Mechanism of protective effect of aminoguanidine on experimental cerebral ischemic injury in rats

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Abstract: AIM To investigate the beneficial effects of aminoguanidine (AG), a selective inducible nitric oxide synthase (iNOS) inhibitor, on cerebral ischemic injury of rats and the possible mechanism. **METHODS** The middle cerebral artery occlusion model was prepared with thread embolism. AG 100 $mg \cdot kg^{-1}$ was injected ip first at 2, 6 and 12 h, respectively, after ischemia, then 2 times a day for 3 consecutive days. The infarct volume of brain tissue was determined with tetrazolium chloride staining. The mitochondria in brain tissue were isolated for measuring integrity of electron transport chain (ETC), mitochondrial swelling, NO and malondialdehyde (MDA) contents, ATPase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. In addition, the neuronal cells of newborn rats were cultured in glucose-free medium with sodium hydrosulfite for cell viability, lactate dehydrogenease (LDH) and NO analysis. **RESULTS** AG significantly reduced infarct volume, ameliorated neuronal ultramicrostructural damages induced by ischemia. And the swelling of mitochondria, the lesions of ETC, the contents of MDA and NO in mitochondria were markedly decreased, the activities of ATPase, SOD and GSH-Px in mitochondria were increased. *In vitro*, compared with the ischemic group, AG (10, 20 and 100 $\mu mol \cdot L^{-1}$) increased the cell viability and reduced the contents of LDH and NO in culture medium. **CONCLUSION** AG has protective effects on cerebral ischemic injury through inhibiting the production of oxygen free radical, increasing antioxidation, ameliorating energy metabolism, and beneficially improving the integrity of structure and function of mitochondria in brain tissue.

Key words: brain ischemia; nitric oxide; aminoguanidine; mitochondria; cells, cultured

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Cerebral ischemia leads to neuronal damage in animals and in man. The exact mechanisms of damage are not fully clear, but several pathways (activation of voltage-gated calcium channels, excitotoxicity, response to free radicals, mitochondrial damage and apoptosis) appear to be involved[1]. The role of nitric oxide (NO) in cerebral ischemia has been investigated. NO is synthesized in the brain from *L*-arginine by a constitutive nitric oxide synthase (cNOS). NOS inhibitors have been examined as possible neuroprotective agents. Earlier studies showed the protective effects of NOS inhibitor in ischemia[2-4]. However, other reports have found that NOS inhibitor did not decrease infarct volume after transient middle cerebral artery occlusion in rats[5,6].

At present, thrombolysis is still the major therapeutic means clinically for acute stroke. As stroke is a multifactorial disease, sometimes some patients after thrombolysis may aggravate cerebral injury. Thus it has been becoming a new subject to research and produce effective neuroprotectors against cerebral ischemic injury. Preliminary studies in our laboratory indicated that treatment with the selective inducible NOS (iNOS) inhibitor, aminoguanidine (AG) reduced focal cerebral ischemic damage[7]. In this paper, we have examined the effects of administration of AG on infarction volume, mito-

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chondrion of brain after focal cerebral ischemia in rats and primary cultures of newborn rat cerebral neurons *in vitro*. The protective effect of AG on ischemic damage of brain tissue and the possible mechanism were further observed.

1 MATERIALS AND METHODS

1.1 Preparation of middle cerebral artery occlusion model and administration

Male Sprague-Dawley rats (Grade Ⅱ, Certificate № 04036) weighing 250 – 290 g were supplied by Experimental Animal Center of Hebei Province. Rats were anesthetized with 10% chloral hydrate. A nylon monofilament with a round tip was inserted into the external carotid artery and advanced into the internal carotid artery until a slight resistance was felt. Such resistance indicated that the filament had reached the circle of Willis. In this surgery, animal body temperature was maintained at 37℃ with a controlled infrared lamp. Rats were randomly divided into 7 groups ($n = 8$ for each): sham-operated animals, the external carotid artery of rat was surgically separated, but the filament was not inserted; 3 vehicle-treated ischemic groups and 3 AG (Sigma, USA)-treated ischemic groups. In vehicle-treated and AG-treated groups, normal saline (NS) 10 mL·kg^{-1} or AG 100 mg·kg^{-1} was injected ip first at 2, 6 and 12 h, respectively, after ischemia, then 2 times a day for 3 consecutive days. Rats were sacrificed for measurement of ischemic injury volume and the mitochondrial function in brain.

1.2 Measurement of ischemic injury volume[7]

The brains were carefully sliced to 5 pieces, 1.5-mm thick every slice. The slices were stained with tetrazolium chloride (Sigma, USA) for 30 min at 37℃ and then photographed to calculate the infarction volume with Image analysis software. The results were expressed as a percentage of the infarction volume in whole cerebrum volume.

1.3 Assay of mitochondrial function

The left hemisphere of the brain was rapidly separated and placed into an ice cold separating medium. All procedures were carried out on ice. The tissue was suspended into the medium and homogenized. The homogenate was immediately centrifuged at 2000 × g for 3 min at 4℃. The supernatant was decanted and centrifuged at 12 500 × g for 8 min. The resulting supernatant was discarded and the pellet was re-suspended in 3% Ficoll (Pharmacia, USA) medium. The suspension was re-suspended into 6% Ficoll medium(10 mL) and centrifuged at 10 400 × g for 30 min. The pellets were then suspended in 5 mL separating medium and centrifuged at 12 100 × g for 10 min. The mitochondrial pellets were then suspended in 1 mL separating medium to give a final protein concentration of approximate 5 – 10 g·L^{-1}. Ultrastructure of mitochordria was examined by electronic microscope. Mitochondrial protein was measured by protein assay kit (Jiancheng, Nanjing, China).

Measurement of mitochondrial swelling which reflects increment of membrane permeability[8]: mitochondrial protein was quantitated by the method of Lowry *et al* and 0.5 g·L^{-1} of mitochondrial protein was suspended in buffer (mmol·L^{-1}: cane sugar 250, KH_2PO_4 5, sodium succinate 3, pH 7.2). The swelling of mitochondria was measured by spectrophotometer and expressed as absorbance at 540 nm ($A_{540\ nm}$). Measurement of the integrity of mitochondrial electron transport chain (ETC) by MTT method[9]: 100 μL suspension of mitochondria was added MTT (5 g·L^{-1}) 40 μL. The reaction was monitored at 30℃ for 30 min. Then 100 μL avantin was also added. The mitochondrial ETC integrity was measured by spectrophotometer after 20 min and expressed as $A_{570\ nm}$.

Activities of ATPase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and the contents of NO and malondialdehyde (MDA) in mitochondria were measured by ATPase, SOD, GSH-Px, NO and MDA assay kits (Jiancheng, Nanjing, China). The SOD activity was expressed as difference of nitrite amount between control in test kit and

sample. The nitrite amount was calculated according to the standard curve.

1.4 Cell culture[10]

The newborn Wistar rats of 1 – 3 d old were supplied by Experimental Animal Center of Hebei Province (Grade Ⅱ, certificate №. 04070001). After disinfection, the brain hemispheres were dissected and soaked into cold D-Hanks solution. After removal of meninges and blood vessels, the tissues were mechanically dissociated by gentle triturate 20 – 30 times with a polished pipette. The whole solution was filtered through nylon mesh (200 mesh, hole width 95 μm). The filtrate was centrifuged at 3000 × *g* for 10 min, then the sediment was resuspended in DMEM containing 15% fetal bovine serum with a cell density about 1.5×10^9 L^{-1}. The cells were grown on a 24-well plate, which was previously coated with poly-*L*-lysine 10 mg·L^{-1} for 24 h, at 37℃ in 95% air/5% CO_2. Arabinosylcytosin 10 mg·L^{-1} was added at 72 h to prevent the growth of non-neuronal cells. After 48 h, the medium was changed to the normal medium and refreshed every 2 – 3 d. Purity was identified with neuronspecific enolase and neurofilament (Zhongshan, China) by immuocytochemistry.

On d 9 of cell culture, the original culture medium was replaced by Earle′s fluid (with or without sugar). The culture plates were divided into 5 groups at random: normal group, the culture was incubated by Earle′s fluid containing glucose; ischemic model group, the cells were incubated with sodium hydrosulfite in glucose-free Earle′s fluid. After 10 h, the medium was replaced by DMEM and cultivated for 14 h; AG treated groups: AG (10,20 and 100 μmol·L^{-1}) was first added prior to sodium hydrosulfite in glucose-free Earle′s fluid, then added again to replaced medium DMEM, and the culture was incubated at 37℃ for total 24 h.

After the culture was incubated at 37℃ for 20 h, MTT 0.5 g·L^{-1} was added to the medium. After additional 4 h, the formed formazan crystal was separated from the medium and dissolved in 1 mL Me_2SO. The absorbance at 570 nm was measured by ELISA plate reader. Lactate dehydrogenease (LDH) activity in the medium was measured by LDH assay kit (Jiancheng, Nanjing, China) at 440 nm. NO release from neuronal cells was measured by NO assay kit (Jiancheng, Nanjing, China).

1.5 Data analysis

Values were expressed as $\bar{x} \pm s$. All data were analyzed with one-way ANOVA and Dunnett′s *t* test by SPSS 11.5. $P < 0.05$ was considered to be significant.

2 RESULTS

2.1 Effects of AG on the mitochondrial function after ischemia

In ischemic 2, 6 and 12 h groups, $A_{540\ nm}$ decreased, indicating the increased swelling of mitochondria, and $A_{570\ nm}$ decreased, indicating some lesions in mitochondrial ETC. Compared with ischemic group, the swelling and lesions in ETC of mitochondria decreased after administration of AG (Tab 1). The contents of mitochondria NO were markedly increased in ischemic 2, 6 and 12 h groups. Compared with ischemic group, the contents of NO were decreased after administration of AG. The activities of ATPase, SOD and GSH-Px in mitochondria were markedly decreased, and the content of MDA in mitochondria was markedly increased in ischemic group. Compared with ischemic group, the activities of ATPase, SOD and GSH-Px in mitochondria were distinctly increased and the contents of MDA in mitochondria were distinctly decreased in AG group (Tab 2). Electronic microscope showed the mitochondria swollen, the cristae disrupted, dissolved or disappeared in ischemic rats. The above changes were not observed in sham group. Administration of AG could ameliorate the injury induced by cerebral ischemia in rats (Fig 1).

2.2 Effect of AG on infarcted volume of brain after ischemia

The infarcted tissue displayed white color,

Tab 1. Effects of aminoguanidine (AG) on mitochondrial swelling and integrity of mitochondrial electron transport chain (ETC) after ischemia

Group	Mitochondrial swelling ($A_{540\ nm}$)			Mitochondrial ETC ($A_{570\ nm}$)		
	2	6	12 (h)	2	6	12 (h)
Sham			0.279 ± 0.043			0.553 ± 0.040
I + NS	0.204 ± 0.024$^{\#\#}$	0.198 ± 0.027$^{\#\#}$	0.186 ± 0.026$^{\#\#}$	0.461 ± 0.048$^{\#\#}$	0.460 ± 0.035$^{\#\#}$	0.454 ± 0.037$^{\#\#}$
I + AG	0.256 ± 0.031*	0.252 ± 0.030*	0.253 ± 0.036*	0.544 ± 0.033*	0.530 ± 0.034*	0.524 ± 0.027*

Ischemic model (I) was prepared by middle cerebral artery occlusion with thread embolism. Normal saline (NS) 10 mL·kg^{-1} or AG 100 mg·kg^{-1} was injected ip first at 2, 6 and 12 h, respectively, after ischemia, then 2 times a day for 3 consecutive days. Mitochondrial swelling was measured by spectrophotometer. Integrity of mitochondrial ETC was determined by MTT assay. $\bar{x} \pm s$, $n = 8$. $^{\#\#}P < 0.01$, compared with sham group; $^{*}P < 0.05$, compared with corresponding I + NS group.

Tab 2. Effects of AG on NO and malondialdehyde (MDA) contents, ATPase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in rat brain mitochondria after ischemia

Group	Time after ischemia/h		
	2	6	12
	NO/μmol·g^{-1} protein		
Sham			2.11 ± 0.52
I + NS	3.86 ± 0.49$^{\#\#}$	3.40 ± 0.65$^{\#\#}$	3.32 ± 0.58$^{\#\#}$
I + AG	2.69 ± 0.91*	2.22 ± 0.21*	2.17 ± 0.52*
	ATPase/mmol Pi·h^{-1}·g^{-1} protein		
Sham			4.30 ± 0.30
I + NS	3.03 ± 0.30$^{\#\#}$	2.77 ± 0.38$^{\#\#}$	2.74 ± 0.40$^{\#\#}$
I + AG	3.72 ± 0.46$^{*\#\#}$	3.61 ± 0.38$^{**}_{\#\#}$	3.47 ± 0.29$^{**}_{\#\#}$
	SOD/mmol·min^{-1}·g^{-1} protein		
Sham			1.60 ± 0.14
I + NS	1.09 ± 0.11$^{\#\#}$	1.08 ± 0.16$^{\#\#}$	1.03 ± 0.26$^{\#\#}$
I + AG	1.46 ± 0.16**	1.52 ± 0.14**	1.43 ± 0.20**
	GSH-Px/mmol·min^{-1}·g^{-1} protein		
Sham			0.33 ± 0.05
I + NS	0.23 ± 0.03$^{\#\#}$	0.23 ± 0.04$^{\#\#}$	0.21 ± 0.04$^{\#\#}$
I + AG	0.32 ± 0.04**	0.30 ± 0.04*	0.29 ± 0.04*
	MDA/μmol·g^{-1} protein		
Sham			2.28 ± 0.36
I + NS	3.38 ± 0.79$^{\#}$	3.43 ± 0.85$^{\#}$	3.47 ± 0.52$^{\#}$
I + AG	2.25 ± 0.30*	2.37 ± 0.46*	2.44 ± 0.41*

See legend of Tab 1 for treatment. $\bar{x} \pm s$, $n = 8$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with corresponding I + NS group.

and nonischemic tissue displayed red color. When treated with AG for 3 d, the infarction volume of brain was markedly decreased compared with that of NS-treated groups respectively (Fig 2).

2.3 Effect of AG on ischemic injury in cultured rat cerebral neurons

NO content and LDH release remarkably increased, and cell viability decreased in ischemic group compared with the normal group. Administration of AG (10, 20 and 100 μmol·L^{-1}) increased the cell viability and reduced the contents of LDH and NO (Tab 3). In ischemic group, part of gathered nervous cells, cellular necrosis, cellular swelling, and nontypical morphology were observed. Administration of AG could inhibit above changes.

3 DISCUSSION

The model of focal cerebral infarction has been widely used to assess the potential role of experimental therapeutics for stroke because it is both reproducible and responsive to pharmacological agents. In our previous study, endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS mRNA reached the maximum at 2, 6 and 12 h after ischemia, respectively[11]. Therefore, AG was injected at 2, 6 and 12 h following ischemia to observe its effect.

Giulivi *et al*[12] demonstrated that mitochondria are a source of NO, the production of which may affect energy metabolism, O_2 con-

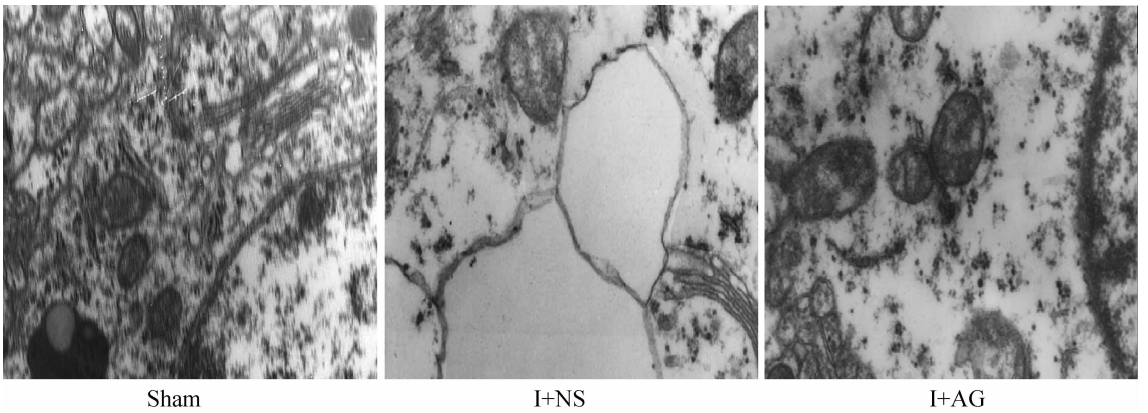

Fig 1. Ultrastructural changes in neuronal mitochondria when AG was administrated at 12 h after ischemia. ×20 000. See legend of Tab 1 for treatment. Sham: normal neuronal cells were showed; I + NS: the mitochondria swollen, the cristae disrupted, dissolved or disappeared; I + AG: mitochondria showed light matrix, and loss of matrix granules.

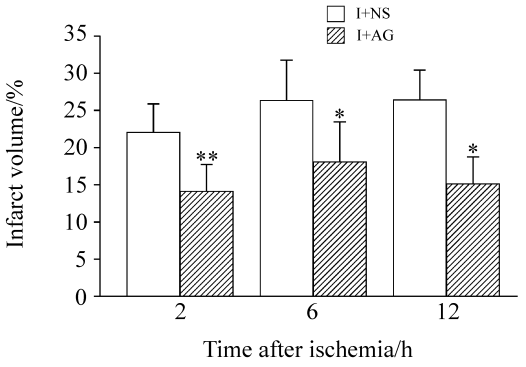

Fig 2. Effect of AG on infarct volume after ischemia. See legend of Tab 1 for treatment. $\bar{x} \pm s$, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with corresponding I + NS group.

Tab 3. Effect of AG on the cell injury induced by ischemia in neuronal cell cultures

Group		Cell viability/%	NO/μmol·g^{-1} protein	LDH/μmol·min^{-1}·L^{-1}
Normal		100 ± 9	55 ± 4	0.107 ± 0.019
I		53 ± 7$^{\#\#}$	188 ± 5$^{\#\#}$	0.192 ± 0.009$^{\#\#}$
I + AG	10	63 ± 6$^{*\#\#}$	120 ± 11$^{**}_{\#\#}$	0.174 ± 0.009$^{*\#\#}$
	20	66 ± 6$^{**}_{\#\#}$	110 ± 7$^{**}_{\#\#}$	0.173 ± 0.008$^{*\#\#}$
	100	76 ± 7$^{**}_{\#\#}$	104 ± 8$^{**}_{\#\#}$	0.157 ± 0.015$^{**}_{\#\#}$

Ischemia was prepared by incubating neuronal cells with sodium hydrosulfite in glucose-free Earle's fluid for 10 h, then in DMEM for 14 h. AG (10, 20 and 100 μmol·L^{-1}) was first added prior to sodium hydrosulfite in glucose-free Earle's fluid, then added again to DMEM. The cultures were incubated at 37℃ for 24 h. Cell viability was determined by MTT assay. $\bar{x} \pm s$, $n = 8$. $^{\#\#}P < 0.01$, compared with normal group (incubated with Earle's fluid containing glucose); $^{*}P < 0.05$, $^{**}P < 0.01$, compared with I group.

sumption, and oxygen free radical formation. Our results also agree with this hypothesis when the content of NO was increased after ischemia, the activities of ATPase, SOD and GSH-Px in mitochondria were decreased and MDA content was increased. There are escalating evidences showing that mitochondria play a key role in both necrotic and apoptotic neuronal cell death after acute cerebral ischemia[13-15]. Early classic ultrastructural studies on ischemic neurons concluded that the earliest site of cell damage was at the mitochondria, as evidenced by varying degrees of mitochondrial matrical swelling. The mitochondrial swelling reflects increment of membrane permeability transition initiated by a variety of stimuli. These data strongly encourage investigators to design pharmacological strategies for neuroprotection based on the target mitochondria to develop adjunct therapeutics to improve the therapeutic effect of currently established thrombolytic measures for acute stroke. Our results showed by electronic microscope significant mitochondrial damage in the form of increased swelling with cristal disruption, intracristal dilation, and loss of matrix density after cerebral ischemia. In our focal ischemia model, it appeared that ischemia produced rapid mitochondrial injury with increased swelling and some lesions in mitochondrial ETC. Administration of AG ameliorated these

mitochondrial injuries induced by cerebral ischemia in rats, suggesting that AG have protective effect by inhibiting the production of NO, beneficially improving the integrity of structure and function, and ameliorating energy metabolism of mitochondria after focal cerebral ischemia in rats.

We describe here a cell culture model of ischemia *in vitro*, in which cerebral neurons were rendered by sodium hydrosulfite resistant to injury induced by subsequent longer glucose deprivation. Using the experimental leverage gained in such an ischemic model system, part of gathered nervous cells, cellular necrosis, cellular swelling, and non-typical morphology were observed. NO content and LDH release were remarkably increased, and cell viability was decreased in ischemic group compared with the normal group. Administration of AG markedly inhibited this damage, indicating that AG can protect neurons against free radical insults.

The role of NO in the mechanisms of cerebral ischemia is multifaceted. NOS can be divided into two distinct classes: cNOS and iNOS. In the initial stages after ischemia, NO produced by cNOS is beneficial to the brain tissue[16]. In the late stages after cerebral ischemia, NO produced in large amounts by iNOS induction in the postischemic tissue contributes to the progression of the tissue damage. AG is the relatively selective iNOS inhibitor[2] without affecting brain cNOS activity. Therefore, the brain tissue was protected by NO produced by cNOS in the initial stages, and was better protected from the neurotoxic consequences of NO production by iNOS when AG was administrated after ischemia in our study, which support the hypothesis that postischemic NO production by iNOS is neurotoxic[17]. However, we could not be able to find the significant difference in beneficial effects, when AG was injected at 2, 6 and 12 h following ischemia. The detailed mechanism remains to be clarified further.

Despite the mechanism of AG-mediated cerebroprotection is uncertain, administration of AG can ameliorate the ischemic injury. Moreover, AG can be used both orally and intravenously, it is well absorbed after oral administration and is relatively nontoxic. The significant cerebroprotective effects of AG now suggest that it be plausible to investigate further the use of AG in stroke. It is anticipated that future experimental and clinical studies will provide a better understanding of the underlying cerebroprotective mechanism of AG and possibly add an additional therapeutic target in stroke pathogenesis.

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氨基胍对大鼠缺血性脑损伤的保护作用及其机制

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摘要：目的　观察选择性一氧化氮合酶抑制剂氨基胍(AG)保护大鼠缺血性脑损伤的可能机制。**方法**　采用线栓法复制大鼠大脑中动脉梗塞模型，缺血后 2，6 或 12 h 开始给予 AG(100 $mg\cdot kg^{-1}$，ip，每天 2 次，给药 3 d)治疗。测定脑梗死体积，脑线粒体肿胀度和呼吸链的完整性，脑线粒体内 NO 和丙二醛(MDA)含量，总 ATP 酶、超氧化物歧化酶(SOD)和谷胱甘肽过氧化物酶(GSH-Px)活性；培养大鼠神经元细胞，观察 AG(10，20 和 100 $\mu mol\cdot L^{-1}$)对神经元细胞形态、活力及乳酸脱氢酶(LDH)释放和 NO 含量的影响。**结果**　AG 显著降低脑缺血后脑梗死体积，改善缺血后神经元超微结构变化，减轻脑线粒体肿胀度和呼吸链损伤；降低 NO 和 MDA 含量，增加总 ATP 酶、SOD 和 GSH-Px 活性。AG 使体外培养的缺血神经细胞损伤程度明显减轻，NO 含量降低，LDH 释放减少，细胞活力增加。**结论**　AG 可能通过抑制氧自由基生成，增加线粒体抗氧化作用，改善线粒体能量代谢和保护线粒体形态与功能的完整而对大鼠脑缺血损伤产生保护作用。

关键词：脑缺血；一氧化氮；氨基胍；线粒体；细胞，培养的

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