### Distinct Alteration in Brain Endothelin A and B Receptor Characteristics Following Focal Cerebral Ischemia in Rats

Authors

Affiliation

S. Bhalla, M. G. Leonard, S. Briyal, A. Gulati

Chicago College of Pharmacy, Midwestern University, Downers Grove, United States

Key wordscerebral ischemia

- endothelin
- receptor binding
- ET<sub>A</sub> receptors
- ET<sub>B</sub> receptors

received 25.03.2015 accepted 11.08.2015

#### Bibliography

DOI http://dx.doi.org/ 10.1055/s-0035-1559779 Published online: September 23, 2015 Drug Res 2016; 66: 189–195 © Georg Thieme Verlag KG Stuttgart - New York ISSN 2194-9379

#### Correspondence A. Gulati. MD. PhD

Associate Dean and Professor Chicago College of Pharmacy Midwestern University 555 31<sup>st</sup> St. Downers Grove IL 60515-1235 Tel.: +1/630/971 6417 Fax: +1/630/971 6097 AGULAT@midwestern.edu

### Abstract

Manipulation of the central endothelin (ET) system via either ET<sub>A</sub> antagonists or ET<sub>B</sub> agonists following experimental cerebral ischemia has been shown to be beneficial in previous experimental studies. In order to further explore the involvement of these receptors in cerebral ischemia, we determined changes in binding affinity (K<sub>d</sub>) and density  $(B_{max})$  of  $ET_A$  and  $ET_B$  receptors in the rat brain at 24h and 1 week following middle cerebral artery occlusion (MCAO). Rats subject to MCAO exhibited significant neurological and motor function deficit as well as large infarct volumes (126.41±40.12 and 152.82±21.67 mm<sup>3</sup> on days 1 and 7, respectively). B<sub>max</sub> increased (P < 0.01) and  $K_d$  decrease<sub>d</sub> (P < 0.01) for  $ET_A$ receptors in the infarcted right cerebral hemisphere compared to sham 24 h post MCAO. However, after 7 days of MCAO, B<sub>max</sub> of ET<sub>A</sub> receptors was similar, while K<sub>d</sub> in the infarcted hemisphere increased (P<0.05) compared to sham. Binding characteristics for brain ET<sub>B</sub> receptors were not altered 24h post MCAO. However, 7days following MCAO, there was a significant decrease (P < 0.001) in K<sub>d</sub> values and an increase (P < 0.001)in B<sub>max</sub> values for ET<sub>B</sub> receptors in the ischemic cerebral hemisphere. The initial increase in ET<sub>A</sub> receptors is damaging and may be aggravating cerebral ischemia due to its vasoconstrictive actions. On the other hand, since ET<sub>B</sub> receptors have been shown to enhance brain angiogenesis, it is possible that an increase in binding characteristics of these receptors is part of a natural defense mechanism to repair the ischemic brain.

### Introduction

Stroke is a leading cause of death and long-term disability worldwide. In addition to a loss of mental and motor capabilities, multiple deleterious pathways are initiated within the brain tissue itself. Coincident with neuronal apoptosis, there is a rise in oxidative stress as well as an increase in the expression of endothelin A  $(ET_A)$  receptor and its endogenous ligand ET-1 [1,2].

Endothelin (ET) has been implicated in numerous physiological and pathological phenomena within the body. Acting upon 2 distinct receptors,  $ET_A$  and  $ET_B$ , ET influences a range of processes from regulation of blood pressure to neurotransmitters [3–6]. ET receptors are widespread throughout the body, including the brain, where they are located on neurons and glial cells as well as the cerebovas-culature [7]. Although previous studies focused on selectively antagonizing the  $ET_A$  receptor following cerebral ischemia have led to some promising results, non-selective antagonism of both  $ET_A$  and

 $ET_B$  receptors has not been as effective and has led to conflicting reports [8–12].

Initial studies in our laboratory demonstrated that selective  $ET_B$  receptor stimulation via IRL-1620 significantly improved neurological and motor function while drastically decreasing infarct volume at both 24 h and 7 days following permanent middle cerebral artery occlusion (MCAO) in rats [2,13]. These studies also demonstrated that MCAO rats treated with IRL-1620 presented with reduced oxidative stress as well as increased angiogenesis and neurogenesis as compared with the vehicle-treated animals, indicating a further neuroprotective and neuroreparatory role for  $ET_B$  receptors following cerebral ischemia [14].

While these initial studies have clearly demonstrated that the ET system is involved in the pathophysiology of ischemic stroke, it is of interest to determine how the binding characteristics of the ET receptors are altered at different stages of cerebral ischemia in order to optimize potential treatments. Therefore, the purpose of this study was determine the effects of permanent MCAO on the binding characteristics of both  $ET_A$  and  $ET_B$  receptors in the rat brain during the acute and sub-acute phases of cerebral ischemia.

### **Methods and Materials**

### Animals

Male Sprague Dawley rats weighing 250–300 g were used. The animals were housed 2 per cage with controlled temperature  $(23\pm1^{\circ}Celsius)$ , humidity  $(50\pm10^{\circ})$  and 12-h light/dark cycle (6:00 AM to 6:00 PM). Food and water were made available *ad libitum*. Animal care and use of experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Midwestern University.

### **Drugs and chemicals**

 $ET_A$  antagonist, BQ123 (American Peptide Co., Sunnyvale, CA), and  $ET_B$  agonist, IRL1620 (Tocris Pharmaceuticals Inc., Ellisville, MO), were dissolved in distilled deionized pyrogen-free water and dilutions were made in Tris buffer (50 mM, pH 7.4). [<sup>125</sup>I] ET-1 (specific activity, 2200Ci/mmol, Perkin Elmer, Billerica, MA) and [<sup>125</sup>I]IRL1620 (specific activity, 2200Ci/mmol, Perkin Elmer, Billerica, MA) were diluted in Tris buffer (50 mM, pH 7.4).

### **Experimental protocol**

In order to determine the progression of changes in binding characteristics of ET receptors during both acute and sub-acute phases of stroke, 2 endpoints were chosen: 24 h and 7 days following cerebral ischemia. Rats for each endpoint were randomly divided into 2 groups. Animals in group 1 were subject to a sham operation, and animals in group 2 were subject to MCAO. A total of 32 rats were used for this study, with n = 4 per group, per endpoint. 2 separate groups of 16 animals each were used for either binding studies or immunoblotting. No animals were excluded from the study.

### Middle cerebral artery occlusion

Permanent middle cerebral artery occlusion (MCAO) was performed according to the method of Koizumi, et al [15]. Rats were anesthetized with ketamine and xylazine. Rectal core temperature was measured with a Cole Palmer Animal Monitoring Thermometer colonic probe (Vernon Hills, IL) and maintained throughout surgery at 37±1°C using the thermo-controlled base of the operating table. With the anesthetized rat in a secure supine position, a midline incision was made and the right common, internal, and external carotid arteries were exposed. A 4.0 monofilament nylon filament (CP Medical, Portland, OR) with a flame-rounded tip was advanced from the external carotid artery into the lumen of the internal carotid artery until resistance is felt (~20-22 mm), indicating occlusion of the middle cerebral artery. In order to create a permanent model of cerebral ischemia, the filament was securely tied and allowed to remain in place until the end of the experiment. The incision was closed with 3.0 silk surgical sutures (Ethicon, Inc.). In sham-operated animals, the common and external right carotid arteries were exposed and the incision was sutured without touching the internal carotid artery. Rats were monitored twice daily to assess appearance, activity, and behavior. Proper and intact placement of the filament was verified in all animals at the time of sacrifice.

### Motor performance tests

4 assessments were used to determine neurological and motor deficit following permanent MCAO – neurological evaluation, grip test, foot fault error test, and rota rod. Animals were subject to blinded assessments 15 min prior to occlusion to establish a baseline and at 1, 4 and 7 days post occlusion to determine the effects of ischemia.

### Neurological evaluation

The neurological evaluation was based on a 6 point scale as described by Tatlisumak, et al. [16]. The scoring was as follows: 0=no deficits, 1=failure to fully extend left forepaw, 2=circling to the left, 3=paresis to the left, 4=no spontaneous walking, 5=death.

### Grip test

The grip test for muscular strength consisted of a string elevated 40 cm above a flat surface pulled taut between 2 vertical supports spaced 50 cm apart. The animal was placed on the string midway between the supports and evaluated according to a 6 point scale [17]. The scoring was as follows: 0 = falls off, 1 = hangs on by 2 forepaws, 2 = hangs on by 2 forepaws and attempts to climb on, 3 = hangs on by 3 + paws, 4 = hangs on by all paws plus tail, and 5 = escapes.

### Foot fault error test

Animals were placed on an elevated grid floor with a mesh size of 30 mm<sup>2</sup> for one minute to acclimate. They were then observed for one minute and evaluated for foot fault errors (i.e. a misplaced limb falling through the grid) compared with paired steps as follows [18]:

% foot fault error=(number of faults/number of paired steps)×100

### Rota rod

Animals were acclimated to the rotating spindle of the rota rod (Rota-Rod 47700, Ugo Basile, Italy) prior to occlusion. For acclimatization, animals were placed on the rotating spindle, set to a constant 8 rotations per minute (RPM), until they demonstrated the ability to remain on the spindle for 60s. Animals were then subject to a baseline test trial on the accelerating spindle (4–40 RPM) over 5 min. The acceleration trial was repeated at 1, 4 and 7 d post occlusion, and the time (in sec) at which the animals fell off was recorded [19].

### Assessment of Cerebral Infarct Volume

Animals were euthanized by decapitation at 24 h or one week following MCAO, and the brains were removed for assessment of infarct volume. The brains were washed in chilled saline at 4°C for 5 min and then cut into 2 mm thick slices using a Brain Matrix (Harvard Apparatus, Holliston, MA). The sections were incubated at 37°C for 15 min in 2% 2,3,5-triphenyltetrazolium (TTC, Sigma, St. Louis, MO) dissolved in saline. The stained sections were then stored in 10% formalin at 4°C for further analysis [20]. Infarct volume was calculated by sampling each side of the coronal sections with a digital camera (Nikon). The infarct area, outlined in white, was measured by image analysis software (Adobe Photoshop CS4). Edema was determined by taking the percent increase in size of the ischemic over the contralateral hemisphere [21]. Total infarct size is expressed as infarct volume in mm<sup>3</sup> as the sum of infarct areas in each slice, corrected for edema.

# Estimation of [<sup>125</sup>I]ET-1 and [<sup>125</sup>I]IRL1620 binding in the rat brain

Rats were sacrificed and brains were quickly excised. The cerebellum was removed and remainder of the brain was washed in chilled saline. The brain was homogenized in 30 ml of Tris-HCl buffer (50 mM, pH 7.4), using a Brinkman polytron homogenizer (setting 6 for 30 s). The homogenate was centrifuged at 49.000 × *g* for 20 min in a refrigerated Sorvall RC-5B centrifuge (4 °C). The supernatant was discarded and the pellet was resuspended in 30 mL of Tris-HCl buffer and centrifuged again at 49.000 × *g* for 20 min. The pellet was re-suspended in 30 mL of Tris-HCl buffer, homogenized again, and centrifuged for the third time at 49.000 × *g* for 20 min. The supernatant was discarded and the pellet used for binding studies. The amount of protein used in each tube was 40–50 µg. Concentration of protein in the samples was determined by using the Precision Red Assay [23].

# Estimation of binding to ET<sub>A</sub> receptors in the rat brain using [<sup>125</sup>I]ET-1

The binding of [125I]ET-1 to ET<sub>A</sub> receptors was carried out in a total volume of 0.50 mL, which contained 0.35 mL of homogenate (containing 40-50µg protein) and 50 mM Tris-HCl buffer (pH 7.4). The final concentration of [125I]ET-1 was 0.5 nM in each tube [22]. The concentration range for BQ123, the ET<sub>A</sub> receptor antagonist, was 0-885.74 nM and a high concentration of 15 µM BQ123 was used as the displacer to determine non-specific binding. All binding assays were done in triplicate and samples were incubated at 37°C for 60 min. Binding was terminated by rapidly filtering the contents of the incubation tubes through Whatman GF/B glass fiber filters under reduced pressure using a Brandell cell harvester (model M-24R). The filters were washed 3 times with 5 mL of 50 mM Tris-HCl buffer (pH 7.4). The filters were transferred to plastic tubes and the radioactivity in all samples was determined using a Perkin Elmer gamma counter (model U5003). Total binding for ET<sub>A</sub> receptors was defined as binding observed in presence of 0-885.74nM of BQ123 and expressed as fmol/mg protein (mean ± SEM). Non-specific binding was determined in presence of high concentration of BQ123 (displacer 15µM). Specific binding was calculated by subtracting non-specific binding from total binding.

## Estimation of binding to $ET_B$ receptors in the rat brain using [<sup>125</sup>I]IRL-1620

The binding of  $[^{125}I]$ IRL1620 and  $[^{125}I]$ ET-1 was carried out in a total volume of 0.50 mL, which contained 0.35 mL of homogenate (40–50 µg protein) and 50 mM Tris-HCl buffer (pH 7.4). The final concentration of  $[^{125}I]$ IRL1620 was 0.39 nM in each tube [22]. The concentration range for IRL1620 was 0–32 nM and a

high concentration of 1  $\mu$ M IRL1620 was used as the displacer to determine non-specific binding. All binding assays were conducted using the procedure in the subsection on ET<sub>A</sub> receptor binding. Total binding for ET<sub>B</sub> receptors was defined as binding observed in presence of 0–32 nM of IRL1620 and expressed as fmol/mg protein (mean ± SEM). Non-specific binding was determined in presence of high concentration of IRL1620 (displacer 1  $\mu$ M). Specific binding was calculated by subtracting non-specific binding from total binding.

# Calculation of binding affinity ( $K_d$ ) and receptor density ( $B_{max}$ ) in the rat brain

The binding affinity ( $K_d$ , the dissociation constant commonly used to describe the affinity between a ligand and a receptor) was determined using the following equation:

The receptor density ( $B_{max}$ , the total density or concentration of receptors in the brain tissue was determined using the following equation:

$$B_{max} = \frac{Top - Bottom}{Fractional \ Occupancy} = \frac{Top - Bottom}{[Radioligand]/(K_d + [Radioligand])}$$

### Estimation of ET receptors in the rat brain

ET<sub>A</sub> and ET<sub>B</sub> receptor protein levels in the infarcted brain were measured via Western blotting. Animals were decapitated at either 24h or 7 days post MCAO, and the brains, sectioned into right (infarcted) and left (non-infarcted) hemispheres, were flash frozen and stored at -80°C. The tissue was homogenized in 10x (w/v) RIPA lysis buffer, and protein concentration was determined according to the Lowry method, using bovine serum albumin as standard [24]. Protein (20µg) was denatured in Laemmli sample buffer and resolved in 10% SDS-PAGE, and then transferred onto nitrocellulose membrane. After blocking, The membranes were incubated with rabbit polyclonal anti-ET<sub>A</sub> and anti-ET<sub>B</sub> antibodies (1:1000) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C, followed by 1.5 h incubation with by HRP-conjugated secondary antibodies (1:2000; Cell Signaling Technology, Inc., MA) at room temperature. β-actin (1:2000; Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control. The labeled proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) using the Kodak Gel Logic 1500 Imaging System (Carestream Health Inc., New Haven, CT). Protein expression was analyzed using Image [ (NIH) software.

 Table 1
 Neurological and motor deficits at baseline, Day 1, Day 4, and Day 7 following induction of cerebral ischemia. Values are expressed as Mean±S.E.M.,

 N = 4 each group. \*\*\* P<0.001 significant change compared to sham (control) group. \*\* P<0.01 significant change compared to sham (control) group. \* P<0.05 significant change compared to sham (control) group.</td>

	Group	Baseline	Day 1	Day 4	Day 7	
Neurological Evaluation	Sham	0±0	0 ± 0	0 ± 0	0 ± 0	
	MCAO	0±0	2.25±0.25***	2.75±0.25***	3.00±0.41 ***	
Grip Test	Sham	4.25±0.25	$4.00 \pm 0.00$	$4.00 \pm 0.41$	$3.75 \pm 0.48$	
	MCAO	3.75±0.25	1.50±0.50 **	1.50±0.65 **	0.50±0.50 ***	
Foot Fault Error (%)	Sham	4.24±1.16	$4.55 \pm 0.80$	$3.09 \pm 1.50$	$3.50 \pm 2.36$	
	MCAO	$5.42 \pm 2.08$	47.68±11.19 ***	43.54±6.53 ***	35.27±6.71**	
Rota Rod Duration (sec)	Sham	103.25±11.10	145.25±10.70	146.25±11.99	124.25±13.62	
	MCAO	111.75±6.14	26.25±9.94***	56.50 ± 26.61 * *	47.50 ± 28.99 *	

### Statistical analysis

Statistical analysis of binding affinity ( $K_d$ ) and receptor density ( $B_{max}$ ) was conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical differences between groups for receptor binding studies were analyzed using Students t-test. Statistical differences between groups for behavioral studies were analyzed using a one-way ANOVA followed by post-hoc test (Bonferroni's Test). A level of P<0.05 was considered significant.

### Results

### Effect of MCAO on neurological and motor function

The induction of permanent cerebral ischemia resulted in a marked neurological and motor function deficit (**• Table 1**). Animals undergoing right MCAO presented with paresis and weakness of their left limbs lasting up to 7 days following ischemia as measured by the neurological and grip tests. Similarly, motor function and coordination were impaired in animals following MCAO as demonstrated by an over 35% foot fault error (P<0.01) and a decreased ability to remain on the rotating spindle of the rota rod (P<0.05).

### Infarct Volume

Animals subject to MCAO presented with significant infarct volumes as detected by TTC staining (**• Fig. 1**). After 24 h, occluded animals had infarct volumes of 126.41±40.12 mm<sup>3</sup>. The size of the infarct increased in animals subject to 7 days of permanent MCAO, with volumes of 152.82±21.67 mm<sup>3</sup>.

### Effect of MCAO on ET<sub>A</sub> receptor binding characteristics

After 24h of permanent cerebral ischemia, the density ( $B_{max}$ ) of ET<sub>A</sub> receptors increased significantly in the infarcted hemisphere (**• Fig. 2a**; P<0.01). At the same time, the K<sub>d</sub> for ET<sub>A</sub> receptors decreased from 16.38±2.15 nM to 7.69±1.05 nM (P<0.01) in the infarcted hemisphere of the sham and MCAO groups, respectively. Conversely, by day 7 of permanent MCAO, the density of ET<sub>A</sub> receptors had returned to normal levels, while the K<sub>d</sub> was increased (**• Fig. 2b**; P<0.05) in the infarcted hemisphere of occluded animals as compared to sham.

### Effect of MCAO on ET<sub>B</sub> receptor binding characteristics

There was no significant difference in either  $B_{max}$  or  $K_d$  of  $ET_B$  receptors after 24h of permanent cerebral ischemia (**© Fig. 3a**). By 7 days post MCAO, however,  $B_{max}$  for  $ET_B$  receptors in the



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**Fig. 2** Binding affinity ( $K_d$ ) and receptor density ( $B_{max}$ ) of ET<sub>A</sub> receptors in the left and right cerebral hemisphere in male Sprague Dawley rats **a** 24-h and **b** 7 days following MCAO. Values are expressed as Mean ± S.E.M, N = 4 each group. # P<0.01 significant change compared to sham (control) group.



**Fig. 3** Binding affinity ( $K_d$ ) and receptor density ( $B_{max}$ ) of ET<sub>B</sub> receptors in the left and right cerebral hemisphere in male Sprague Dawley rats **a** 24-h and **b** 7 days following MCAO. Values are expressed as Mean ± S.E.M, N=4 each group. \* P<0.05 significant change compared to sham (control) group. #P<0.0001 significant change compared to stroke (MCAO) left hemisphere.

infarcted hemisphere of the stroked animals was significantly higher (**• Fig. 3b**; P<0.001) than that of sham animals. K<sub>d</sub> in the infarcted hemisphere was  $3.30\pm0.03$  nM, significantly lower than  $9.60\pm0.20$  nM of the comparable hemisphere in the sham group (P<0.001). Interestingly, the contralateral hemisphere of MCAO animals 7 days following occlusion showed a similar, albeit lesser pattern in both density and binding as the ipsilateral hemisphere with a rise in B<sub>max</sub> (P<0.05) and a decrease in K<sub>d</sub> (P<0.001) as compared to sham. The changes in the right hemisphere (ischemic) were highly significant compared to left hemisphere (non-ischemic) of the rats with MCAO.

### Effect of MCAO on ET receptor protein expression

Protein expression of brain  $ET_A$  receptors was significantly increased in the infarcted hemisphere at 24 h after permanent MCAO (**• Fig. 4**; P<0.001). By 7 days of permanent cerebral ischemia, however,  $ET_A$  receptor expression in the infarcted hemisphere returned to normal levels, showing no significant difference when compared to either the sham group or the contralateral hemisphere of the stroked group. Brain  $ET_B$  receptor protein expression did not vary significantly between either the sham and stroked animals or the infarcted/contralateral hemispheres at both 24 h and 7 days post MCAO (**• Fig. 5**).

### Discussion

▼

This is the first study to show how the binding characteristics of ET receptors alter over time in a brain subject to ischemia. Specifically, the density and binding affinity of  $ET_A$  receptors are high in the infarcted areas of the brain at 24h post ischemia, while  $ET_B$  receptor binding characteristics are unaltered compared to sham. After 7 days of permanent ischemia, however,  $ET_A$  receptor binding characteristics are similar to sham, whereas  $ET_B$  receptor density and binding affinity significantly increase. These differences in the pathophysiology of the central ET system at various stages of ischemic stroke may help to optimize novel treatments for this disease.

In the present study, we examined the binding characteristics of both  $ET_A$  and  $ET_B$  receptors in the brain during both the acute (24h) and sub-acute (7 days) stages of ischemic stroke. Whereas the acute phase is hallmarked by a strong oxidative stress reaction, the sub-acute phase is characterized by microvascular damage, inflammation and a breakdown in the blood brain barrier, resulting in significant cerebral edema [25,26]. During this stage, the brain also begins its long road to recovery, initiating angiogenesis, neurogenesis and neuroblast migration towards the ischemic boundary [27]. It has previously been reported that



**Fig. 4** ET<sub>A</sub> receptor protein expression in the left and right cerebral hemisphere in male Sprague Dawley rats 24-h (left panel) and 7 days (right panel) following MCAO. Lane 1 – Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – MCAO (LH); Lane 5 – MCAO (RH). LH = Left hemisphere; RH = Right hemisphere. Values are expressed as Mean ± S.E.M., N = 4 each group. #P<0.0001 significant change compared to sham (control) group. \* P<0.0001 significant change compared to stroke (MCAO) LH.



**Fig. 5** ET<sub>B</sub> receptor protein expression in the left and right cerebral hemisphere in male Sprague Dawley rats 24-h (left panel) and 7 days (right panel) following MCAO. Lane 1 – Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – MCAO (LH); Lane 5 – MCAO (RH). LH=Left hemisphere; RH=Right hemisphere. Values are expressed as Mean  $\pm$  S.E.M., N=4 each group. No significant change in ET<sub>B</sub> receptor protein expression was observed between left and right hemispheres in both sham (control) and stroke (MCAO) groups.

ET-1 levels are elevated during the first 24h following an ischemic attack, correlating with the relative size of the infarct [1]. Here, we show that the  $B_{max}$  increases while  $K_d$  decreases for ET<sub>A</sub> receptors in the infarcted hemisphere during the first 24h following MCAO in rats. Additionally, we demonstrate that ET<sub>A</sub> receptor protein expression is significantly elevated after the initial 24 h of cerebral ischemia. Changes in ET<sub>A</sub> receptor characteristics in the first 24h after ischemia along with an increase in ET-1 may lead to severe vasoconstriction of cerebral blood vessels. One week after infarct, ET<sub>A</sub> receptor density returns to normal, with lower binding affinity and a normal protein expression. High levels of ET-1 and its subsequent stimulation of ET<sub>A</sub> receptors have been implicated in delayed hypoperfusion, excitotoxicity, blood brain barrier disruption and inflammation following cerebral ischemia [28]. For this reason, several studies have been conducted using ET<sub>A</sub> antagonists as a treatment for ischemic stroke in animal models [9,12,21]. Although ET<sub>A</sub> antagonists have proven relatively beneficial in these models, combination  $ET_A$  and  $ET_B$  antagonists have not [8]. These results point to the differing roles which ET<sub>A</sub> and ET<sub>B</sub> receptors may play in the pathogenesis and/or recovery of cerebral ischemia.

There was no alteration in the binding characteristics of  $ET_B$  receptors in the rat brain at 24h after cerebral ischemia as compared to sham group. After 7 days, however,  $B_{max}$  was increased and  $K_d$  was decreased for  $ET_B$  receptors, indicating an increase in the density and binding affinity for these receptors during the sub-acute phase of stroke. A small but significant increase in

B<sub>max</sub> and decrease in K<sub>d</sub> values was observed in the contralateral hemisphere. Minor but significant changes observed on the contralateral side could be due to the mild hypoxia from surgery involving manipulation of the common carotid artery to produce an occlusion of the middle cerebral artery. The changes in the ischemic hemisphere were highly significant compared to non-ischemic hemisphere. This indicates that an increase in the density and affinity of ET<sub>B</sub> receptors is specifically due to ischemia and could play an important role in attenuating damage to the brain or assisting in neurovascular recovery. Interestingly, however, we did not observe any change in ET<sub>B</sub> receptor protein expression as measured by Western blot at either 24 h or 7 days post MCAO. While these findings seemingly contradict the results of the binding characteristics, they do coincide with previous reports on ET<sub>B</sub> protein expression following permanent cerebral ischemia [2, 13, 14]. A major difference between binding studies and Western blot analysis is the use of ET<sub>B</sub> agonist, IRL1620. In binding studies, we used IRl1620 to label the receptors and also to displace the radiolabeled IRl1620 from its specific receptors. On the other hand Western blots do not involve use of any ET<sub>B</sub> agonists, and specific antibodies are used to determine the expression of ET<sub>B</sub> receptors.

The lack of change in  $ET_B$  receptor expression in the present Western blot studies can be explained based on the fact that there is no explicit stimulation of the  $ET_B$  receptors by IRL 1620.  $ET_A$  receptors are outer membrane bound, while  $ET_B$  receptors are localized to both the outer membrane and intracellular compartments [29].  $ET_B$  receptors also desensitize faster than  $ET_A$  receptors [29]. Internal  $ET_B$  receptors are recycled to the cell membrane in the presence of  $ET_B$  agonist, IRL1620; therefore, changes in  $ET_B$  receptors can be detected in receptor binding studies when IRL1620 is used as a ligand. In the absence of IRL1620 (such as in the Western blotting studies),  $ET_B$  receptors remain in the intracellular compartments. It is possible that the internal receptors are not fully converted to  $ET_B$  agonist. Similar findings have been previously observed where upon stimulation of  $ET_B$  receptors by IRL1620, the expression of  $ET_B$  receptors is enhanced as detected in Western blot studies [13]. Therefore, it appears that recycling of  $ET_B$  receptors may be responsible for the differences observed in  $ET_B$  receptors in receptor expression and receptor binding studies.

Previous studies conducted in our laboratory have demonstrated that selective stimulation of  $ET_B$  receptors via agonist, IRL-1620, leads to a significant reduction in infarct volume, oxidative stress and neurological and motor deficits during both the acute and sub-acute stages of cerebral ischemia in rats [2,13]. During the sub-acute phase of ischemic stroke, stimulation of  $ET_B$  receptors resulted in an increase in vascular and neuronal growth factors, highlighting the beneficial role these receptors may play in neurovascular repair and recovery following cerebral ischemia [14]. These studies, combined with the present results, indicate that selective  $ET_B$  receptor stimulation may be a novel therapeutic tool in the treatment of ischemic stroke.

#### Conclusions

This is the first study demonstrating that the binding characteristics of ET receptors are altered during the acute and sub-acute stages of cerebral ischemia. Specifically, the binding affinity and density of  $ET_A$  receptors are high following the first 24h of ischemia, but return to normal levels at the later stages.  $ET_B$ receptor binding affinity and density, on the other hand, are not altered in the first 24h, but significantly increase by 7 days of permanent cerebral ischemia, indicating the role these receptors may play in the recovery of the brain following ischemic stroke. We speculate that following cerebral ischemia in the initial phase antagonism of  $ET_A$  receptors and in the later stage stimulation of  $ET_B$  receptors may prove to be beneficial.

**Conflict of interest** 

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The authors have no conflict of interest to disclose.

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