The effect of endothelin receptor A antagonism on basilar artery endothelium-dependent relaxation after ischemic stroke

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Aims: Endothelin (ET) receptor A antagonism decreases neuronal damage in experimental models of stroke. Since large arteries like basilar artery contribute significantly to total cerebrovascular resistance and are major determinants of microvascular pressure, dysregulation of basilar artery function may worsen stroke injury. ET-1 is involved in the regulation of basal constriction. However, whether stroke influences vasoreactivity of basilar artery and to what extent ET-1 contributes to basilar vascular dysfunction after stroke remained unknown. The goal of this study was to test the hypothesis that ET-1 impairs basilar artery vasorelaxation after ischemia/reperfusion (I/R) injury via activation of ETα receptor.

Main methods: Male Wistar rats were subjected to 3 h middle cerebral artery occlusion (MCAO) and 21 h reperfusion. One group received ETα receptor antagonist atrasentan (5 mg/kg, i.p.) at reperfusion. At 24 h, basilar arteries were isolated from control non-stroked, stroked and stroked+atrasentan-treated animals for vascular reactivity measurements using pressurized arteriograph.

Key findings: Acetylcholine (Ach)-induced maximum relaxation (Rmax) was decreased in stroked animals as compared to non-stroked group and ETα antagonism partially restored it. There was also a trend for decreased EC50 value for the antagonist treatment group indicating improved Ach sensitivity.

Significance: These findings suggest that I/R not only affects vessels distal to the occlusion but also impairs relaxation of proximal large vessels. ET-1-mediated basilar artery dysfunction may contribute to neurovascular damage after stroke and early restoration of vascular function by ET receptor antagonism after I/R injury may offer a therapeutic strategy.

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Introduction

Vascular endothelium plays an important role in regulating vascular tone, coagulation and smooth muscle growth. Endothelin-1 (ET-1) generated by vascular endothelial cells and vascular smooth cells is a potent vasoconstrictor with mitogenic and profibrotic properties (Inoue et al., 1989). ET-1 can exert opposing vascular effects via two G protein-coupled receptor subtypes, ETα and ETβ. Activation of ETα receptors on vascular smooth muscle cells lead to vasoconstriction, while stimulation of ETβ receptors on endothelial cells promote vasodilation via stimulation of nitric oxide production (Edvinsson and Povlsen, 2011; Ergul, 2002; Haynes and Webb, 1998). Therefore, abnormal regulation of ET-1 system contributes to the pathophysiology of various cardiovascular diseases including ischemia/reperfusion (I/R) injury (Ergul, 2002). Many studies showed increased ET levels after ischemic stroke both in patients and experimental models (Barone et al., 1994; Thampatty et al., 2011; Ziv et al., 1992) and that ETα receptor antagonists have neuroprotective effects in both mechanical and embolic models of stroke (Matsuo et al., 2001; Patel et al., 1996). Whether this effect is due to direct neuronal protection and/or improvement of vascular function is not fully understood.

Cerebrovascular autoregulation is crucial for maintaining constant blood flow during changes of perfusion pressure (Owens, 2011). In the brain, large extracranial and intracranial arteries (e.g., basilar arteries) contribute significantly to vascular resistance which attenuates changes in downstream microvascular pressure during increases in systemic arterial pressure (Cipolla, 2009). Therefore any alterations in the reactivity of those vessels may lead to disturbance of this protective mechanism and could contribute to cerebrovascular disease. Accordingly, the goals of the current study were to determine: 1) the impact of reperfusion injury on ET-1-mediated vasoconstriction and endothelium-dependent relaxation of basilar arteries; and 2) the role of ETα receptors in this response in an experimental model of stroke.
Materials and methods

Animals and experimental groups

This study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Division of Laboratory Animal Services at the Georgia Health Sciences University. Male Wistar rats (Harlan Laboratories, Indianapolis, IN) were housed four per cage on a 12-h light/dark cycle.

Model of ischemia and drug treatment

Experimental groups included 1. Control (C, no stroke, n = 8), 2. Stroke (S, stroke + vehicle, n = 10), and 3. Atrasentan (A, stroke + atrasentan, n = 8). Transient middle cerebral artery occlusion (MCAO) was induced as previously described (Ergul et al., 2007). The cerebral perfusion was measured with a PIM3 laser Doppler scanning system (Perimed, Stockholm, Sweden) to evaluate basal perfusion as well as perfusion changes following MCAO to confirm successful occlusion. At the end of ischemia (3 h MCAO), the animal was briefly anesthetized, atrasentan (5 mg/kg) was given by intraperitoneal injection, and reperfusion was initiated by filament withdrawal.

Pressurized arteriograph system and basilar artery preparation

At 24 h after ischemia, the animals were anesthetized and perfused with 100 ml of 0.01 M phosphate buffered saline (PBS). Basilar arteries isolated from control, stroked or stroked-treated rats were mounted on glass cannulas in an arteriograph chamber containing 5 ml HEPES buffer (NaCl, 130 mM; KCl 4 mM; MgSO4 1.2 mM; NaHCO3 4 mM; HEPES 10 mM; KH2PO4 1.18 mM; Glucose 5.5 mM) and the vessels were pressurized at 60 mmHg for 1 h. The Living Systems arteriograph chamber was connected to a temperature controller that kept HEPES buffer at a constant temperature 37 ± 0.5 °C during the whole experiment. Lumen diameter was measured with a video dimension analyzer both at the beginning and at the end of the 1 h equilibration.

Vascular reactivity studies

Basilar lumen diameters of control, stroked and stroked rats treated with atrasentan were measured after 2 min of exposure to increasing concentrations of ET-1 (2 × 10^-9, 10^-8, 2 × 10^-8, 10^-7, 1.5 × 10^-7, 2 × 10^-7 and 5 × 10^-7 M) to determine the impact of ischemia and the treatment on the contractility of the basilar artery. At the end of ET dose response, the endothelium dependent relaxation was assessed by measuring the lumen diameter in response to increasing concentrations of acetylcholine (Ach) (2 × 10^-10 - 2 × 10^-5 M). In preliminary experiments, when higher doses of ET-1 were used to reach a plateau, vessels were either unresponsive or did not relax in response to the following Ach treatment. Therefore, dose–response curve to ET-1 was performed up to 5 × 10^-7 M. Maximum response (Rmax) and EC50 values were calculated.

Infarct and edema analysis

At 24 h after ischemia, after the isolation of basilar arteries for vascular function studies, brains were cut into 2-mm slices and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 5 min at 37 °C. The image of the slices was scanned and the infarct volume was analyzed as previously described (Ergul et al., 2007). Total infarct volume was calculated as percent volume of the total ischemic hemisphere. Edema is reported as the percent increase in ischemic hemisphere size to the contralateral hemisphere.

Statistical analysis

A repeated-measures analysis of variances was used to determine group differences (control versus stroked versus stroked-treated) across ET-1 or Ach concentrations. Post-hoc group comparisons at each concentration used a Tukey’s adjustment for the multiple comparisons. Unpaired two-tailed t-test was used to compare infarct size and edema between groups. Effects were considered statistically significant at p < 0.05. Graphpad Prism 5 was used for all statistical tests performed. Results are expressed as the means ± SEM.

Results

Vascular reactivity

The sensitivity (EC50) and the contractile response to at 500 nm ET-1 (maximum dose studied) were similar among the three groups (Fig. 1A, B). Stroked rats exhibited impaired endothelium-dependent relaxation following preconstriction with ET-1. Atrasentan treatment caused a leftward shift and improved relaxation compared to the stroked rats (Fig. 2A). Treatment with atrasentan did not only improve endothelium-dependent relaxation but also increased the maximum response (Fig. 2B). There was also a trend for decreased EC50 value for the antagonist treatment group indicating improved Ach sensitivity (Fig. 2C).

Infarct volume and edema

Treatment with atrasentan had no effect on infarct size or edema as both parameters were similar in both groups (Fig. 3A, B). To ensure that the drop in cerebral blood flow (CBF) at MCAO is similar across the study groups and also to determine whether there are differences in CBF in the post-reperfusion period, the cerebral perfusion was...
measured with a scanning Doppler imaging system at different time points (ischemia, right before reperfusion when antagonist was administered and after reperfusion). Compared to baseline, there was approximately a 50% decrease in cerebral perfusions in both vehicle and atrasentan-treated rats and recovery after reperfusion was similar across the groups (Fig. 3C).

Discussion

The present study demonstrates that while temporary focal brain ischemia does not affect ET-1-mediated vasoconstriction of basilar arteries, it impairs the ability of cerebral arteries to relax. Moreover, acute treatment with atrasentan, an ET_A receptor antagonist, given right before reperfusion restored endothelium-dependent vasodilation. The current study also provides evidence that acute short-term use of atrasentan is not sufficient to reduce infarct size or edema. Taken together, these findings suggest that I/R injury causes cerebrovascular dysfunction of vessels upstream of occlusion in an ET-1-dependent manner and that the role of ET-1 in the pathogenesis of cerebral ischemic injury is complex.

Regulation of cerebrovascular resistance and ultimately CBF is critical for proper brain function (Faraci and Heistad, 1990). Temporary interruption of CBF as occurs in stroke has important implications. The current treatment strategies focus on the reestablishment of CBF as soon as possible to prevent infarct expansion but it is also known that reperfusion causes significant tissue damage (Li et al., 2010; Takahashi et al., 1997). Early hyperperfusion following ischemia contributes to blood brain barrier disruption and edema (Sage et al., 1984). This period is usually followed by post-ischemic hypoperfusion and multiple mechanisms including increased vascular smooth muscle tone of downstream microvessels (Cipolla and Bullinger, 2008; Cipolla et al., 2004; Takahashi et al., 1997). In an early study, Takahashi et al. elegantly demonstrated that vascular smooth muscle of the microvasculature in particular is highly influenced by I/R injury (Takahashi et al., 1997). It is also known that both microvasculature and macrovasculature are equally involved in control of cerebrovascular resistance (Cipolla and Bullinger, 2008;
Faraci and Heistad, 1990). While significant effort was directed towards how ischemia induced by occlusion of a vessel modifies structure and function of the downstream microvasculature and ultimately cerebral perfusion (Cipolla and Bullinger, 2008; Coulson et al., 2002), the impact on upstream vessels is relatively understudied. Therefore, in the current study we investigated the vascular reactivity of basilar arteries after I/R. Given that ET-1 is one of the most potent vasoconstrictor identified to date and that ET receptor antagonism decreases ischemic injury (Gupta et al., 2005; Matsuo et al., 2001; Patel et al., 1996; Zhang et al., 2008), we focused on ET-1-mediated vascular reactivity. While there was no difference in ET-1-mediated vasoconstriction under control (no stroke) and stroke (vehicle or atrasentan-treated) rats, the endothelium-dependent vascular relaxation was significantly impaired by I/R and ET_A receptor antagonism partially prevented this effect. We studied vascular reactivity 21 h after the antagonist administration. Thus, our data suggests that acute ET receptor antagonism has a long-lasting effect on vascular function. It is highly possible that at early time points after drug administration the restoration of vascular relaxation can be greater. Another possibility is the upregulation of ET_B receptors after ischemia. While we did not investigate the effect of ET_B receptor blockade on basilar artery reactivity, several studies demonstrated that ischemia upregulates ET_B receptors on vascular smooth muscle cells (Henriksson et al., 2003; Stensen et al., 2002). Therefore, it is highly possible that combined ET_A and ET_B receptor blockade may fully restore large artery dysfunction following I/R injury and that needs to be tested.

Several lines of evidence suggest that ET-1 contributes to ischemic brain injury. First, direct administration of ET-1 into the brain causes severe vasoconstriction and significant reductions in CBF inducing stroke (Macrae et al., 1993). Second, both experimental and clinical studies reported increased plasma, cerebrospinal fluid and tissue ET-1 levels following I/R (Barone et al., 1994; Lampl et al., 1997; Loo et al., 2002). Not only vasoactivity but also neuronal and glial ET-1 and ET receptors are altered following ischemia (Loo et al., 2002). Third, gain and loss of function approaches indicated an important role of ET-1 in ischemic brain injury. While endothelial over expression of ET-1 exacerbates infarct size and edema (Leung et al., 2009), with the exception of one study (Chuquet et al., 2002), pharmacological blockade of ET receptors in various experimental models of stroke reduces neurovascular injury and improves outcomes. Chuquet and colleagues demonstrated that selective ET_A receptor blockade by direct injection into the brain, reduces CBF and exacerbates neuronal injury (Chuquet et al., 2002). Gupta et al., however, reported that dual ET_A and ET_B antagonist TAK-044 pretreatment reduces ischemic injury and this is associated with reduced oxidative markers and improved functional outcomes (Gupta et al., 2005). Another study demonstrated that selective ET_A blockade given for 24 h starting 10 min, 1 or 3 h after middle cerebral artery occlusion decreases ischemic injury (Matsuo et al., 2001). A more recent study showed that when given with the tissue plasminogen activator (tPA), the only FDA-approved therapeutic for stroke treatment, ET_A antagonism reduces the risk of bleeding and provides neurovascular protection in an embolic model of stroke (Zhang et al., 2008). In all these past studies, ET receptor blockade was administered either before stroke as a preventive measure or continuously during the reperfusion period varying from 22 to 72 h. The current study aimed to determine the acute use of ET receptor blockade and ET_A antagonist was given once right before reperfusion with neurovascular injury being evaluated 21 h later. We did not find any differences in infarct size or edema between the groups. This may be due to the acute and the one-time administration of the antagonist. Use of the receptor antagonist prior to reperfusion may reduce neuronal injury. It is may also be due to the greater magnitude of injury induced by 3 h MCAO used in the current study. In a less severe model of ischemia, differences between vehicle and antagonist-treated groups may be easier to detect.

There are several limitations that need to be addressed. It has to be recognized that the current study used healthy and young animals. Even in this group, I/R injury influenced vascular function. We and others have reported under disease states like diabetes, ET-1 causes hyperactivity of basilar arteries and impairs endothelial function (Harris et al., 2008; Matsumoto et al., 2004). Given that diabetes is a risk factor for stroke, the impact of ischemic injury on cerebrovascular function in diabetes needs to be further investigated. We also reported that diabetes alters ET receptor expression (Kelly-Cobbs et al., 2011). As such, role of both ET_A and ET_B receptors in the regulation of vascular reactivity should be studied. The current study did not include functional outcome of stroke but based on the infarct sizes we did not anticipate any differences in neurological function. We also acknowledge that we did not study the effect of ET receptor blockade on microvascular responses but rather focused on basilar arteries that significantly contribute to the regulation of cerebrovascular resistance. Due to the shorter viability of basilar arteries after ischemic injury, we were able to conduct only ET_A and Ach dose response curves and could not assess endothelium-independent vascular responsiveness in the limited number of vascular segments we can isolate from one animal. Nevertheless, this study provides important information for the use of ET receptor antagonists in the setting of stroke. While the acute use of ET_A receptor blockade is able to restore vascular dysfunction, this does not translate into reduction of neuronal injury suggesting that the role of ET-1 in ischemic injury is more complex. Therefore, extended use of ET_A receptor antagonist in the hours following stroke is more desirable to reduce injury and improve outcomes.

Conclusion

The present study provides evidence that, in this model of I/R injury, ET-1 plays an important role in the impairment of endothelium dependent relaxation of cerebral vessels and that acute treatment with ET_A receptor antagonist could improve vascular function.

Conflict of interest statement

There are no conflicts of interest.

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