat $5’$-flanking region of the genes encoding the ET receptors contains several regulatory elements, such as GATA motifs and E boxes.$^{21–26}$ This indicates that the genes might be activated by, for example, inflammatory components after cerebral ischemia. An enhanced ET$_A$ receptor–mediated contraction of the rat BA has been reported after incubation with the proinflammatory cytokines interleukin-1$\beta$ and tumor necrosis factor-$\alpha$, which supports the hypothesis that inflammatory components might be involved. One possible reason for upregulation of ET receptors might be the changes that occur in perfusion pressure during and after the occlusion.

Cattaruzza et al$^{28}$ (2000) presented a study in which the ET$_A$ receptor mRNA levels in rat aortic smooth muscle cells were increased by up to 10-fold after periodic stretch.

Many studies have implicated a role for ET in the pathophysiology of stroke. Elevated levels of ET-1 have been reported in nonhemorrhagic stroke.$^1$ A marked increase in ET-1 activity in areas of severe ischemic damage has been demonstrated compared with various other vasoactive peptides.$^{29}$ The effect of ET$_A$ receptor blockade on reperfusion after cerebral ischemia has been examined by Patel et al (1996)$^{10}$ and Dawson et al (1999).$^{11}$ They suggested that the 2 selective ET$_A$ receptor antagonists PD156707 and Ro 61-1790 might increase cerebral perfusion and reduce brain damage after focal cerebral ischemia in cat and rat, respectively. The selective ET$_A$ receptor antagonist TA-0201 may prevent cerebral vasospasm after subarachnoid hemorrhage in canine basilar artery.$^{12}$ while 2 other studies showed that Ro 61-1790 and the ET$_A$ receptor antagonist FR139317 did not improve the outcome in focal cerebral ischemia in cat and rat, respectively.$^{30,31}$ The effect of the combined ET$_A$ and ET$_B$ receptor antagonist bosentan has also been tested and proven not to have any effect on perfusion or size of brain damage after cerebral ischemia in rat.$^{13,14}$ In addition, the ETs have been thought to be neuromodulatory,$^{32}$ although subsequent studies have suggested that the detrimental effect of ET-1 after cerebral ischemia is not due to neurotoxicity but to its vasoconstrictive effects.$^{33}$

The present study has focused on the possibility of localized changes in ET$_A$ receptor expression after cerebral ischemia. It suggests that there is a local upregulation of contractile ET$_A$ receptors after cerebral ischemia and that this regulation occurs on a transcriptional level. We also observed that the experimental cerebral ischemia resulted in elevated ET$_A$ receptor mRNA levels in the ipsilateral MCA, while an increased contraction mediated via this receptor could not be observed. We may speculate that either the mRNA is not translated to functional ET$_A$ receptors or there may be an enhanced turnover rate of ET$_A$ receptors in order to eliminate the high levels of ET-1 after cerebral ischemia.$^{1–3}$ ET has long-lasting contractile effects on cerebral vessels, which results in hypoperfusion and may therefore be detrimental in the event of ischemia. An upregulation of contractile ET receptors may exacerbate the ischemic region. Antagonists to ET$_B$ receptor–mediated responses might therefore be potential future therapeutic targets in the aim to limit the extent of neuronal damage after cerebral ischemia.

Acknowledgments

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References


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