Research Report

Effect of aminoguanidine on post-ischemic brain edema in transient model of focal cerebral ischemia

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ABSTRACT

Pervious experimental studies have shown that aminoguanidine (AG) is beneficial in the late phase of cerebral ischemia. Recently, it has been reported that AG reduces cerebral edema in traumatic brain injury. However, the effects of AG on post-ischemic cerebral edema and blood–brain barrier (BBB) permeability are not clear. Under chloral hydrate anesthesia, transient focal cerebral ischemia was induced in rats by 60 min of middle cerebral artery occlusion (MCAO), followed by 23 h of reperfusion. Saline as vehicle or AG at the doses of 75, 150 and 300 mg/kg, i.p., was administered at the beginning or at 1 or 3 h after induction of ischemia. Subsequently, 24 h after MCAO brain edema, BBB permeability and infarct volume were evaluated. Administration of AG (150 mg/kg) at the beginning or at 1 or 3 h after MCAO, significantly reduced cerebral edema (P < 0.001), while AG at the doses of 75 and 300 mg/kg had no effect. Moreover, treatment with AG (150 mg/kg) significantly reduces Evans Blue extravasation by 48% into ischemic brain compared to the saline group (P < 0.001). Additionally, AG at the doses of 75 and 150 mg/kg significantly reduces cortical and striatal infarct volumes (P < 0.001), while AG at the dose of 300 mg/kg did not change striatal infarct volumes (P > 0.05). Our findings show that AG significantly reduced post-ischemic increase of brain edema with a 3-h therapeutic window in the transient model of focal cerebral ischemia. Moreover, it seems that at least part of the anti-edematous effects of AG is due to decrease of BBB disruption.

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1. Introduction

Aminoguanidine (AG) is a small, water-soluble compound currently being used for the prevention of chronic tissue complications of diabetes mellitus in humans (Abdel-Rahman and Bolto, 2002). In experimental studies, AG was shown to prevent diabetic nephropathy (Taguchi et al., 2002), retinopathy (Hammes et al., 1991), and neuropathy (Yagihashi et al., 1992). Moreover, several experimental studies have shown that AG, as an inhibitor of inducible nitric oxide synthase (iNOS), reduces ischemic injuries in animal models of stroke (Zhang et al., 1996; Takizawa et al., 1999; Cash et al., 2001; Sugimoto and Iadecola, 2002). Findings of the said studies mainly have demonstrated that administration of AG between 24 and 96 h after ischemia attenuates post-ischemic iNOS activity and reduces the size of infarct volume (Iadecola et al., 1995a; Zhang and Iadecola, 1998; Nagayama et al., 1998; Takizawa et al., 1999; Sugimoto and Iadecola, 2002). Moreover, a few studies have shown that AG has beneficial effects in the early phase of permanent (Cockroft et al., 1996) or transient model of focal cerebral ischemia (Cash et al., 2001). Recently, it has been reported that administration of AG immediately after ischemia notably reduces cell damage

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in a global model of cerebral ischemia (Danielisova et al., 2004). More recently, Louin et al. (2006) have reported that administration of AG 6 h after post-traumatic brain injury considerably reduces cerebral edema in rat. However, the effects of AG on post-ischemic cerebral edema formation and blood–brain barrier (BBB) permeability have not been investigated. Therefore, the aim of this study was to evaluate the effects of various doses of AG on cerebral edema, BBB permeability and brain injuries in a transient model of focal cerebral ischemia.

2. Results

2.1. Dose-dependent effect of AG on infarct volume

The cortical and striatal infarct volume in saline-treated control animals were 161±11 mm³ and 52±5 mm³, respectively. Peripheral administrations of AG at the doses of 75, 150 and 300 mg/kg at the beginning of middle cerebral artery occlusion (MCAO) significantly reduced cortical infarct volume by 47% (86±12 mm³), 62% (61±9 mm³) and 49% (82±10 mm³), respectively (*P<0.001, Fig. 1). Moreover, AG at the doses of 75 mg/kg (31±5 mm³) and 150 mg/kg (32±4 mm³) significantly reduced striatal infarct volumes (*P<0.01), while a dose of 300 mg/kg did not significantly change striatal infarct volumes (53±4 mm³) compared to the saline group (52±5 mm³) (Fig. 1).

2.2. Dose-dependent and time-course effects of AG on cerebral edema

Measurement of the brain water content (BWC) was used to evaluate cerebral edema. BWC in the left and right hemispheres...
of sham-operated rats was 78.8 ± 0.13% and 78.9 ± 0.12%, respectively. Induction of focal cerebral ischemia in the saline group significantly increased the BWC of the ischemic hemisphere (right) to 82.9 ± 0.36% \((p < 0.001)\), whereas the BWC of the contralateral hemisphere (left) has not been changed significantly (79.1 ± 0.18%) compared to the sham-operated group. As shown in Fig. 3, administration of AG (150 mg/kg) at 1 or 3 h after MCAO significantly reduced the post-ischemic increase of the BWC (80.2 ± 0.3% vs. 82.9 ± 0.36%, \(p < 0.001\)). While AG at the doses of 75 and 300 mg/kg have not significantly changed the BWC of the ischemic hemisphere (81.4 ± 0.32% and 81 ± 0.5% vs. 82.9 ± 0.36%, respectively) \((p > 0.05)\)

As shown in Fig. 3, administration of AG (150 mg/kg) at 1 or 3 h after MCAO significantly reduced the post-ischemic increase of the BWC (80.2 ± 0.3% and 80 ± 0.7% vs. 82.9 ± 0.36%, respectively) compared to the saline group \((p < 0.001)\).

### 2.3. Effect of single dose of AG on blood–brain barrier permeability

Evans Blue (EB) extravasation was used as a marker of BBB breakdown after cerebral ischemia. As shown in Fig. 4, in the saline group, EB content in ischemic hemisphere was 0.94 ± 0.13 \(\mu g/g\) tissue. On the other hand, EB content in the non-ischemic hemisphere was 0.20 ± 0.01 \(\mu g/g\) tissue which is not significantly different from the sham group (0.26 ± 0.02 \(\mu g/g\) tissue). AG administration (150 mg/kg) at the beginning of ischemia reduced EB extravasation into the ischemic brain by 48% compared to the saline group (0.49 ± 0.06 vs. 0.94 ± 0.13 \(\mu g/g\) tissue; \(p < 0.001)\).

### 2.4. Physiological parameters

There were no significant differences in mean arterial pressure, \(P_{CO_2}\), \(P_{O_2}\), and blood pH between saline- (control, 1 ml/kg) and AG- (150 mg/kg) treated groups (Table 1).

### 3. Discussion

The aim of this study was to investigate the effects of AG on cerebral edema, BBB permeability and brain injuries in a transient model of focal cerebral ischemia in rats. Our findings indicated that AG effectively protected the BBB and reduced brain edema formation in a dose-dependent manner. Moreover, this anti-edematous effect of AG persisted for at least up to 3 h after the onset of focal cerebral ischemia.

Results of the first part of this study demonstrated that treatment with AG at the dose of 150 mg/kg at the beginning of ischemia significantly reduced cortical and striatal infarct volume by 62% and 38%, respectively. On the other hand, a higher dose of AG (300 mg/kg) reduced the size of ischemic lesion in cerebral cortex (49%) but had no protective effect on striatum damage. Consisted with our findings, Zhang et al. (1996) have demonstrated that administration of AG at a dose of 300 mg/kg only decreased cortical damage without having any effect on striatum damage in a transient model of focal cerebral ischemia. Furthermore, it has been reported that pre-treatment or early post-treatment with AG at a dose of 160 mg/kg effectively decreased infarct size in a permanent model of focal cerebral ischemia (Cockcroft et al., 1996; Zimmerman et al., 1995). These findings altogether support the results of the present study. Our data show that AG at a middle dose (150 mg/kg) has the best neuroprotective activity but is less effective in a higher dose and might even aggravate ischemic damage. One possible explanation for this finding is that AG at a high dose might inhibit endothelial NOS (eNOS) (Laszlo et al., 1995). Nitric oxide produced by eNOS has beneficial effects in the early stage of cerebral ischemia, probably by producing vasodilatation and by inhibiting platelet aggregation and leukocyte adhesion (Huang et al., 1996). Therefore, eNOS inhibition by AG may lead to a further increase in ischemic area and counteract the protective effect of AG on striatum damage.

In the second part of our study, we exhibited for the first time that the treatment with AG only at a dose of 150 mg/kg significantly reduced post-ischemic increase of brain edema with a 3-h therapeutic window in a transient model of focal cerebral ischemia. This is an interesting finding, indicating that the anti-edematous effects of AG persisted for at least 3 h after post-transient focal stroke. In addition, our data demonstrated that AG at a dose of 150 mg/kg has the best anti-edematous activity, whereas other doses (75 or 300 mg/kg) did not significantly change cerebral edema. These findings are in agreement with those of a recent study showing that AG significantly reduced the cerebral edema in fluid percussion-induced brain trauma (Louin et al., 2006). However, our results are in contrast with another study, indicating that administration of AG 24 h after MCAO did not change neocortical water content (Zhang et al., 1996). In their study, AG (100 mg/kg) was given at 24 h after induction of ischemia and brain edema was evaluated at 96 h after focal cerebral ischemia (Zhang et al., 1996), while in the present study AG’s effects were assessed at 24 h after transient MCAO. A number of experimental studies have reported that brain edema reached its maximum 24 h after ischemia (Hatashita and Hoff, 1990; Lin et al., 1993; Slivka et al., 1995) and brain trauma (Shapira et al., 1988; Louin et al., 2006), and then slowly recuperated. We suggest that part of this discrepancy might be due to the differences in timing of BWC evaluation and in timing of AG administration and applied doses of AG.

The mechanism underlying the anti-edematous effect of AG is not clear. Since AG is a selective inhibitor of iNOS, it is more likely that its anti-edematous effect might relate to its inhibitory effect on the iNOS (Iadecola et al., 1995a). However,

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### Table 1 – Physiological parameters including mean arterial pressure (MAP, mm Hg), \(P_{CO_2}\), \(P_{O_2}\) and \(\text{PaO}_2\) (mm Hg) in saline- (as control) or aminoguanidine- (AG, 150 mg/kg) treated groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>10 min before MCAO</th>
<th>10 min after MCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>AG</td>
<td>Saline</td>
</tr>
<tr>
<td>pH</td>
<td>7.27 ± 0.02</td>
<td>7.28 ± 0.03</td>
</tr>
<tr>
<td>(P_{CO_2})</td>
<td>46 ± 4</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>(P_{O_2})</td>
<td>90 ± 5</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>MAP</td>
<td>79 ± 8</td>
<td>80 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.
it has been demonstrated that iNOS expression does not increase during the first 24 h after focal cerebral ischemia (Cash et al., 2003; Iadecola et al., 1995a; Iadecola et al., 1995b), whereas in this study, AG was administered at the beginning of ischemia and the BWC measured 24 h after ischemia, before iNOS expression increased.

Furthermore, recent studies have reported that nitric oxide produced by iNOS does not participate in the formation of post-traumatic cerebral edema (Louin et al., 2006; Jones et al., 2004). Hence, the anti-edematous effect of AG observed in our study is unlikely related to the inhibition of iNOS and may involve other mechanisms rather than NO pathway. AG has additional pharmacological properties such as free radical scavenging, antioxidant activity (Yildiz et al., 1998; Giardino et al., 1998) and inhibition of diamine oxidase (Nilsson, 1999). It has been demonstrated that free radicals and oxidants have important roles in the development of edema and BBB disruption, and/or expansion of ischemic damage in the animal model of cerebral ischemia (Chan, 1994; Zhang and Ellis, 1990; Nelson et al., 1992; Kondo et al., 1997). Thus, we suggest that part of the anti-edematous effect of AG might be related with these effects. Besides, the effects of AG cannot be a consequence of change in arterial blood pressure, blood gases and rectal temperature because these variables were carefully monitored and no difference was found among the groups that were studied.

We also have shown that treatment with AG (150 mg/kg) given at the start of ischemia significantly reduced the extravasation of EB 24 h after post-ischemic damage. This may indicate that AG reduces brain edema through decreasing BBB permeability, and thus may show the vasogenic origin of the edema. However, further experiments are needed to determine the origin (vasogenic vs. intracellular) of the edema. BBB breakdown occurred in early phase (within 24 h) of focal cerebral ischemia (Ding-Zhou et al., 2002; Kondo et al., 1997; Yang et al., 1999). Mechanism of altered BBB permeability during brain ischemia is not completely clear. There are some evidence indicating that production of free radical and reactive oxygen species in acute phase of cerebral ischemia plays an important role in the destruction of endothelium and the opening of BBB (Kondo et al., 1997). AG is a scavenger of free radical (Yildiz et al., 1998; Giardino et al., 1998) and aldehyde in vivo (Kazachkov et al., in press). This suggests that the beneficial effect of AG may be in part due to the prevention of free radical and oxidant formation and to the prevention of aldehyde-induced cytotoxicity (Kazachkov et al., in press).

Consequently, the protection of the BBB by AG might contribute to reduce cell damage by limiting the entry of potential toxic compounds from blood into brain parenchyma. Finally, more studies are needed to reveal the mechanisms underlying the anti-edematous effect of AG in various models of cerebral ischemia and to consider the possible therapeutic effect of AG in stroke patients.

In conclusion, our study demonstrated that treatment with AG reduces brain edema formation in a dose-dependent manner likely through the protection of the BBB disruption in the acute phase of focal cerebral ischemia. Moreover, the anti-edematous effect of AG persisted for at least up to 3 h after the onset of focal cerebral ischemia.

4. Experimental procedures

4.1. Animals

Male Wistar rats (Pastor Institute, Tehran, Iran) were housed in standard cages in a temperature- (22–24 °C), humidity- (40–60%), and light period- (07:00–19:00 h) controlled environment. Experiments were performed in conformity with the University Research Council Guidelines for Conducting Animal Studies. Animals were randomly assigned to the different treatment groups and the investigator who performed animal surgery was blinded to the treatment of groups.

4.2. Transient focal cerebral ischemia

Middle cerebral arterial occlusion was induced by intraluminal filament method as described previously (Vakili et al., 2005; Vakili and Zahedi Khorasani, 2007). Briefly, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and the right common carotid artery (CCA) and the external carotid artery were exposed. A nylon thread (3-0) was carefully inserted into the internal carotid artery and was advanced towards the origin of the middle cerebral artery until a light resistance was felt. Such resistance indicates that the tip of the nylon thread was wedged at the beginning of anterior cerebral artery (20–22 mm from CCA bifurcation), resulting in MCAO. After 60 min of MCAO, reperfusion was accomplished by withdrawing the nylon filament. Animals were then recovered from anesthesia and kept in single cages for 24 h. Rectal temperature was measured by a thermometer and maintained at 37±0.5 °C throughout the experiment using an electrical blanket.

4.3. Measurement of infarct volume

Infarct volume was evaluated 24 h after MCAO in four different groups of rat. Group 1 (n=8) was the control group which received saline as vehicle (1 ml/kg, i.p.) at the beginning of MCAO. Group 2 (n=8), Group 3 (n=8) and Group 4 (n=8) were the treatment groups which received AG (Sigma, Germany) at the doses of 75, 150 and 300 mg/kg, i.p., at the start of MCAO, respectively.

To calculate infarct volume, 24 h after MCAO, rats were deeply anesthetized and were killed by cervical dislocation. Subsequently, brains were removed and sectioned coronally into seven 2-mm-thick slices using a Brain Matrix. Afterwards, slices were immersed in 2% triphenyltetrazolium chloride solution (Sigma, Germany), and kept at 37 °C in a water bath for 15 min. The slices were then transferred to 10% buffered formalin (Merck, Germany). 24 h later, slices were photographed using a digital camera connected to a computer (Cannon-Japan). Infarct areas were first measured using an Image Analyzer Software (NIH Image Analyzer). The infarct volume of each slice was calculated by multiplying the infarct area of the slice by its thickness. The total infarct volume of each brain was calculated as the sum of the infarct volumes of the seven brain slices. The contribution of edema to the infarct volume was corrected by applying the following formula as described previously (Swanson et al., 1990): corrected infarct volume=left hemisphere size– (right hemisphere size – measured infarct size).
4.4. **Evaluation of cerebral edema**

Cerebral edema was evaluated by determining BWC (Vakili et al., 2005). For a dose-dependent study, animals were randomly assigned into five different groups. Group 1 was sham-operated (n=8), Group 2 (n=8) was the control group which received saline (1 ml/kg), and Group 3 (n=8), Group 4 (n=8) and Group 5 (n=8) were treatment groups that received AG at the doses of 75, 150 and 300 mg/kg at the beginning of MCAO, respectively. For a time-course study, two separated groups received AG (150 mg/kg) at 1 h (n=8) or 3 h (n=8) after MCAO, respectively.

In all of these groups, 24 h after MCAO, rats were killed and the brains removed. Then the pons and olfactory bulb were removed and the brains were weighted to obtain their wet weight (WW). Subsequently, brains were dried at 110 °C for 24 h to determine their dry weight (DW). BWC was calculated by using the following formula: \((\text{WW}−\text{DW})/\text{WW}×100\).

4.5. **Evaluation of blood–brain barrier permeability**

BBB permeability was measured at 24 h after MCAO in three different groups: Group 1 was sham-operated (n=6), Group 2 (n=10) was the control group which received saline (1 ml/kg) at the beginning of MCAO and Group 3 (n=14) was the treatment group which received AG at a dose of 150 mg/kg, i.p., at the beginning of MCAO. In these groups, EB (2% in saline, 1 ml/kg) was injected from tail vein at the beginning of reperfusion. In all of these groups, BBB permeability was evaluated by measuring EB extravasations (Kucuk et al., 2002). Briefly, 20 h after MCAO, rats were deeply anesthetized, chest wall was opened, and transcardially perfused with 250 ml saline solution to clear cerebral circulation of EB. After decapitation, the brains were removed and hemispheres were separated and weighed. Each hemisphere was placed in the 2.5 ml phosphate buffer saline, was homogenized and then 2.5 ml of 60% trichloroacetic acid (Merck, Germany) was added to precipitate protein. Samples were centrifuged for 30 min at 3500 rpm. The supernatants were measured at 610 nm for absorbance of EB using a spectrophotometer (UV-visible–single beam; Apel, PD-303UV-Japan). The results were expressed as μg/g tissue of wet weight of brain tissue calculated against a standard curve.

4.6. **Measurement of physiological parameters**

Physiological parameters were assessed in three separate experimental groups: Group 1 (n=6) was sham-operated, Group 2 (n=7) was the control group which received saline (1 ml/kg) and Group 3 (n=7) was the treatment group which received AG (150 mg/kg, i.p.) at the beginning of MCAO. In these groups of animals, the left femoral artery was cannulated and the mean arterial pressure was recorded continuously from 60 min before MCAO until 60 min after reperfusion. Arterial blood gases were measured in 0.2-ml arterial blood samples taken 10 min before MCAO and 10 min after reperfusion.

4.7. **Statistical analysis**

Data are presented as means ±S.E.M. For time-course effect of AG on brain edema and infarct volume, comparison among multiple groups was performed using one-way analysis of variance (ANOVA), followed by Student–Newmann–Keuls’ and Dunnett’s tests as post hoc analyses. For the dose-dependent effect of AG on brain edema and BBB permeability, comparison among multiple groups was performed by nonparametric Kruskal–Wallis one-way ANOVA on ranks followed by Dunns’ test (SigmaStat 2.0, Jandel Scientific, Erkrath, Germany). Differences were considered significant at P<0.05.

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